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Exploring the Capacity of Bacteria for Natural Product Biosynthesis

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EXPLORING THE CAPACITY OF BACTERIA FOR NATURAL PRODUCT BIOSYNTHESIS

by

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ABSTRACT

Exploring the capacity of bacteria for natural product biosynthesis

by

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Bacteria are one of the richest sources of pharmaceutically relevant compounds. Nevertheless, the production level of those important compounds in the original hosts is often quite low, and sometimes it is so low that we cannot detect them with current analytical techniques. Researchers have developed various solutions, such as heterologous expression, co-cultivation of different bacteria, optimization of fermentation conditions, discovery of new species, engineering of biosynthetic enzymes, and manipulating regulatory elements. The main goal of my doctoral dissertation research is to engineer natural product biosynthetic pathways for the generation of novel compounds and enhanced production of known medicinally valuable compounds.

I first investigated the glycosylation steps in the biosynthesis of two antifungal angucyclines, Sch47554 and Sch47555 using two different strategies. The heterologous co-expression of the aglycone and sugar biosynthetic genes with *schS7* in *Streptomyces lividans* K4 led to the production of a novel C-glycosylated rabelomycin derivative. Gene inactivation of *schS9* and *schS10* revealed that subsequent glycosylation steps take place in a sequential manner in which SchS9 first attaches either a L-aculose or L-amicetose

moiety to the 4'-OH of the C-glycosylated aglycone, then SchS10 transfers a L-aculose moiety to the 3-OH of the angucycline core. In this research, I isolated two novel angucycline derivatives and gained new insights into the glycosylation steps in the biosynthesis of Sch47554 and Sch47555.

Next, I engineered the regulatory elements in *Streptomyces* sp. SCC-2136 through overexpression and targeted gene disruption approaches for enhanced production of pharmaceutically important angucyclines. Gene disruption of *schA4* and *schA16* led to a significant increase in the titer of Sch47554, while the titer was dramatically decreased in *Streptomyces* sp. SCC-2136/Δ*schA21*. Overexpression strains produced consistent results with the gene disruption strains. The highest titer of Sch47554 was achieved in *Streptomyces* sp. SCC-2136/Δ*schA4* (27.94 mg/L), which is significantly higher than the wild type. Further, I confirmed the functions of these three regulatory elements. SchA4 and SchA16 are repressors, while SchA21 acts as an activator.

Last, I isolated a carotenoid-producing endophytic bacterium from the leaves of *Taxus chinensis*, which was identified as *Pseudomonas* sp. 102515 based on the 16S rRNA gene sequence. Analysis of its secondary metabolites revealed that this endophytic strain produces a major product zeaxanthin diglucoside, a promising antioxidant natural product that belongs to the family of carotenoids. Based on the genome of a closely related *Pseudomonas* strain, I amplified a complete carotenoid (*Pscrt*) biosynthetic gene cluster from *Pseudomonas* sp. 102515. The functions of PsCrtI and PsCrtY in the biosynthesis of zeaxanthin diglucoside were characterized as phytoene desaturase and lycopene cyclase, respectively. The entire *Pscrt* biosynthetic gene cluster was successfully reconstituted in *E. coli* BL21(DE3) and *Pseudomonas putida* KT2440. The

engineered strain of *P. putida* KT2440 produced zeaxanthin diglucoside at 144 ± 4 mg/L in SOC medium supplemented with 0.5% glycerol at 23 °C, while the titer of zeaxanthin diglucoside in *E. coli* BL21(DE3) was very low. The production of zeaxanthin diglucoside in *Pseudomonas* sp. 102515 was improved through the optimization of fermentation conditions such as medium, cultivation temperature and growth time. The highest titer under the optimized conditions reached 206 ± 6 mg/L. To further enhance the production, I introduced an expression plasmid that harbors the *Pscrt* biosynthetic gene cluster into *Pseudomonas* sp. 102515, yielding an efficient producing strain of zeaxanthin diglucoside. The titer in this engineered strain reached 380 ± 12 mg/L, which is 85% higher than the wild type.

(215 pages)

PUBLIC ABSTRACT

Exploring the capacity of bacteria for natural product biosynthesis

Ozkan Fidan

This dissertation is focused on exploring the potential of bacteria for the biosynthesis of natural products with the purposes of generating novel natural product derivatives and of improving the titer of pharmaceutically important natural products.

A wide variety of compounds from various sources have been historically used in the treatment and prevention of diseases. Natural products as a major source of new drugs are extensively explored due to their huge structural diversity and promising biological activities such as antimicrobial, anticancer, antifungal, antiviral and antioxidant properties. For instance, penicillin as an early-discovered antimicrobial agent has saved millions of lives, indicating the historical importance of natural products. However, the alarming rise in the prevalence of drug resistance is a serious threat to public health and it has coincided with the decreasing supply of new antibiotics. Bacteria with a tremendous undiscovered potential have still been one of the richest sources of bioactive compounds to tackle the growing threat of antibiotic-resistant pathogens. Nevertheless, the production level of those important compounds is often quite low, and often undetectable using current analytical techniques. To expand the chemical repertoire of nature and to increase the titer of the natural products, researchers have developed various strategies, such as heterologous expression, co-cultivation of different bacteria, optimization of fermentation conditions, discovery of new species, engineering of biosynthetic enzymes,

and manipulating regulatory elements. Thus, in my dissertation research, I have exploited a few of these strategies. First, I heterologously expressed some of the biosynthetic genes from the *sch* biosynthetic gene cluster, resulted in the production of a novel glycosylated angucycline. I was also able to generate another new glycosylated derivative of angucycline through gene disruption of tailoring enzymes. In this research, I isolated two novel angucycline derivatives and gained new insights into the glycosylation steps in the biosynthesis of Sch47554 and Sch47555. Next, I engineered the regulatory elements in *Streptomyces* sp. SCC-2136 through the overexpression and targeted gene disruption approaches for enhanced production of pharmaceutically important angucyclines. The highest titer of Sch47554 was achieved in *Streptomyces* sp. SCC-2136/Δ*schA4* (27.94 mg/L), which is significantly higher than the wild type. This work thus provides an initial understanding of functional roles of regulatory elements in the biosynthesis of Sch47554 and Sch47555 and several engineered strains with enhanced production of Sch47554. Last, I isolated a carotenoid-producing endophytic bacterium from the leaves of the yew tree and optimized the fermentation conditions for an improved yield of zeaxanthin diglucoside up to 206 ± 6 mg/L. With the introduction of an additional copy of the *Pscrt* gene cluster through an expression plasmid, the engineered strain *Pseudomonas* sp. 102515/pOKF192 produced zeaxanthin diglucoside at 380 ± 12 mg/L, which is 85% higher than the parent strain. This strain holds a great potential for the production of pharmaceutically important antioxidant agent, zeaxanthin diglucoside.

DEDICATION

I would like to dedicate my doctoral dissertation to my beloved wife for her tireless support and encouragement along the way and to my children.

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I would like to express my deepest gratitude to my major advisor, Dr. Jixun Zhan, for his expert guidance, generous support and constant encouragement throughout all of this dissertation. Without his support and mentoring, it would have been impossible for me to get so much work done and finish this dissertation. From you, I acquired a lot of knowledge not just about the research but also how to become a better mentor. Thanks Dr. Zhan for those invaluable things that you contributed to my life and career. I would also like to thank my committee members, Dr. David Britt, Dr. Charles Miller, Dr. Jon Takemoto and Dr. Cheng-Wei Tom Chang, for their helpful advice and feedback to complete my research. I wish to extend my gratitude to my colleagues in the Metabolic Engineering Laboratory and to the staff in the Department of Biological Engineering, especially Kami McNeil for her help.

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CHAPTER I INTRODUCTION

Background

A wide variety of compounds from various sources, particularly from microorganisms, have been historically used to treat diseases. In particular, natural products as a source of medicine have long been attractive due to the huge structural diversity and promising biological activities such as antimicrobial, anticancer, antitumor, antifungal, antiviral, antiparasitic, antioxidant, antihypertensive properties, and so on

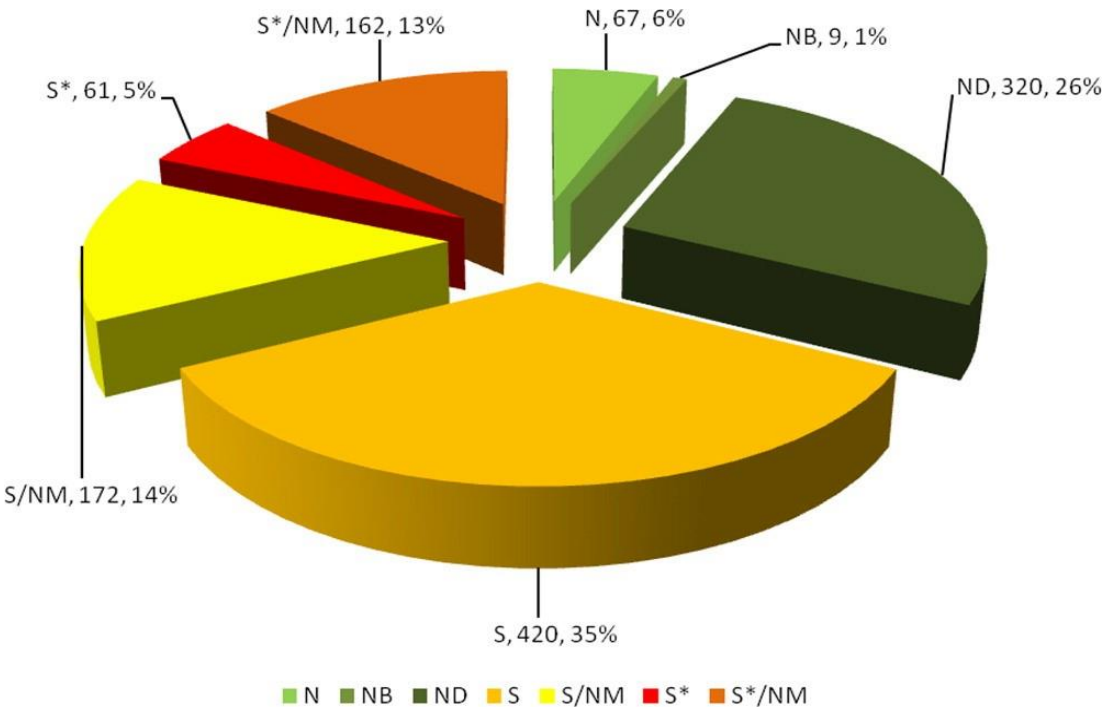

FIG. 1. All small-molecule approved drugs 1981–2014s; n=1211. Major categories are as follows: “N”, natural product; “NB”, natural product botanical drug; “ND”, derived from a natural product and usually a semisynthetic modification; “S”, totally synthetic drug often found by random screening/modification of an existing agent; “S*”, made by total synthesis, but the pharmacophore is/was from a natural product. The subcategory is as follows: “NM”, natural product mimic (4).

(1,2). For instance, penicillin as an early-discovered antimicrobial agent have saved the lives of millions of individuals, indicating the historical importance of natural products (3). In the past 30 years, natural products and their derivatives occupy a significant market share in pharmaceutical industry (Fig. 1) (4).

Natural products are derived from various natural sources such as bacteria, fungi, animals, and plants. There are successful examples of bioactive molecules from each of these natural sources. Actinomycetes, particularly Streptomycetes, have been one of the richest sources of biologically active secondary metabolites and have possessed a huge potential to produce novel, pharmaceutically useful secondary metabolites with a wide variety of biological activities (Fig. 2) (5). However, the deployment of any novel antibiotic has been followed by the observance of clinically significant resistance to that antibiotic in as short as a few years. The Infectious Disease Society of America predicts that 70% of hospital-acquired infections in the United States exhibit resistance to one or more antibiotics (6). Yet, there have been no new classes of clinically relevant antibiotics discovered in over 40 years (7). This discovery void makes the challenge even more severe. In addition, the production titers of many pharmaceutically important compounds are often low, and sometimes it is too low to detect the products with current analytical techniques (8,9). A well-known example is paclitaxel (Taxol®), which has a total market value of over $1 billion per year and is used in the treatment of various cancers. For the doses needed to treat a single patient, two to four fully–grown trees (less than 0.5 g accumulated Taxol per tree) of *Taxus brevifolia* have to be sacrificed because of the low level accumulation of paclitaxel (10,11).

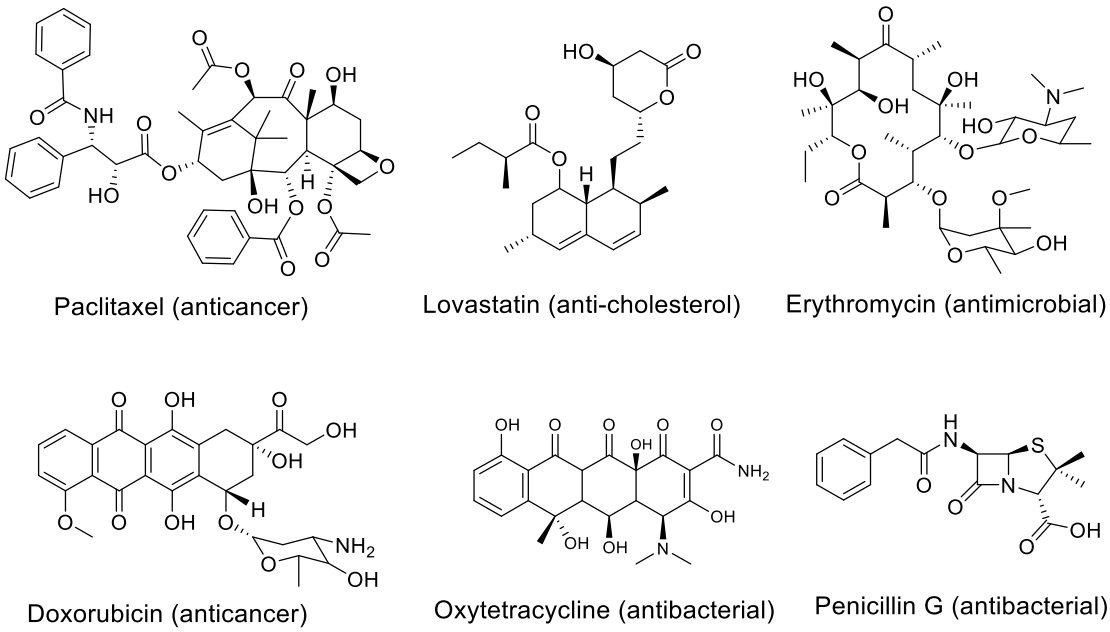

FIG. 2. Chemical structures of some natural drugs with different biological activities.

Technologies to expand chemical diversity or improve the production yields of natural products

To expand Nature's chemical repertoire and to increase the titers of known natural products, researchers have come up with various solutions: heterologous expression, co-cultivation, fermentation engineering, isolation of new species and strain engineering (12-14). In particular, strain engineering can be conducted through both metabolic engineering of biosynthetic pathways and engineering regulatory networks of secondary metabolites in native strains in which secondary metabolite biosynthesis is tightly regulated by global and/or pathway-specific regulatory proteins (8,14-18). In this dissertation, I have studied some of these approaches in order to understand natural product biosynthesis in bacteria with the purpose of generating novel compounds and

enhancing the production of known compounds. I will provide some of the successful examples for those strategies.

Co-cultivation

In Nature, microbes always co-exist within a complex microbial communities and compete with other microorganisms for the limited resources and antagonism. This natural characteristic of microorganisms favors various defense mechanisms that mainly lead to the production of bioactive secondary metabolites (19). Co-cultivation of two or more different microorganisms is to mimic the natural ecological settings in a laboratory environment. Many natural products are discovered through co-cultivation strategy (16). For instance, co-culture of a marine-derived fungus *Libertella* sp. and a marine-derived bacterium *Thalassopia* sp. led to the production of novel diterpenoids, libertellenones A-D (20). The new fungal polyketides, fumicyclines A and B were produced in a mixed cultivation of *Aspergillus fumigatus* and *Streptomyces rapamycinicus* (21). In another example, a known compound, holomycin, was produced from the co-cultivation of *S. clavuligerus*, which does not produce holomycin under normal laboratory condition, with methicillin resistant *Staphylococcus aureus* N315 through exploiting adaptive laboratory evolution (22). Therefore, the co-cultivation strategy not only produces known natural products with potentially higher yields, but also provides a potential mean to activate silent biosynthetic gene clusters for the discovery of new natural products.

Heterologous expression

Microbes, particularly *Escherichia coli* and *Saccharomyces cerevisiae*, have proven to be useful microorganisms for heterologous biosynthesis of medicinally

important natural products (15). They have been engineered for the enhanced production of natural products as well as the discovery of novel natural products (23). Heterologous expression also helps researchers understand and engineer the biosynthetic pathways for the generation of derivatives of known natural products, which further diversifies the chemical repertoire of Nature (24,25). Artemisinic acid, precursor of antimalarial drug artemisinin, has been heterologously produced in yeast with a quiet high titer (25 g/L) (26). Another example is the heterologous expression of erythromycin and its derivatives in *E. coli* by reconstituting a complex polyketide pathway (27-29). The heterologous expression strategy was also utilized for the activation of silent biosynthetic gene clusters from various sources, leading to the production of known or novel natural products in the host microorganisms (30). *Streptomyces* is also used for heterologous expression of natural products. For instance, a novel thioviridamide derivative was heterologously biosynthesized in *S. lividans* TK23 (31). Recently, a new diol-containing polyketide, lavendiol, was discovered by heterologous expression of a silent biosynthetic gene cluster from *S. lavendulae* FRI-5 in the heterologous host, *S. avermitilis* SUKA22 (32). All these examples suggest that heterologous expression can be utilized for the discovery of new compounds through activation of silent biosynthetic gene clusters and enhanced production of medicinally important compounds. It can also be used to understand and manipulate the biosynthetic pathways for the diversification of the chemical repertoire.

Metabolic engineering

Metabolic engineering is the introduction of rational changes in the genetic makeup of an organism to alter the metabolic profile or improve biosynthetic capabilities

(33). It has attracted an increasing interest as a mean to develop high yield bioprocesses and produce novel natural and "unnatural" natural products (14). Recent advances in polyketide biosynthesis by the techniques of metabolic engineering and systems biology established the foundation for combinatorial biosynthesis. The combinatorial biosynthesis approach is based on the understanding of the biosynthetic pathway to regulate, delete, add, substitute, and recombine the genes from different sources in a host microorganism. It has been used to alter the biosynthetic pathways of many natural products, resulting in the formation of many new "unnatural" products with new structures and biological activities (34). The insertion of module 2 of rapamycin PKS into the mutable site of DEBS1-TE led to the production of two novel tetraketides (35). Since chemical modification and/or derivatization of natural products are quite difficult due to structural complexity, metabolic engineering techniques such as heterologous expression, gene disruption and combinatorial biosynthesis are widely-used techniques and have been utilized for the diversification of natural products (14). Mithramycin SK, a novel antitumor drug with improved therapeutic index, was produced in the mithramycin producer *S. argillaceus* through combinatorial biosynthesis (36). Another simple approach for structural derivatization is the disruption of a particular gene that acts in a downstream pathway (usually a tailoring enzyme) (37). A typical example is the generation of a macrolide derivative without the epoxide functional group by the targeted gene disruption of a P450 epoxidase in the pimaricin gene cluster (38). In addition, heterologous co-expression of a set of genes with glycosyltransferases (GTs) in *S. lividans* led to the generation of a new tetracenomycin derivative (39). In this dissertation, I have exploited the latter two strategies (gene disruption and heterologous

expression) in order to generate novel derivatives and gain new insights into the glycosylation steps in the biosynthesis of angucyclines, Sch47554 and Sch47555.

GTs catalyze the transfer of activated sugar moieties to the acceptor molecules and are commonly involved in the biosynthesis and modification of many pharmaceutically significant natural products (40). Regio- and stereo-specifically attached sugar moieties play important roles in both binding of drugs to biological targets and biological activity of many natural products (41,42). For instance, landomycin A with a hexasaccharide side chain exhibits much stronger antitumor activity compared to its analog, landomycin E with a trisaccharide side chain (43). GTs hold huge potential for helping diversify pharmaceutically important drugs through the combination of chemoenzymatic and *in vivo* biosynthetic methods (44). For this purpose, heterologous expression and in-frame gene deletion approaches have been implemented in many studies. For example, Rodriguez et al. generated hybrid elloramycin analogs by combinatorial biosynthesis using genes from anthracycline-type and macrolide biosynthetic pathways (45). Tang and McDaniel also benefited from combinatorial biosynthesis to produce desosaminylated macrolactones and paved the way for the production of 'unnatural' natural product libraries (46). Trefzer et al. were able to produce novel glycosylated urdamycin derivatives by overexpressing GT genes from landomycin-producing strain of *S. cyanogenus* S136 in a mutant of urdamycin-producing strain of *S. fradiae* Tü 2717 (47). With a similar strategy, Ostash et al. generated novel landomycins in a mutant strain (43). Künzel et al. and Luzhetskyy et al. performed targeted gene inactivation including the inactivation of genes encoding GTs, leading to the formation of urdamycins I, J, K and landomycins M and O, respectively (48,49). All

these studies indicate that novel and/or unnatural natural products can be generated by the inactivation of GT genes and heterologous expression.

Strain engineering

With the recent advances in genome sequencing and recombinant DNA technologies, rational strain improvement through metabolic engineering has become a powerful tool for increasing titers of natural products. Some of widely-used metabolic engineering techniques for yield improvement are to increase the precursor supply, delete or tune the competing pathways or unwanted by-products, heterologous expression of the entire biosynthetic pathway, overexpression of rate-limiting enzymes and manipulation of regulatory genes (14,37). Typically, the best yields are achieved through a combination of several approaches. The production of artemisinin precursor, amorphadiene is a very successful example of metabolic engineering for yield improvement (15). Keasling and co-workers engineered an artemisinic acid-producing yeast strain in three steps: (i) engineering an farnesyl diphosphate (FPP) biosynthetic pathway to increase FPP production and reduce FPP use for sterol biosynthesis, (ii) introducing an amorphadiene synthase (ADS) gene from *Artemisia annua* into an efficient FPP producing yeast, and (iii) expressing a novel cytochrome P450 from *A. annua* (CYP71AV1), oxidizing amorphadiene to artemisinic acid in three steps, in an efficient amorphadiene-producing yeast (50). In this study, engineered *S. cerevisiae* with the ADS gene only produced low amounts of amorphadiene (4.4 mg/L). The overexpression of a truncated 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (tHMGR), which is a rate-limiting step in the mevalonate pathway, increased amorphadiene production by five-fold. They also

overexpressed a global transcription factor, UPC2, which regulates sterol biosynthesis in yeast, but this approach had a modest impact on amorphadiene production. However, the combination of this approach with the down regulation of ERG9 encoding squalene synthase, which itself increased amorphadiene production two-fold, led to the production of amorphadiene at 105 mg/L. In addition, they integrated another copy of tHMGR, resulting in the production of amorphadiene at 149 mg/L. The combination of all aforementioned modifications in the heterologous host led to the production of 153 mg amorphadiene/L that is approximately 500-fold higher than previously reported sesquiterpene production (50,51). Moreover, an artemisinic aldehyde Δ11(13) double bond reductase (DBR2) was co-expressed with FPPS, ADS, CYP71AV1 and P450 reductase, leading to the production of both artemisinic acid and dihydroartemisinic acid (52). Additionally, an aldehyde dehydrogenease (ALDH1) and artemisinic alcohol dehydrogenase (ADH1) were identified from *A. annua*. The expression of ALDH1 gene in yeast increased artemisinic acid production substantially. The combined heterologous expression of ALDH1 and ADH1 in *S. cerevisiae* with improvements in the fermentation process gave the result of the highest artemisinic acid production, 25 g/L (26). In this example, heterologous expression of the entire biosynthetic gene cluster, tuning the competing pathway and overexpression of bottle-neck enzymes led to a significant increase in the yield of amorphadiene. Moreover, manipulation of regulatory genes through overexpression of activator genes and/or deletion of repressor genes has been successfully performed to improve the yields of natural products (53). For instance, overexpression of an *Streptomyces* antibiotic regulatory protein (SARP) from the mithramycin biosynthetic gene cluster in *S. argillaceus* led to a 16-fold increase in the

mithramycin titer (54). Also, inactivation of a GntR family transcriptional repressor increased the yield of platensimycin and platencin by 100-fold compared to the wild type strain (55).

Pathway-specific regulatory genes can either activate or repress the expression of certain genes in the gene clusters. Bacterial pathway-specific regulators are classified into approximately 50 families based on the sequence alignment, structural and functional criteria (56). Among the known regulators in *Streptomyces*, the LuxR family transcriptional factors and SARPs are mainly activators as in the examples of GdmRI, GdmRII, ActII-orf4 and RedD (57,58). The TetR family transcriptional regulators mainly function as repressor as in the examples of TdrK, AlpZ and SAV576 (59-61). However, some of the TetR family regulators were characterized as activators, such as GdmRIII, SlgR1 and SAV3818 (62-65). Similar to the TetR family regulators, the AraC family transcriptional regulators can function as either activator or repressor (66-68). Knowledge of the function of regulatory proteins at molecular level is of great interest and would potentially increase the yields of secondary metabolites through either inactivation of repressors or overexpression of activators and a combinatorial approach of both inactivation and overexpression.

Streptomyces sp. SCC-2136 (ATCC 55186) produces Sch47554 and Sch47555 that exhibit antifungal activity against various yeasts and dermatophytes (69). In addition, Sch47554 and Sch47555 might potentially possess antitumor and antimicrobial activities, and enzyme inhibitory and agonistic activities due to their structural similarity to other angucycline metabolites (70). The diverse and promising biological activities of angucyclines excite the attentions of researcher to study their biosynthesis and to enhance

the titer of these pharmaceutically significant compounds. The biosynthetic gene cluster (*sch*) for these angucyclines contains several open reading frames (ORFs) that may produce genes with a potential regulatory function. Two putative regulatory genes, *schA4* and *schA21*, were predicted as the TetR family transcriptional regulators, while *schA16* shared significant similarity to the AraC family transcriptional regulators (71). In this dissertation, I have manipulated the pathway-specific regulatory genes in the *sch* biosynthetic gene cluster for an improved yield of angucyclines in *Streptomyces* sp. SCC-2136.

Discovery of new species/strains

The need for novel drugs for the treatment of human diseases is ever increasing. The field of drug discovery has regained the interest due to the rapid development of drug-resistant microbes, the discovery of new cases of life-threatening infections and the constant recurrence of diseases (72). In addition to aforementioned strategies for the production of novel natural products, isolation of new species is another important strategy because there is an estimated more than 99 per cent of species left undiscovered in the world (73). In particular, endophytes hold a huge potential for the discovery of natural products with pharmaceutical importance (74). Endophytes, microorganisms (bacteria and fungi) that live in the tissues of living plants without causing any apparent disease symptoms in the host, are relatively understudied as potential sources of novel natural products for exploitation in medicine, agriculture, and industry (75,76). For example, the ecomycins, a family of novel lipopeptides, consist of some unusual amino acids including homoserine and β-hydroxy aspartic acid. The endophytic bacterium,

Pseudomonas viridiflava produces ecomycins (77). Another example is pseudomycins, which represent a group of peptide antifungal compounds isolated from *Pseudomonas syringae,* a plant-associated bacterium (11). New species can sometimes lead to the rediscovery of known natural products as happened in the example of camptothecin, a potent antineoplastic agent. Camptothecin was originally isolated from the wood of *Camptotheca acuminate*. Interestingly, several endophytes such as *Entrophospora infrequens* and *Nodulisporium* sp. were reported to produce camptothecin (76), representing an alternative and potential mean for the production of this pharmaceutically important molecule. In this dissertation, I isolated an endophytic bacterium that produces carotenoids, predominantly zeaxanthin diglucoside.

Fermentation engineering

One of the main limiting factors for drug discovery is the ability to produce sufficient amounts of bioactive molecules for clinical applications after their discovery (78) since these compounds are just produced by bacteria to defend their resources and territories against invaders and competitors (79). Often, bacteria only need the bioactive metabolites to fulfill the purpose of defense and thus do not produce in large amounts (80). Fermentation engineering of natural product-producing bacteria is a strong strategy to enhance the yield of natural products. Clavulanic acid is a successful example for the fermentation engineering to maximize the production. The yield of clavulanic acid was enhanced from microgram level (400 μg/L) up to gram level (1.8 g/L) in *S. clavuligerus* through the optimization of fermentation conditions, carbon sources, amino acid supplementation and fed-batch fermentation (17). In another study, ribosome engineering

and fermentation optimization led to the improved production (40-fold higher) of tiancimycin A, a potent anticancer agent, in *Streptomyces* sp. CB03234 (81). In our lab, indigoidine production in *E. coli* was significantly increased up to approximately 4 g/L through optimization of fermentation conditions and co-expression of Sc-IndC and Sc-IndB (82). These examples clearly indicate fermentation engineering has a huge potential to increase the titer of natural products in wild type or engineered microorganisms. In my dissertation, I will test to increase the yield of zeaxanthin diglucoside through the optimization of fermentation conditions.

Objectives

The main goal of my doctoral dissertation research is to engineer bacterial natural product biosynthetic pathways for the generation of novel compounds and improved production of known medicinally important compounds. Additionally, I isolate endophytic bacteria with the purpose of discovering new drugs and providing new means to produce known natural products.

Specific objectives include:

1- Investigation of glycosylation steps in the biosynthesis of Sch47554 and Sch47555
   a) Heterologous expression of minimal PKS enzymes (SchP4-P10) with a glycosyltransferase (SchS7)
   b) Targeted gene disruption of SchS9 and SchS10
2- Engineering regulatory elements in the *sch* biosynthetic gene cluster for the improved production of Sch47554

3- Isolation of a carotenoid-producing endophytic bacterium and optimization of culture conditions to improve the yield

A guide to the dissertation

This dissertation contains five chapters. The first chapter provides a background literature review and the objectives of this dissertation. The second chapter is about the glycosylation steps in the biosynthesis of antifungal angucyclines with the purpose of understanding the pathway and generating novel compounds. Briefly, I heterologously expressed some of the biosynthetic genes from the *sch* biosynthetic gene cluster, which is responsible for the biosynthesis of angucyclines in *Streptomyces* sp. SCC-2136. That led to the production of a novel glycosylated derivative of rabelomycin. I was also able to generate another new glycosylated derivative of angucycline through metabolic engineering of biosynthetic pathways in the wild type strain. In Chapter 3, I engineered the regulatory elements in *Streptomyces* sp. SCC-2136 through overexpression and targeted gene disruption approaches. Two engineered strains led to the approximately 4 times higher yields compared to the wild type strain. Chapter 4 describes the isolation of an endophytic bacterium from a tree leaf. This endophytic strain produces carotenoids, particularly zeaxanthin diglucoside. This chapter also includes the optimization of the fermentation conditions for an improved yield of zeaxanthin diglucoside. The last chapter summarizes this dissertation research and its engineering value, and discusses the future work.

A number of research techniques are used in these studies, including molecular cloning, genetic engineering, metabolic engineering, microbial fermentation, protein

expression, natural products purification, and spectroscopic analyses for structure determination, DNA sequence analysis, aseptic techniques, laboratory skills, and others. A detailed explanation of each chapter is shown in the following chapters.

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CHAPTER II NEW INSIGHTS INTO THE GLYCOSYLATION STEPS IN THE BIOSYNTHESIS OF SCH47554 AND SCH47555[*]

Abstract

Sch47554 and Sch47555 are antifungal compounds from *Streptomyces* sp. SCC-2136. The availability of the biosynthetic gene cluster made it possible to track down the genes encoding biosynthetic enzymes responsible for the structural features of these two angucyclines. Sugar moieties play important roles in the biological activities of many natural products. Investigation of glycosyltransferases (GTs) may potentially help diversify pharmaceutically significant drugs through combinatorial biosynthesis. Sequence analysis indicated that SchS7 is a putative C-GT, while SchS9 and SchS10 were proposed to be O-GTs. In this study, we characterized the roles of these three GTs in the biosynthesis of Sch47554 and Sch47555. Co-expression of the aglycone and sugar biosynthetic genes with *schS7* in *Streptomyces lividans* K4 resulted in the production of C-glycosylated rabelomycin, which revealed that SchS7 attaches a D-amicetose moiety to the aglycone core structure at the C-9 position. Gene inactivation studies revealed that subsequent glycosylation steps take place in a sequential manner in which SchS9 first attaches either a L-aculose or L-amicetose moiety to the 4'-OH of the C-glycosylated aglycone, then SchS10 transfers a L-aculose moiety to the 3-OH of the angucycline core.

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INTRODUCTION

Angucycline antibiotics are a group of biologically active natural products synthesized by type II polyketide synthase (PKS) complexes through a sequence of reactions (1,2). Angucyclines exhibit diverse biological activities including antiviral (SM 196 B), antibacterial (marangucycline A, vineomycin A1), antitumor (landomycin A, urdamycin A, and marangucycline B) and antifungal properties (Sch47554 (**1**) and Sch47555 (**2**), Fig. 3) (3-8). Sch47554 and Sch47555 are produced by *Streptomyces* sp. SCC-2136 (ATCC 55186) and exhibit antifungal activity against various yeasts and dermatophytes (3). The entire biosynthetic gene cluster for the biosynthesis of Sch47554 and Sch47555 has been previously reported (9). The availability of their biosynthetic gene cluster (NCBI accession number AJ628018) made it possible to track down the genes encoding biosynthetic enzymes responsible for the structural features of these two angucyclines. The Sch47554 and Sch47555 (*sch*) biosynthetic gene cluster includes enzymes responsible for the biosynthesis of the core polyketide backbone (SchP6-P8) and nucleotidyl-activated sugar moieties (SchS1-S6). It additionally encodes some tailoring enzymes such as aromatase (SchP4), ketoreductase (SchP5), cyclase (SchP9), oxygenase (SchP10), and three putative glycosyltransferases (GTs) (SchS7, SchS9, and SchS10) (9).

GTs catalyze the transfer of activated sugar moieties to the acceptor molecules and are commonly involved in the biosynthesis and modification of many pharmaceutically significant natural products (10). Regio- and stereo-specifically attached sugar moieties play important roles in both binding of drugs to biological targets and biological activity of many natural products (11,12). For instance, landomycin A with a

hexasaccharide side chain exhibits much stronger antitumor activity compared to its derivative, landomycin E with a trisaccharide side chain (13).

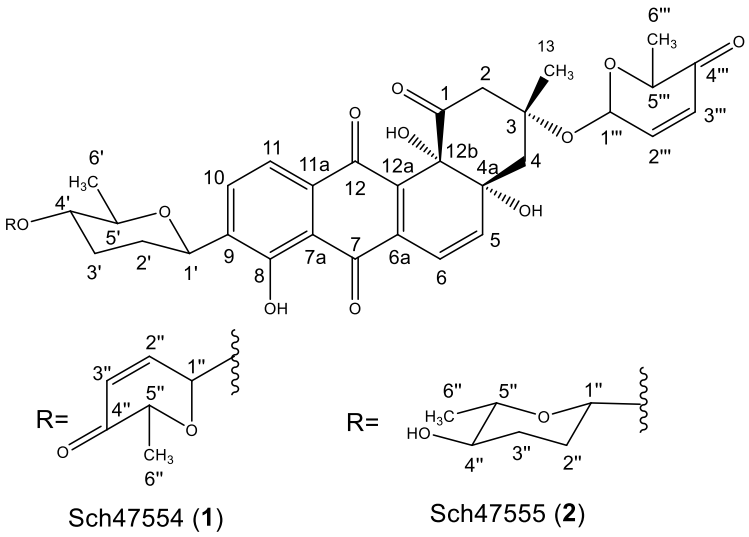

FIG. 3. Structures of Sch47554 (**1**) and Sch47555 (**2**).

GTs hold huge potential for helping diversify pharmaceutically important drugs through the combination of chemoenzymatic and *in vivo* methods (14). For this purpose, heterologous expression and in-frame gene deletion approaches have been implemented in many studies. For example, Rodriguez et al. generated hybrid elloramycin analogs by combinatorial biosynthesis using genes from anthracycline-type and macrolide biosynthetic pathways (15). Tang and McDaniel also benefited from combinatorial biosynthesis to produce desosaminylated macrolactones and paved the way for the production of 'unnatural' natural product libraries (16). Trefzer et al. were able to produce novel glycosylated urdamycin derivatives by overexpressing GT genes from landomycin-producing strain of *S. cyanogenus* S136 in a mutant of urdamycin-producing strain of *S. fradiae* Tü 2717 (17). With a similar strategy, Ostash et al. generated novel

landomycins in a mutant strain (13). Künzel et al. and Luzhetskyy et al. performed targeted gene inactivation including the inactivation of genes encoding GTs, leading to the formation of urdamycins I, J, K and landomycins M and O, respectively (18,19). All these studies indicate that novel and/or unnatural natural products can be generated by the inactivation of GT genes and combinatorial biosynthesis.

In the present study, we elucidated the roles of three GTs involved in the biosynthesis of **1** and **2**. We have used two aforementioned approaches to functionally characterize GTs in the *sch* biosynthetic gene cluster. The function of *schS7* was characterized through heterologous expression, while we performed targeted gene inactivation for *schS9* and *schS10*. These two approaches not only revealed the function of GTs, but also helped to diversify the pharmaceutically relevant molecules through the production of two novel compounds.

RESULTS AND DISCUSSION

Sequence analysis of the putative GTs in the *sch* biosynthetic gene cluster

The *sch* biosynthetic gene cluster contains three putative GT genes (*schS7*, *schS9*, and *schS10*). Multiple amino acid sequence analysis of SchS7 using Clustal Omega revealed its homology to known C-GTs: 72.33% identity with UrdGT2 from *S. fradiae*, 68.78% identity with SaqGT5 from *Micromonospora* sp. Tü 6368, and 55.97% identity with SimB7 from *S. antibioticus* Tü6040, as well as to a known O-GT, LanGT2 from *S. cyanogenus* with 58.71% identity (14,20-22). Multiple amino acid sequence analysis of SchS9 revealed its similarity to several characterized O-GTs: 58.91% identity with UrdGT1c from *S. fradiae*, 58.21% identity with SaqGT4 from *Micromonospora* sp. Tü

6368, and 54.38% identity with LanGT1 from *S. cyanogenus* (17,21,23). Similarly, multiple amino acid sequence analysis of SchS10 revealed its homology to a few O-GTs: 54.50% identity with SaqGT2 from *Micromonospora* sp. Tü6368, 49.40% identity with LanGT4 from *S. cyanogenus*, and 48.82% identity with UrdGT1a from *S. fradiae* (17,21,23). Clustal Omega multiple sequence alignments for each GTs (Figs. B1a-c) indicated that SchS7 is expected to possess the function of C-GT, whereas SchS9 and SchS10 are predicted as O-GTs.

Functional characterization of *schS7* through heterologous expression

Based on the sequence analysis, SchS7 is predicted to be responsible for the attachment of a sugar moiety to C-9 of the aglycone backbone via the formation of a C-C bond. To verify the function of this putative C-GT, we first aimed at the expression of minimal PKS genes of *sch* gene cluster (*schP6-P8*) and of *oxy* gene cluster (*oxyABC*) to test the putative functions of SchP6, SchP7, and SchP8, which led to the production of SEK15, the same product from the co-expression of OxyABC from the oxytetracycline biosynthetic pathway (data not shown) (24). We then expressed more genes in an attempt to produce a stable aglycone for GT studies. To this end, *schP4-P10* genes were ligated into the pRM5 vector, an *Escherichia coli*/*Streptomyces* shuttle vector to yield pOF10, which was then expressed in *S. lividans* K4. HPLC analysis revealed that a major product, **3**, was generated by *S. lividans* K4/pOF10 through heterologous expression of *schP4-P10* genes (pOF10) in *S. lividans* K4 (Fig. 4A). The ESI-MS spectrum of **3** showed the $[M+H]^+$ ion peak at *m/z* 339.0 (Fig. B2), indicating that its molecular weight is 338, which is same as that of rabelomycin, a common intermediate in angucycline biosynthesis. The structure

of **3** was confirmed to be rabelomycin (Fig. 4B) based on a comparison of its ^{1}H NMR data for **3** with previously reported data (25).

Using the same approach, we introduced more genes including *schS7* and the genes involved in the biosynthesis of activated sugar moieties (*schS1-S6* and *schS8*) to pOF10 to yield pGG31. Expression of this set of genes in *S. lividans* K4 yielded another major product **4**, as shown in Fig. 4A. The ESI-MS spectrum of **4** revealed the $[M+H]^+$ ion peak at *m/z* 453.1 (Fig. B2), suggesting a molecular weight of 452. **4** is 114 mass units larger than **3**, indicated that **4** is a D-amicetosylated derivative of **3**. To elucidate the structure of **4**, HR-MS analysis was conducted and showed a $[M+H]^+$ ion peak at *m/z* 453.1561, which is consistent with $C_{25}H_{25}O_8$ (cacld. 453.1505) (Fig. B6a). The formula of **4** revealed that it has 25 carbons, which has 6 more carbons than **3**, further indicating that a D-amicetose moiety had been added. This was supported by 1D and 2D NMR analysis (Table 1, Fig. 5 and Figs. B3a-d). The NMR data of **4** are similar to those of **3**, except that it has extra signals that belong to the D-amicetose moiety. The ^{13}C NMR spectrum showed 25 carbon signals. The chemical shifts of C-6 (δ 152.3) and C-8 (δ 157.9) indicated that they are hydroxylated carbons on the aromatic rings. Additionally, an oxygenated quaternary carbon signal at δ 71.3 was assigned to C-3. These signals are consistent with those in rabelomycin. The six carbon signals at δ 73.0, 33.2, 32.0, 79.2, 71.2 and 18.2, and another hydroxylated CH signal belong to the D-amicetose moiety, which further confirmed the presence of this sugar moiety in **4**. The HMBC correlation of the anomeric proton H-1 at δ_H 4.79 to C-9 (δ_C 139.8) confirmed the linkage of the D-amicetose moiety to the C-9 position (Fig. 5). Accordingly, the structure of **4** can be identified as 9-D-amicetosyl-rabelomycin and this compound was named as GG31.

Therefore, the function of SchS7 was identified as a C-GT in the biosynthesis of Sch47554 and Sch47555 (Fig. 4B).

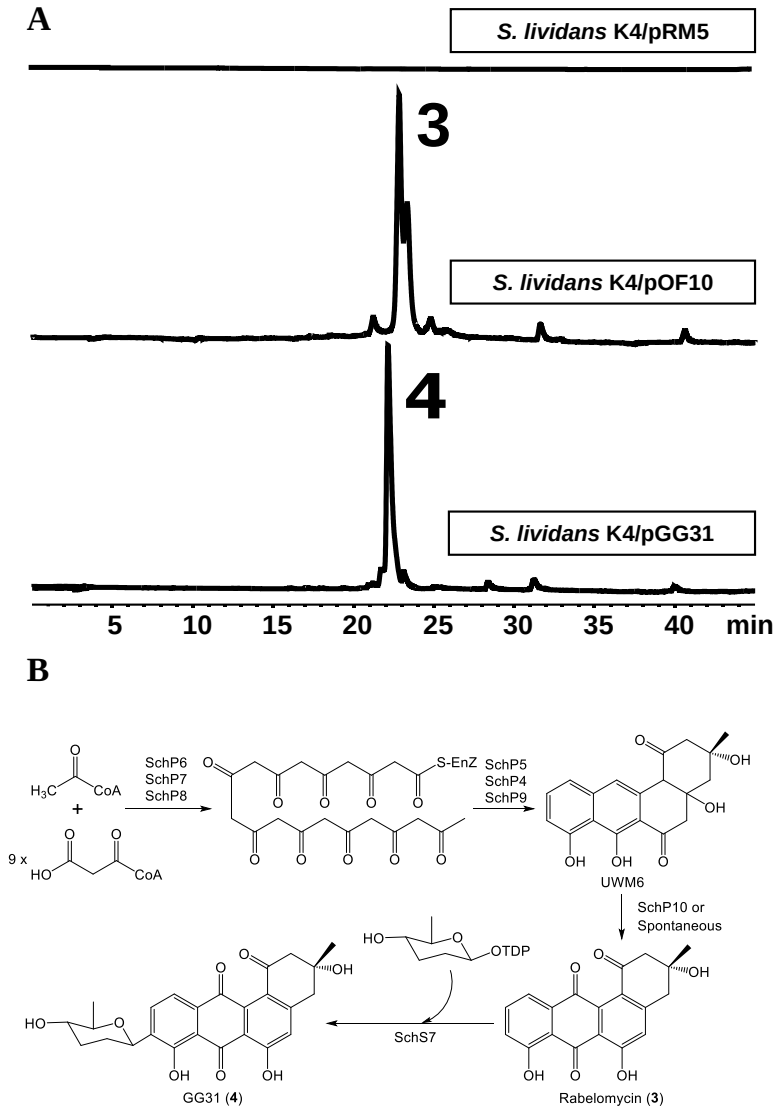

FIG. 4. Production of rabelomycin (**3**) and 9-D-amicetosyl-rabelomycin (GG31, **4**) in *S. lividans* K4 through heterologous expression. (A) HPLC analysis (420 nm) of the products of *S. lividans* K4 harboring pRM5 (upper), pOF10 (middle) and pGG31 (bottom). (B) Biosynthetic pathway of **3** and **4**.

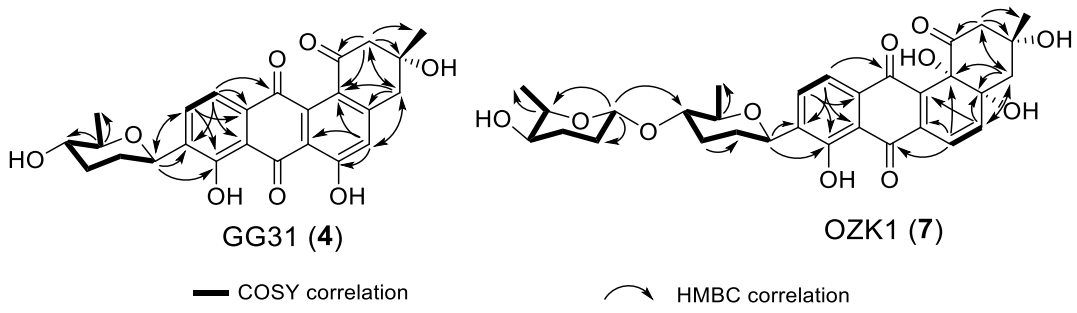

FIG. 5. Selected ^{1}H-^{1}H COSY and HMBC correlations for **4** and **7**.

Targeted gene inactivation of *schS9* and *schS10*

Sequence analysis of SchS9 and SchS10 suggested that they are responsible for O-glycosylations at 3-OH and 4'-OH of **1** and **2**. We inactivated both genes through homologous recombination to generate mutants lacking corresponding O-GTs (Fig. 6A). Apramycin resistant exconjugants were obtained from intergenic conjugation between *Streptomyces* sp. SCC-2136 and *E. coli* ET12567 respectively harboring pRY9 and pRY10. The confirmation of the single crossover events was performed by PCR using vector- (RV-M and M13-47) and genome-specific primers (Fig. 6B). As illustrated in Figs. 6A and 6B, the 2.2 kb and 2.1 kb PCR products were amplified from *Streptomyces* sp. SCC-2136/Δ*schS9* genome, whereas *Streptomyces* sp. SCC-2136 wild type genome did not yield these fragments. Similarly, we amplified the 2.6 kb and 2.8 kb fragments from the genome of *Streptomyces* sp. SCC-2136/Δ*schS10*, while we did not obtain these expected fragments from the genome of wild type strain. Thus, these PCR results confirmed the targeted gene inactivation for *schS9* and *schS10*.

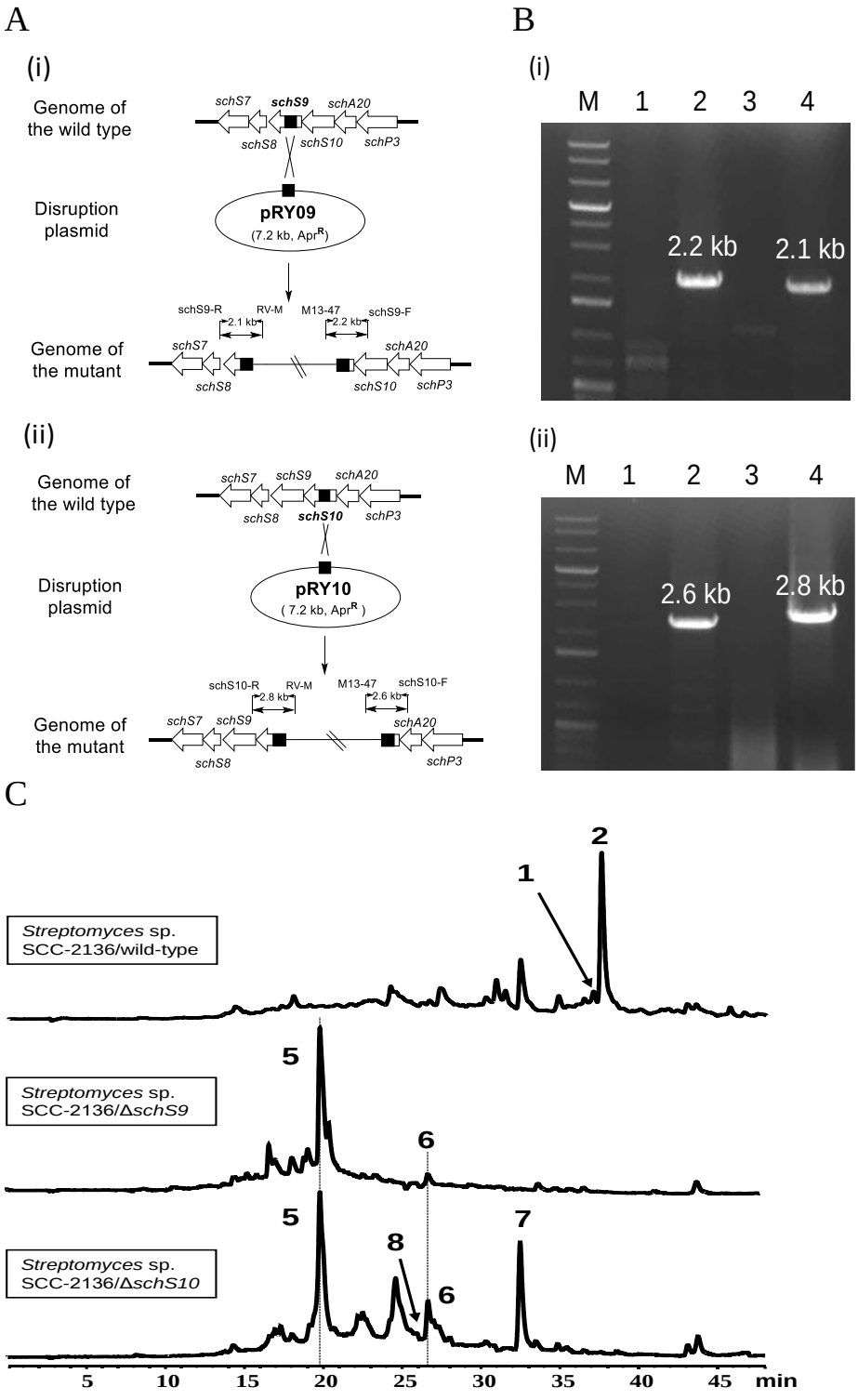

FIG. 6. Gene disruption of *schS9* and *schS10* and HPLC traces for the cultures

Streptomyces sp. SCC-2136/wild-type, Δ*schS9* and Δ*schS10.* (A) Single crossover recombination strategy for disruption of *schS9* (i) and *schS10* (ii) in *Streptomyces* sp. SCC-2136. (B) PCR confirmation of the *Streptomyces* sp. SCC-2136/Δ*schS9* (i) and *Streptomyces* sp. SCC-2136/Δ*schS10* (ii) single crossover mutants. (i) Confirmation of the insertion of pRY09 into the genome of *Streptomyces* sp. SCC-2136. M: 1 kb plus DNA ladder; 1: primers sch9-F/M13-47 with the wild type as the template; 2: primers sch9-F/M13-47 with the *ΔschS9* mutant as the template; 3: primers sch9-R/RV-M PCR with the wild type as the template; 4: primers sch9-R/RV-M PCR product with the Δ*schS9* mutant as the template. (ii) Confirmation of the insertion of pRY10 into the genome of *Streptomyces* sp. SCC-2136. M: 1 kb plus DNA ladder; 1: primers sch10-F/M13-47 PCR with the wild type as the template; 2: primers sch10-F/M13-47 with the Δ*schS10* mutant as the template; 3: primers sch10-R/RV-M with the wild type as the template; 4: primers sch10-R/RV-M with the Δ*schS10* mutant as the template. (C) HPLC traces (at 420 nm) for the cultures *Streptomyces* sp. SCC-2136/wild-type (upper), Δ*schS9* (middle) and Δ*schS10* (bottom).

Upon confirmation of gene inactivation, the positive exconjugants were grown on YM plates supplemented with apramycin for product analysis. HPLC analysis of the extracts of the cultures of *Streptomyces* sp. SCC-2136/Δ*schS9* and *Streptomyces* sp. SCC-2136/Δ*schS10* at 420 nm showed two same products (**5** and **6**) at 19.6 and 26.5 min in both extracts. *Streptomyces* sp. SCC-2136/Δ*schS10* produced two additional compounds (**8** and **7**) at 26.1 and 32.5 min (Fig. 6C). The ESI-MS spectra of **5, 6, 7**, and **8** showed the $[M-H]^-$ ion peaks at *m/z* 373.0, 469.1, 583.1, and 579.1, respectively (Fig. B4), indicating

that their molecular weights are 374, 470, 584, and 580, respectively. We purified all these compounds for chemical structure elucidation, except **8** due to the low titer. We confirmed the chemical structures of **5** and **6** based on the ^{1}H NMR data as listed in Table1. We, additionally, ran ^{1}H, ^{13}C, HSQC, and HMBC NMR as well as HR-MS for OZK1 (**7**) (Table 1, Fig. 5 and Figs. B5a-f) to elucidate the chemical structure of this new compound. NMR data analysis for **5** and **6** confirmed that the structures of **5** and **6** matched with the previously reported compounds of urdamycin X and 3'-deoxyaquayamycin, respectively (3,26). Urdamycin X was reported to be a surprising shunt product in *Streptomyces fradie*/Δ*urdQ/R*, while 3'-deoxyaquayamycin is a common C-glycosylated intermediate compound in angucycline biosynthesis.

Compounds **7** and **8**, on the other hand, are O-glycosylated intermediate compounds. HR-MS data for **7** indicated a $[M-H]^-$ ion signal at *m/z* 583.2559, which was consistent with the calculated exact mass of 583.2224 (Fig. B6b); this indicated that the molecular formula of **7** was $C_{31}H_{36}O_{11}$, as supported by the NMR spectroscopic data (Table 1), which contained 31 carbon signals. The chemical shift of C-8 (δ 159.3) indicates a hydroxy group attached to the aromatic ring. In addition, similar to **6**, compound **7** contains tertiary hydroxy groups at the C-4a (δ 132.1) and C-12b (δ 133.9) positions and an anomeric hydroxy group at the C-4′ position (δ 72.14). As suggested by the molecular weight of **7**, we found an additional anomeric proton signal (CH-1″) at δ_C 98.4 and δ_H 4.91; this indicates that **7** has one more amicetose sugar moiety. HBMC correlation of H-1″ to C-4′ revealed the linkage of the second amicetose moiety to 4′-OH of **6**. Thus, compound **7** was characterized as L-amicetosyl-3′-deoxyaquayamycin. All signals are assigned according to the 1D and 2D NMR spectroscopic spectra (Table 1).

Based on our findings, we proposed the glycosylation steps in the biosynthesis of **1** and **2** as illustrated in Fig. 7. Glycosylation occurs in a sequential manner, in which SchS7 first C-glycosylates the polyketide aglycone, followed by O-glycosylation of 3′-deoxyaquayamycin by SchS9. Lastly, SchS10 attaches the third sugar moiety to the C-3 position, which leads to the production of **1** and **2**.

DISCUSSION

Many natural products are produced through the assembly of their aglycone core structure followed by decoration with sugar moieties. The biological activities of many natural products, including medicinally significant antibiotics and anticancer drugs, depend on the regio- and stereospecifically attached sugar moieties (11,27). Chemical synthesis of sugar moieties is excessively difficult to perform and impractical to establish on a large scale (14). Thus, GTs are useful alternative tools for chemoenzymatic and *in vivo* approaches to develop glycosylated natural products (27). Many GT genes have been isolated from antibiotic-producing microorganisms and functionally characterized, mostly by gene inactivation experiments and in a few cases by heterologous expression. Among these GTs are *lanGT1*, *lanGT2*, *lanGT3*, and *lanGT4* in the landomycin gene cluster (14,17,28,29); *urdGT1a*, *urdGT1b*, *urdGT1c*, and *urdGT2* in the urdamycin gene cluster (20,23,30,31); *saqGT1*, *saqGT2*, *saqGT3*, *saqGT4*, *saqGT5*, and *saqGT6* in the saquayamycin Z gene cluster (21); *mtmGI*, *mtmGII*, *mtmGIII*, and *mtmGIV* in the mithraycin biosynthetic gene cluster (32,33); *oleG1* and *oleG2* in the oleandomycin gene cluster (34,35); and *dnrS* in the daunosamine biosynthetic gene cluster (36).

TABLE 1 ^{1}H NMR data for **3** (in $CDCl_3$), **4** (in $(CD_3)_2CO$), **5** (in CD_3OD), **6** (in DMSO-*D_6*), and **7** (in $CDCl_3$) and ^{13}C NMR data for **4** and **7**.

Position	**Rabelomycin (3)** [a]	**GG31 (4)** [a]	**Urdamycin X (5)** [a]	**3'-Deoxyaquayamycin (6)** [a]	**OZK1 (7)** [a]	**GG31 (4)**[b]	**OZK1 (7)**[b]
1	-	-	-	-	-	195.1	174.4
2	2.99 (d, 1H, 15.1) 3.07 (d, 1H, 15.2)	2.80 (d, 1H, 14.6) 2.95 (d, 1H, 14.7)	1.95 (dd, 1H, 13.9, 3.0) 2.33 (d, 1H, 13.8)	2.35 (d, 2H, 15.0)	2.62 (d, 2H, 14.8)	53.4	44.8
3	-	-	-	-	-	71.3	72.16
4	3.12 (s, 2H)	3.14 (d, 2H, 11.9)	2.20 (d, 1H, 10.5) 2.03 (dd, 1H, 10.5, 3.3)	2.90 (d, 1H, 13.0) 3.02 (d, 1H, 13.0)	3.08 (dd, 2H, 29.1, 13.7)	44.0	41.2
4a	-	-	-	-	-	129.7	132.1
5	7.04 (s, 1H)	7.08 (s, 1H)	1.67 (m, 1H) 2.11 (m, 1H)	7.81 (d, 1H, 7.8)	7.64 (d, 1H, 7.7)	121.5	139.5
6	-	-	2.61 (td, 1H, 12.1, 3.4) 2.85 (td, 1H, 11.9, 5.6)	7.72 (d, 1H, 7.7)	7.82 (d, 1H, 7.7)	152.3	119.1
6a	-	-	-	-	-	116.8	115.8
7	-	-	-	-	-	193.1	188.3
7a	-	-	-	-	-	114.8	115.3
8	-	-	-	-	-	157.9	159.3
9	7.30 (d, 1H, 2.6)	-	7.25 (dd, 1H, 6.3, 2.7)	-	-	134.5	139.8

10	7.72 (d, 1H, 4.7)	7.94 (d, 1H, 7.7)	7.59 (d, 1H, 7.7)	7.86 (d, 1H, 7.9)	7.92 (d, 1H,7.9)	134.1	133.4
11	7.70 (d, 1H, 4.8)	7.56 (d, 1H, 7.6)	7.58 (d, 1H, 7.6)	7.79 (d, 1H, 6.3)	7.85 (d, 1H, 7.9)	118.2	119.6
11a	-	-	-	-	-	138.1	131.5
12	-	-	-	-	-	182.9	188.0
12a	-	-	-	-	-	138.2	161.0
12b	-	-	-	-	-	128.6	133.9
13	1.52 (s, 3H)	1.47 (s, 3H)	1.54 (s, 3H)	1.16 (s, 3H)	1.34 (s, 3H)	29.6	27.2
1'	-	4.79 (d, 1H, 10.8)	-	4.70 (d, 1H, 10.6)	4.85 (d, 1H, 10.1)	73.0	73.2
2'	-	1.61 (m, 2H)	-	1.39 (m, 1H) 2.09 (m, 1H)	1.46 (m, 2H)	33.2	31.7
3'	-	2.14 (m, 2H)	-	1.55 (m, 1H) 2.02 (m, 1H)	2.27 (m, 2H)	32.0	31.8
4'	-	3.23 (m, 1H)	-	3.12 (m, 1H)	3.32 (m, 1H)	79.2	79.7
5'	-	3.40 (m, 1H)	-	3.33 (m, 1H)	3.55 (m, 1H)	71.16	77.6
6'	-	1.33 (d, 3H, 6.0)	-	1.26 (d, 3H, 6.0)	1.35 (d, 3H, 6.1)	18.2	18.5
1"	-	-	-	-	4.91 (s, 1H)	-	98.4
2"	-	-	-	-	1.90 (m, 2H)	-	27.7
3"	-	-	-	-	1.80 (m, 2H)	-	30.5
4"	-	-	-	-	3.31 (m, 1H)	-	72.14
5"	-	-	-	-	3.76 (m, 1H)	-	69.9
6"	-	-	-	-	1.27 (d, 3H, 6.2)	-	17.8

[a] The parentheses for ^{1}H NMR data are in the order of multiplicity, intensity, coupling constant (Hz). [b] ^{13}C NMR data for **4** and **7**

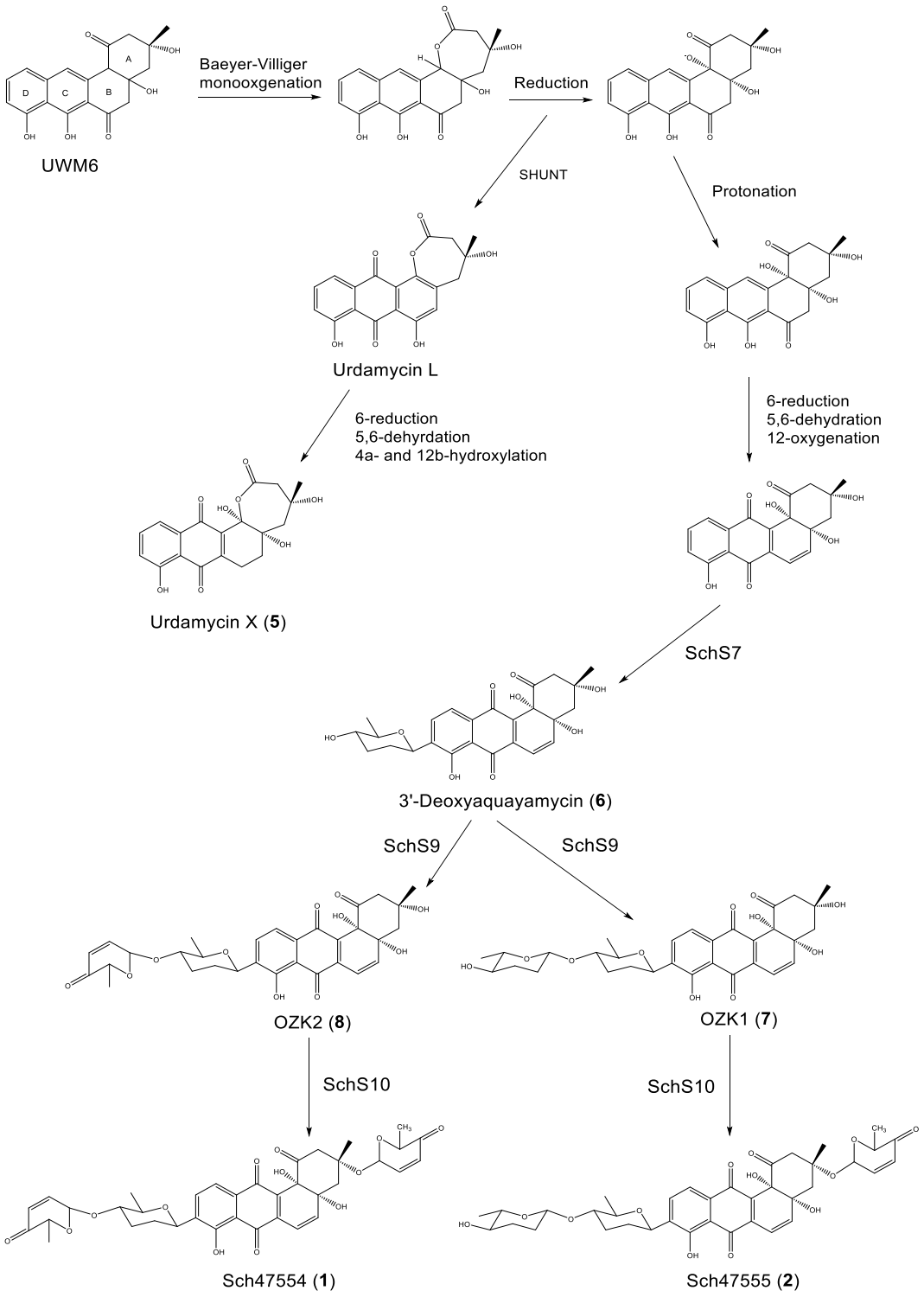

FIG. 7. Proposed glycosylation steps in the biosynthesis of **1** and **2**.

Compounds **1** and **2** are two antifungal angucyclines that possess three sugar moieties. However, how these sugars are added to the structures remains unknown. The *sch* gene cluster contains three putative GT genes (*schS7*, *schS9*, *schS10*) (9). Based on a multiple sequence comparison of the deduced amino acid sequences of GTs in the *sch* biosynthetic gene cluster, SchS7 showed high homology to several known C-GTs including UrdGT2, SaqGT5, and SimB7. SaqGT5 attaches an activated D-olivose moiety directly to the C-9 position of angucycline in the case of saquayamycin Z and of tetracenequinone in the case of galtamycin B (21). SimB7 and UrdGT2 are also responsible for the attachment of an activated D-olivose to C-9 of polyketide aglycone (22,30). In particular, UrdGT2 attaches an activated D-olivose to C-9 of 2-hydro-3-hydroxy-prejadomycin (UWM6) or terangulol (14,30). The examples of SaqGT5 and UrdGT2 show the substrate flexibility that will facilitate the synthesis of derivative compounds using those C-GTs. UrdGT2 also functions as O-GT and transfers the activated D-olivose sugar moiety to 1,2-dihydroxyanthraquinone (20). Surprisingly, multiple sequence comparison also revealed high homology of SchS7 to a known O-GT, LanGT2 from *S. cyanogenus* which catalyzes the first glycosylation step by attaching activated D-olivose to 8-OH of the aglycone (14). Interestingly, an engineered version of LanGT2 was able to act as C-GT, showing activity for both C- and O-glycosylation similar to UrdGT2 (20,37). Therefore, we expected SchS7 to be responsible for the attachment of D-amicetose to C-9 of the polyketide aglycone. To characterize its function, we have used a heterologous expression approach to create two expression plasmids, pOF10 and pGG31. pOF10 contains the minimal PKS genes (*schP6*, *schP7*, and *schP8*) and aromatase, ketoreductase, cyclase, as well as oxygenase genes (*schP4*,

schP5, *schP9*, and *schP10*), while pGG31 additionally includes *schS7* and genes required for the biosynthesis of activated sugar moieties (*schS1*, *schS2*, *schS3*, *schS4*, *schS5*, *schS6*, and *schS8*). *S. lividans* K4 harboring pOF10 and pGG31 respectively produced two major peaks of rabelomycin (**3**) and a novel compound, 9-D-amicetosyl-rabelomycin (**4**), proving that SchS7 is responsible for the attachment of a D-amicetose sugar moiety to the C-9 position of **3**.

Sequence analysis of SchS9 and SchS10 revealed that they are homologous to known O-GTs such as UrdGT1a, UrdGT1c, SaqGT2, SaqGT4, LanGT1, and LanGT4. In particular, SchS9 is highly homologous to SaqGT4 and UrdGT1c which attach activated L-rhodinose to the first sugar moiety in the biosynthesis of saquayamycin Z and urdamycin A, respectively. UrdGT1c transfers a L-rhodinose sugar moiety to urdamycinone B, 100-2, and aquayamycin at 3'-OH, while SaqGT4 attaches the third and fifth sugar moieties at 3-OH of the saccharide chain (21,23). This indicates the substrate flexibility of these enzymes and SchS9 might potentially recognize different substrates. Additionally, SchS9 has high homology to LanGT1 from the landomycin biosynthetic gene cluster that is responsible for the attachment of D-olivose to 4'-OH of the first sugar moiety (17). For SchS10, a multiple sequence alignment showed that it is highly homologous to SaqGT2 and UrdGT1a that are responsible for the attachment of L-rhodinose at 12b-OH and 3-OH, respectively (21,23). The amino acid sequence of SchS10 is also similar to that of LanGT4 which, in contrast to SaqGT2 and UrdGT1a, attaches the third sugar moiety (L-rhodinose) to the saccharide chain at C-9 (17). Therefore, both SchS9 and SchS10 are expected to catalyze the O-glycosylation steps in Sch47554 and Sch47555 biosynthesis. To confirm their functions, we disrupted the

corresponding genes through targeted gene deletion. HPLC analysis of the extracts of *Streptomyces* sp. SCC-2136/Δ*schS9* and *Streptomyces* sp. SCC-2136/Δ*schS10* mutants revealed that both mutants produced **5** and **6** in common. *Streptomyces* sp. SCC-2136/Δ*schS10* produced two additional compounds (**7** and **8**) (Fig. 6C). Except **8** which was only characterized with LC-MS due to the very low titer, all compounds were purified and characterized by LC-MS and NMR. Production of these compounds in the mutant strains indicates that O-glycosylation takes place in a sequential manner in which SchS9 first attaches either L-amicetose or L-aculose moiety to 4'-OH of **6**, then SchS10 transfers a L-aculose moiety to 3-OH of **7** or **8** to yield **1** and **2** (Fig. 7). SchS9 has relatively relaxed substrate specificity toward sugar donors as shown by its ability to attach two different sugar moieties, while SchS10 is flexible with sugar acceptors and can recognize both **7** and **8** as substrate. Compound **8** might be a more favorable substrate for SchS10 than **7** due to a higher titer of **1** compared to **2** in the wild type, whereas more efficient production of **7** than **8** in *Streptomyces* sp. SCC-2136/Δ*schS10* mutant indicates that SchS9 favorably attaches an activated L-amicetose moiety to 4'-OH of **6**. Additionally, the presence of **6** in both *Streptomyces* sp. SCC-2136/Δ*schS9* and Δ*schS10* extracts suggests that SchS7 is the dedicated C-GT that catalyzes the first glycosylation with 9-deolivosyl aquayamycin as the sugar acceptor substrate. Compounds **3** and **4** were not detected in the wild type and mutant strains of *Streptomyces* sp. SCC-2136. The enzymes involved in 6-reduction, 5,6-dehydration, and 12-oxygenation may not recognize a glycoside substrate, such as **4**. The production of **4** through heterologous expression in *S. lividans K4* indicated that SchS7 has relaxed specificity towards the sugar acceptors.

In addition, both mutant strains produced **5** that was reported as a shunt product. Similar to **5**, urdamycin L was detected in a Δ*urdM* mutant of *S. fradiae* during the Baeyer-Villiger monooxygenation process in the biosynthesis of urdamycin A (25). Compound **5** is different from urdamycin L due to the almost saturated ring B (additional OH groups at C-4a and C-12b), whereas urdamycin L possesses a phenolic arene ring B with an OH group at C-6 (Fig. 7) (26). However, both compounds are related to each other by an unusual ε-lactone as ring A. Urdamycin L is derived from the Baeyer-Villiger monooxygenation reaction through spontaneous oxidation, whereas **5** is an overoxidized analogue of urdamycin L (25,26). In the *sch* biosynthetic gene cluster, there are oxygenases, such as *schP3* and *schP10*, with high homology to *urdM* (64 %) and *urdE* (84 %), respectively. These enzymes might be responsible for the oxygenation of the angucycline ring. It is surprising that **5**, as a shunt product, was produced in larger quantities than the biosynthetic intermediate **6** in the *schS9* and *schS10* disruption mutants (Fig. 6C). Because *schS7* is located downstream of *schS9* and *schS10*, there is a possibility that a polar effect occurs if the disruption plasmids are inserted into the genome. This polar effect may cause a low-level expression of C-GT, SchS7, and thus, the overall attenuation of the biosynthetic route of **6**. This will in turn lead to the increased accumulation of **5**.

CONCLUSION

We used two different approaches to characterize the functions of three GTs in the *sch* biosynthetic gene cluster; heterologous expression for SchS7 and targeted gene disruption for SchS9 and SchS10. As expected from multiple sequence alignment

analysis of these GTs with their homologues, we found that the glycosylation process in the biosynthesis of **1** and **2** occurred in a sequential manner. First, SchS7, which is a C-GT, attaches a D-amicetose moiety to the polyketide aglycone, followed by the attachment of the second sugar moiety to 4′-OH of the first sugar moiety by SchS9. In the last step, SchS10 transfers an L-aculose to C-3 of the glycosylated polyketide aglycone, which leads to the biosynthesis of major products **1** and **2**. We isolated two novel compounds, **4** and **7**, from these two different approaches, respectively, diversifying the pharmaceutically relevant compounds. Through elucidating the functions of three GTs, we may combine them with other GTs to change the saccharide chain, which possibly enhances the antifungal activity, and creates structural diversity in angucyclines.

EXPERIMENTAL SECTION

Bacterial strains, media, and culture conditions

S. lividans K4 strains harboring pOF10 and pGG31 were maintained on R5 agar plates supplemented with thiostrepton at 28 °C for 10 days. *Streptomyces* sp. SCC-2136/Δ*schS9* and *Streptomyces* sp. SCC-2136/Δ*schS10* mutants were grown on YM agar plates supplemented with apramycin at 28 °C for 10 days. *Streptomyces* sp. SCC-2136 was grown in YM broth for 5 days for genomic DNA extraction. *E. coli* XL1-Blue and ET12567, which were routinely grown in LB broth at 37 °C, were used for general genetic manipulations and intergenic conjugation, respectively. MS and ISP4 media were used for conjugation and single crossover homologous recombination, respectively. Apramycin (50 μg/mL), thiostrepton (50 μg/mL), chloramphenicol (25 μg/mL), ampicillin (50 μg/mL), and nalidixic acid (25 μg/mL) were supplemented, if appropriate.

General genetic manipulation and PCR

Standard molecular biology protocols were performed as previously described (38). Genomic DNA samples of *Streptomyces* sp. SCC-2136 and its mutants were extracted using a ZR Fungal/Bacterial DNA Miniprep Kit (Irvine, CA, USA). Plasmid DNA extraction from *E. coli* cells was performed using a Thermo Scientific GeneJET Plasmid Miniprep Kit (Logan, UT, USA). PCR reactions were performed with an Arktik Thermal Cycler (Thermo Scientific) using Phusion DNA polymerase (Thermo Scientific). Oligonucleotide primers were ordered from Sigma–Aldrich and dissolved in TE buffer to a concentration of 100 ng/mL.

Construction of plasmids for heterologous expression and gene disruption

For the heterologous expression approach, *schP4*, *schP5*, *schP6*, *schP7*, *schP8*, *schP9*, and *schP10* genes were PCR-amplified from *Streptomyces* sp. SCC-2136 genomic DNA using the oligonucleotide primers listed in Table B1. Specific forward and reverse primers contained compatible restriction enzymes, such as XbaI, SpeI, and NheI. The PCR products were first ligated into the pJET1.2 cloning vector for DNA sequencing using Sanger's method (Eton Bioscience, San Diego, CA, USA). Each combination of genes was then constructed by the subsequent addition of individual genes into the cloning vector and correct ligation was confirmed by restriction enzyme digestions. Additionally, each group of genes was ligated into a pRM5-derived vector. The gene cassette containing *schP6*, *schP7*, and *schP8* was excised from pOF1 using PacI and NheI and ligated into pRM5 between the same sites to yield pOF3. Subsequently, the *schP5* gene was excised from pOF2 using NheI and SpeI and ligated into pOF3 between

the same sites to yield pOF4. *schP4*, *schP9*, and *schP10* were also excised from pOF5-7 and inserted in the same sequential manner to yield pOF8-10, respectively. Similarly, *schS1*, *schS2*, *schS3*, *schS4*, *schS5*, *schS6*, *schS7*, and *schS8* were PCR-amplified from the genomic DNA of *Streptomyces* sp. SCC-2136 and subsequently inserted into pJET1.2 to yield pKN57. The gene cassette containing these genes was excised from pKN57 using the NheI and SpeI restriction sites, and ligated into pOF10 digested with the same restriction enzymes to yield pGG31. pOF10 and pGG31 plasmids were introduced into *S. lividans* K4 through poly(ethylene glycol) (PEG)-mediated protoplast transformation for product analysis.

For the targeted gene disruption approach, a 672 bp fragment of *schS9* and a 763 bp fragment of *schS10* were PCR-amplified from the genomic DNA of *Streptomyces* sp. SCC-2136 using the primers listed in Table B1. The PCR products for *schS9* and *schS10* were first ligated into the pJET1.2 cloning vector by using T4 DNA ligase (New England Biolabs) to yield pRY7 and pRY8, respectively. These plasmids were sequenced to ensure that the genes were correct and free of mutations. The inserts were excised from pRY7 and pRY8 with XbaI and HindIII (New England Biolabs) and ligated to the thermally sensitive plasmid pKC1139 between the same sites to yield pRY9 and pRY10, respectively, which were subsequently used for gene inactivation of *schS9* and *schS10* in *Streptomyces* sp. SCC-2136 through intergenic conjugation with *E. coli* ET12567. Positive exconjugants for *Streptomyces* sp. SCC-2136/Δ*schS9* and Δ*schS10* mutants were transferred to ISP4 plates supplemented with apramycin and nalidixic acid, and incubated at 37 °C for about 10 days to allow the plasmids to integrate into the genome and yield the single crossover mutants. The positive mutants were shuttled back and forth to YM

and ISP4 plates supplemented with apramycin for two generations to guarantee the positive mutants. Positive exconjugants after genome integration were subjected to product analysis and PCR confirmation of gene knockouts using primers listed in Table B1. The details of PEG-mediated protoplast transformation and intergenic conjugation protocols are described in the literature (39).

Production, extraction, purification, and characterization of compounds

S. lividans K4, harboring pOF10 and pGG31, and *Streptomyces* sp. SCC-2136/Δ*schS9* and *Streptomyces* sp. SCC-2136/Δ*schS10* mutants were grown for 10 days at 28 °C on R5 and YM agar plates supplemented with appropriate antibiotics, respectively. The cultures were chopped and extracted with a mixture of solvent consisting of 89 % ethyl acetate, 10 % methanol, and 1 % acetic acid (v/v). The resulting extracts were dried *in vacuo*, and the residues were redissolved in methanol for LC-MS analysis. All crude extracts were separated on a silica gel 60 column using different ratios of chloroform–methanol. The fractions with target compounds were further separated by HPLC with an Agilent Eclipse XDB-C18 column (5 μm, 250×4.6 mm). Low-resolution ESI-MS spectra were obtained on an Agilent 6130 LC-MS. A Waters GCT high-resolution mass spectrometer, with EI/CI and LIFDI capabilities (University of California, Riverside), was used to obtain the accurate mass spectra for the new compounds. 1D and 2D NMR spectra were recorded on a JEOL ECX-300 or a Bruker Avance III HD Ascend-500 NMR spectrometer.

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CHAPTER III IMPROVED PRODUCTION OF ANTIFUNGAL ANGUCYCLINE SCH47554 BY MANIPULATING THREE REGULATORY GENES IN *STREPTOMYCES* SP. SCC-2136[†]

Abstract

Sch47554 and Sch47555 are two angucyclines from *Streptomyces* sp. SCC-2136 with antifungal activities against various yeasts and dermatophytes. The *sch* gene cluster contains several putative regulatory genes. Both *schA4* and *schA21* were predicted as the TetR family transcriptional regulators, while *schA16* shared significant similarity to the AraC family transcriptional regulators. Although Sch47554 is the major product of *Streptomyces* sp. SCC-2136, its titer is only 6.72 mg/L. This work aimed to increase the production of this promising antifungal compound by investigating and manipulating the regulatory genes in the Sch47554 biosynthetic pathway. Disruption of *schA4* and *schA16* led to a significant increase in the production of Sch47554, whereas the titer was dramatically decreased when *schA21* was disrupted. Overexpression of these genes gave opposite results. The highest titer of Sch47554 was achieved in *Streptomyces* sp. SCC-2136/Δ*schA4* (27.94 mg/L), which is significantly higher than the wild type. Our results indicate that SchA4 and SchA16 are repressors, while SchA21 acts as an activator. This work thus provides an initial understanding of functional roles of regulatory elements in the biosynthesis of Sch47554. Several efficient producing strains of Sch47554 were

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constructed by disrupting or overexpressing particular regulatory genes, which can be further engineered for industrial production of this medicinally important molecule.

INTRODUCTION

Gram-positive, filamentous, soil-inhabiting bacteria of *Streptomyces* belong to the phylum of Actinobacteria and have been recognized as one of the richest sources of biologically active secondary metabolites. They have a complex life cycle of morphological differentiation, which begins with spore germination in order to form branched vegetative hyphae (1). The subsequent differentiation results in the formation of sporogenic aerial hyphae, followed by the chains of unigenomic spores (2). During the initiation of morphological differentiation, most of the *Streptomyces* strains produce an extraordinary diversity of bioactive secondary metabolites, including a wide variety of pharmaceutically important natural products with various biological activities such as antimicrobial, anticancer, antifungal, antiviral and anti-cholesterol properties (3-6). The morphological differentiation and secondary metabolite production are tightly controlled by a complex network of regulators that senses and responds to plenty of physiological and environmental stimuli, including environmental stress, nutrition depletion, growth rate and small signaling molecules such as γ-butyrolactone and ppGpp (7-10). The regulators function at several hierarchical levels, starting from the highest level of regulation (also known as global or pleiotropic regulators), which involves pleiotropic genes that play significant roles in morphological differentiation as well as secondary metabolite production. The lowest level of regulation utilizes pathway-specific regulatory genes that are located in the biosynthetic gene clusters and act as a master switch for the

biosynthesis of corresponding secondary metabolites (8,9,11,12).

Pathway-specific regulatory genes can either activate or repress the expression of certain genes in the gene clusters. Biosynthetic gene clusters might have different numbers of pathway-specific regulatory genes. For instance, there is one in the actinorhodin biosynthetic gene cluster (13), whereas the daunorubicin gene cluster has three regulators (14). Some gene clusters, such as that for tylosin biosynthesis (15), contain both activators and repressors in the same gene cluster. Bacterial pathway-specific regulators are classified into approximately 50 families based on the sequence alignment, structural and functional criteria (11). Among the known regulators in *Streptomyces*, the LuxR family transcriptional factors and *Streptomyces* antibiotic regulatory proteins (SARPs) are mainly activators as in the examples of GdmRI, GdmRII, ActII-orf4 and RedD (13,16). The TetR family transcriptional regulators often have the repression function as in the examples of TdrK, AlpZ and SAV576 (17-19). However, some of the TetR family regulators were characterized as activators, such as GdmRIII, SlgR1 and SAV3818 (20-23). Similar to the TetR family regulators, the AraC family transcriptional regulators can also function as either activator or repressor (24-26). Knowledge of the function of regulatory proteins at molecular level is of great interest. It would potentially enhance the yield of secondary metabolites through either inactivation of repressors or overexpression of activators and a combinatorial approach of both inactivation and overexpression.

Streptomyces sp. SCC-2136 (ATCC 55186) produces Sch47554 and Sch47555 that exhibit antifungal activity against various yeasts (*Candida albicans*, *C. tropicalis* and *C. stellatoidea*) and dermatophytes (*Trichophyton mentagrophytes*, *T. rubrum*, *T.*

tonsurans and *Microsporum canis*) (27). In addition to reported antifungal activities, Sch47554 and Sch47555 (Fig. 3) might potentially possess antitumor and antimicrobial activities, as well as enzyme inhibitory and agonistic activities due to their structural similarity to other angucycline metabolites (28). The diverse and promising biological activities of angucyclines excite the attentions of researcher to study the biosynthesis of these pharmaceutically important natural products as well as to enhance the production of these compounds.

The initial sequence analysis of the biosynthetic gene cluster (*sch*) for Sch47554 and Sch47555 revealed a total of 55 open reading frames (ORFs) in a region of 77.5 kb (NCBI accession number AJ628018). In our previous work, we proposed the biosynthetic pathway in which minimal PKS enzymes (SchP6, SchP7, and SchP8) can synthesize SEK15 from acetyl-CoA and nine units of malonyl-CoA. The addition of ketoreductase (SchP5), aromatase (SchP4) and cyclase (SchP9) to the mimimal PKS produced UWM6, which was subsequently converted to rabelomycin by an oxygenase (SchP10). Upon another oxygenation step by SchP3, the aglycone backbone was synthesized and further modified by three glycosyltransferases, resulting in the biosynthesis of Sch47554 and Sch47555 (29,30). Additionally, based on the sequence similarity analysis, the *sch* gene cluster contains several ORFs that may produce genes with a potential regulatory function. Two putative regulatory genes, *schA4* and *schA21*, were annotated as the TetR family transcriptional regulators, while *schA16* shared significant similarity to the AraC family transcriptional regulators. In addition to the well-known families of regulatory genes, Basnet et al. predicted the putative functions of *schA25* and *schA27* as repressor-response regulator and transcriptional factor, respectively (29).

With the recent advances in genome sequencing and recombinant DNA technologies, rational strain improvement through metabolic engineering and synthetic biology has become a powerful tool to increase the titers of natural products for industrial purposes. Some of widely-used metabolic engineering techniques for yield improvement are to increase the precursor supply, delete or tune the competing pathways or unwanted by-products, heterologous expression of the entire biosynthetic pathway, overexpression of rate-limiting enzymes, and manipulation of regulatory genes (31,32). Typically, the best yields are achieved through a combination of several approaches. Moreover, the manipulation of regulatory genes through overexpression of activator genes and/or deletion of repressor genes has been successfully performed in order for the improved yield of natural products (33). For instance, overexpression of a SARP from the mithramycin biosynthetic gene cluster in *S. argillaceus* led to a 16-fold increase in the mithramycin titer (34). In addition, the inactivation of a GntR family transcriptional repressor increased the yield of platensimycin and platencin by 100-fold compared to the wild type strain (35). The aim of the present study was to identify the functional roles of the TetR and AraC family transcriptional regulators in the *sch* gene cluster in *Streptomyces* sp. SCC-2136 for the improved production of the major antifungal compound Sch47554. The results presented in this study indicate that SchA4 and SchA16 are repressors, while SchA21 is an activator based on the inactivation and overexpression of corresponding regulatory genes. Manipulation of these regulatory genes significantly increased the production of Sch47554. This study paves the way for further engineering of this producing strain for enhanced production of these medicinally important angucyclines.

MATERIALS AND METHODS

Plasmids, strains, media and growth conditions

Plasmids and bacterial strains used in this study are listed in Table 2. *Streptomyces* sp. SCC-2136 was used as the parent strain for gene disruption and overexpression of regulatory proteins. *Streptomyces* sp. SCC-2136 was grown in YM broth (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose) for 5 days for genomic DNA extraction. *Streptomyces* sp. SCC-2136 and its derivative strains were grown at 28 °C for 10 days on YM agar plates (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose, and 20 g/L agar) for product analysis. MS (20 g/L mannitol, 20 g/L soy flour, 20 g/L agar and 10 mM $MgCl_2$) and ISP4 (Difco^{TM}) media were used for conjugation and single crossover homologous recombination, respectively. *Escherichia coli* XL1-Blue was used as the cloning host for plasmid construction and amplification. *E. coli* ET12567 was used for the introduction of gene knockout and overexpression plasmids into *Streptomyces* sp. SCC-2136 through intergenic conjugation. *E. coli* strains were routinely grown in LB medium at 37 °C. Apramycin (50 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), ampicillin (50 μg/mL) and nalidixic acid (25 μg/mL) were supplemented when appropriate.

Standard molecular biology protocols were performed as previously described (38). Genomic DNA samples of *Streptomyces* sp. SCC-2136 and its derivative strains were extracted with a ZR Fungal/Bacterial DNA Miniprep Kit (Irvine, CA, USA). Plasmid DNA extraction from *E. coli* cells was performed using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Thermo Scientific). PCR reactions were performed with

an Arktik™ Thermal Cycler (Thermo Scientific) using Phusion DNA polymerase (Thermo Scientific). Oligonucleotide primers were ordered from Sigma-Aldrich and dissolved in TE buffer to the concentration of 100 ng/mL. Restriction enzymes used in this study were purchased from New England BioLabs.

TABLE 2 Bacterial strains and plasmids used in Chapter 3.

Strains	**Description**	**Source**
E. coli XL1-Blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*	Agilent Technologies
E. coli ET12567 (pUZ8002)	F- dam-13::Tn9 dcm-6 hsdM hsdR zjj-202::Tn10 recF143 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44	(30)
Streptomyces sp. SCC-2136/WT	*Streptomyces sp*. SCC-2136 wild type strain	ATTC 55186
Streptomyces sp. SCC-2136/Δ*schA4*	*Streptomyces sp.* SCC-2136 *schA4* knockout strain	This study
Streptomyces sp. SCC-2136/Δ*schA16*	*Streptomyces sp.* SCC-2136 *schA16* knockout strain	This study
Streptomyces sp. SCC-2136/Δ*schA21*	*Streptomyces sp.* SCC-2136 *schA21* knockout strain	This study
Streptomyces sp. SCC-2136/OE-SchA4	*Streptomyces sp.* SCC-2136 *schA4* overexpression strain	This study
Streptomyces sp. SCC-2136/OE-SchA16	*Streptomyces sp.* SCC-2136 *schA16* overexpression strain	This study
Streptomyces sp. SCC-2136/OE-SchA21	*Streptomyces sp.* SCC-2136 *schA21* overexpression strain	This study
Plasmids	**Description**	**Source**
pJET1.2	Cloning vector	Thermo Fisher
pKC1139	*E.coli-Streptomyces* shuttle plasmid contains a *Streptomyces* temperature-	(36)

	sensitive origin of replication	
pSET152	ϕC31 int + attP integrative expression plasmid	(37)
pRY15	Partial fragment of *schA4* gene in pJET1.2	This study
pRY16	Partial fragment of *schA16* gene in pJET1.2	This study
pRY17	Partial fragment of *schA21* gene in pJET1.2	This study
pRY19	Partial fragment of *schA4* gene (from pRY15) in pKC1139	This study
pRY20	Partial fragment of *schA16* gene (from pRY16) in pKC1139	This study
pRY21	Partial fragment of *schA21* gene (from pRY17) in pKC1139	This study
pOKF7	*schA4* gene in pJET1.2	This study
pOKF8	*schA16* gene in pJET1.2	This study
pOKF9	*schA21* gene in pJET1.2	This study
pOKF10	*schA4* gene (from pOKF8) in pSET152	This study
pOKF11	*schA16* gene (from pOKF9) in pSET152	This study
pOKF12	*schA21* gene (from pOKF10) in pSET152	This study

Plasmid construction for targeted gene disruption and overexpression of regulatory genes

Targeted gene disruption plasmids were constructed based on the temperature-sensitive vector pKC1139 (36). Firstly, we PCR-amplified 456 bp, 538 bp, and 508 bp fragments of *schA4*, *schA16* and *schA21*, respectively, from the genomic DNA of

Streptomyces sp. SCC-2136 using the primers listed in Table 3. These PCR products were ligated into the pJET1.2 cloning vector to yield pRY15, pRY16 and pRY17 (Table 2), respectively. The plasmids were sent out for DNA sequencing using Sanger's method (Eton Bioscience). Subsequently, the partial DNA fragments of *schA4*, *schA16*, and *schA21* genes were excised from pRY15, pRY16 and pRY17 using XbaI and HindIII, and then ligated into pKC1139 between the same sites to yield pRY19, pRY20 and pRY21 (Table 2), respectively. These plasmids were subsequently used for targeted gene disruption of *schA4, schA16* and *schA21* in *Streptomyces* sp. SCC-2136 through intergenic conjugation using *E. coli* ET12567. Positive exconjugants for *Streptomyces* sp. SCC-2136/Δ*schA4*, Δ*schA16* and Δ*schA21* mutants were transferred to ISP4 plates supplemented with apramycin and incubated at 37 °C for about 10 days to allow the plasmids to integrate into the genome and yield the single-crossover mutants. The positive mutants were shuttled back and forth to YM and ISP4 plates supplemented with apramycin for two generations to guarantee the positive mutants. Positive exconjugants after genome integration were subjected to product analysis and PCR confirmation of gene knockouts using primers listed in Table 3. The details of intergenic conjugation protocols were described in the literature (39).

For overexpression of regulatory genes, an integrative expression plasmid for *Streptomyces*, pSET152, was used (37). We firstly amplified *schA4*, *schA16* and *schA21* genes by PCR from the genomic DNA of *Streptomyces* sp. SCC-2136 using the primers listed in Table 3. These PCR products were inserted into pJET1.2 cloning vector to yield pOKF7, pOKF8 and pOKF9 (Table 2), respectively. After sequencing, each regulatory gene was excised from pOKF7, pOKF8 and pOKF9 with XbaI and subsequently ligated

into pSET152 using the same site, yielding pOKF10, pOKF11 and pOKF12 (Table 2), respectively. The direction of the genes was double-checked using the NdeI restriction site, which was designed in the primers for direction check. These plasmids were transformed into *Streptomyces* sp. SCC-2136 through intergenic conjugation using *E. coli* ET12567. Positive exconjugants were streaked on YM plates supplemented with apramycin for product analysis.

TABLE 3 Oligonucleotides used in Chapter 3.

Primers	**Oligonucleotides**
schA4-SCKO-F-HindIII	aaAAGCTTacgtcgccacgcacgagttc
schA4-SCKO-R-XbaI	aaTCTAGAggaagaagcagaacgagctgat
schA16-SCKO-F-HindIII	aaAAGCTTggcacgcgaacgaggcgatc
schA16-SCKO-R-XbaI	aaTCTAGAtcggcgtacgcgctgacgct
schA21-SCKO-F-HindIII	aaAAGCTTaacgcctcaaccgccccgca
schA21-SCKO-R-XbaI	aaTCTAGAttcgcgctggaccagccctg
OE-schA4-F-XbaI-NdeI	aaTCTAGAgggagccCATATGtcagtccgccgagcggtcc
OE-schA4-R-XbaI	aaTCTAGAatgaccagcgtcgaagaaccgg
OE-schA16-F-XbaI-NdeI	aaTCTAGAggaggagccCATATGgcggacataagcaccca
OE-schA16-R-XbaI	aaTCTAGAtcagccctgaacgccgccgg
OE-schA21-F-XbaI-NdeI	aaTCTAGAgggagccCATATGtcagtccgccgagcggtcc
OE-schA21-R-XbaI	aaTCTAGAatggccactcggagaaaagaggagaagc
SchA4-RY19-check-F	aaatgacgtagccgacgacg
SchA4-RY19-check-R	aagtgacatcggtgttgtgc
SchA16-RY20-check-F	aacagacggtgtggtcgaag
SchA16-RY20-check-R	aaagaccacgaccgactctg
SchA21-RY21-check-F	aagtgagccagtcgcagtgc
SchA21-RY21-check-R	aacagtcgatcgcgttcttcag

Extraction, purification and product analysis

Streptomyces sp. SCC-2136 and engineered strains were grown for 10 days at 28 ºC on YM agar plates supplemented with apramycin. The plates were chopped and extracted with a mixture of solvents consisting of 89% ethyl acetate, 10% methanol, and 1% acetic acid (v/v). The resulting extracts were dried *in vacuo*, and the residues were redissolved in methanol for HPLC analysis. We purified the major compound Sch47554 by HPLC with an Agilent Eclipse XDB-C18 column (5 μm, 250 mm × 4.6 mm) to prepare a standard curve for the product analysis of engineered strains. This purified compound was characterized by LC-MS and NMR (Figs. C1 and C2). Low-resolution and high-resolution ESI-MS spectra were obtained on Agilent 6130 and 6210 LC-MS, respectively. Proton NMR was recorded on a Bruker AvanceIII HD Ascend-500 NMR instrument.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, J = 7.8 Hz, 1H), 7.64 (d, J = 7.8 Hz, 1H), 6.94 (d, J = 9.8 Hz, 1H), 6.85 (dd, J = 10.2, 3.5 Hz, 1H), 6.71 (dd, J = 10.2, 3.5 Hz, 1H), 6.43 (d, J = 9.8 Hz, 1H), 6.13 (d, J = 10.2 Hz, 1H), 6.10 (d, J = 10.2 Hz, 1H), 5.61 (d, J = 3.4 Hz, 1H), 5.35 (d, J = 3.5 Hz, 1H), 4.83 (d, J = 10.0 Hz, 1H), 4.76 (q, J = 6.7 Hz, 1H), 4.62 (q, J = 6.8 Hz, 1H), 3.58 (dd, J = 9.1, 6.1 Hz, 1H), 3.43 (ddd, J = 14.5, 10.1, 4.8 Hz, 2H), 3.25 (dd, J = 13.4, 3.0 Hz, 1H), 2.56 (d, J = 13.3 Hz, 1H), 2.49 (dd, J = 15.6, 2.9 Hz, 1H), 2.40 – 2.31 (m, 2H), 2.30 – 2.24 (m, 1H), 1.90 – 1.85 (m, 1H), 1.83 (d, J = 15.5 Hz, 1H), 1.49 (s, 3H), 1.46 (d, J = 6.7 Hz, 3H), 1.41 (d, J = 6.8 Hz, 3H), 1.39 (d, J = 6.1 Hz, 3H).

The titers of Sch47554 for all strains were calculated using the standard curve prepared with the purified compound. Three replicates were analyzed for production

formation for all strains. Depending on the production level, the crude extracts of all strains were diluted with different ratios in order to be in the range of the standard curve and then analyzed by HPLC.

RESULTS AND DISCUSSION

Actinomycetes have possessed a huge potential to produce novel, pharmaceutically useful secondary metabolites with a wide variety of biological activities (40). Particularly, Streptomycetes have been recognized as one of the richest sources of biologically active secondary metabolites (41). However, the production titers of those important compounds are often low, and sometimes it is too low to detect the products with current analytical techniques (42,43). To expand Nature's chemical repertoire and to increase the titers of known natural products, researchers have come up with various solutions: heterologous expression, co-cultivation, fermentation engineering and strain engineering (8). In particular, strain engineering can be conducted through both metabolic engineering of rate-limiting and/or competing pathways, and engineering regulatory networks of secondary metabolites in native strains in which secondary metabolite biosynthesis is tightly regulated by global and/or pathway-specific regulatory proteins (40,42,44-47). In this study, we investigated the roles of three regulatory genes in the *sch* biosynthetic gene cluster with the purpose of enhancing the production of Sch47554 in *Streptomyces* sp. SCC-2136.

Sequence analysis of three putative regulatory proteins in the *sch* biosynthetic gene cluster

BLAST analysis revealed that SchA4 and SchA21 are putative TetR family

transcriptional factors, while SchA16 is a putative AraC family transcriptional factor. SchA4 and SchA21 exhibit high sequence homology to LanK (72% and 76%, respectively) and SimR (80% and 63%, respectively), both of which are regulatory proteins in the biosynthetic gene clusters of landomycin A and simocyclinone (48-50). SchA16 is highly similar to some of the characterized AraC family transcriptional factors such as AdpA (78%), RapG (72%), NanR4 (75%) and SAV742 (78%) (24-26,51). We also performed a multiple amino acid sequence alignment for those regulatory genes against some of the characterized regulatory proteins from various *Streptomyces* strains in order to identify the conserved domains (Fig. 8). The overall conserved structure of the TetR family transcriptional factors consists of nine α helices, and the DNA-binding domain is composed of helices 1 to 3 (52). Both SchA4 and SchA21 consist of conserved DNA-binding domains similar to other reference TetR family transcriptional factors (Figs. 8A and 8B). On the other hand, the AraC family transcriptional factors (Fig. 8C) possess a C-terminal conserved helix-turn-helix (HTH) DNA-binding domain with the consensus of 15 amino acid residues (AxxxxxSxxxLxxxFxxxxGxxxxxxxxxxRxxx AxxxLxxxxxxxxxI/VxI/VxxxxG(F/K)xxxxxxFxxxF(R/K)xxxxGxP, where x is any amino acid) (53). SchA16 has high similarity to this consensus sequence. All in all, BLAST and Clustal Omega multiple sequence alignments for these regulatory proteins indicated that SchA4 and SchA16 belong to the TetR family, while SchA16 is a member of the AraC family transcriptional factors.

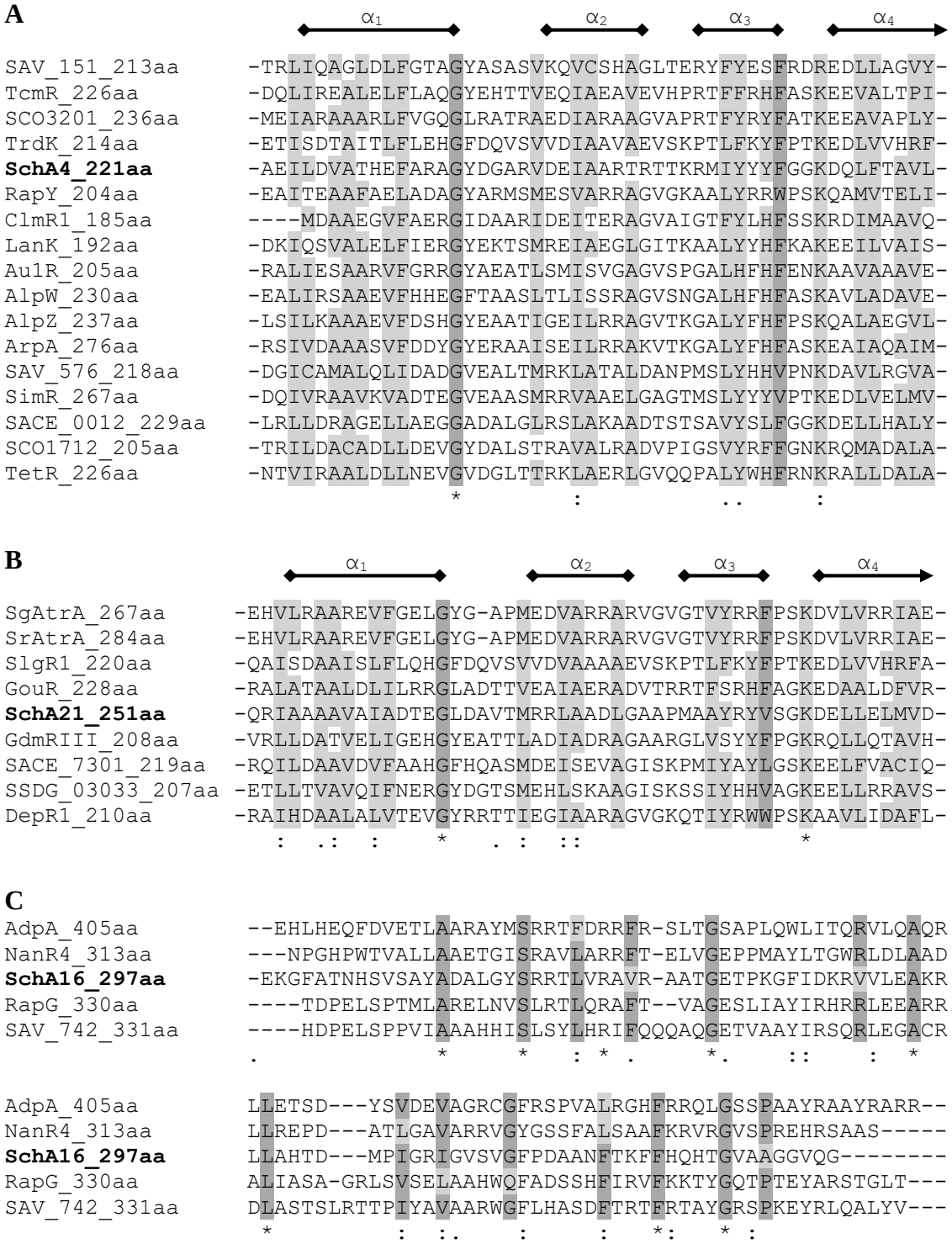

FIG. 8. Multiple amino acid sequence alignment for SchA4 (A), SchA21 (B), and

SchA16 (C) with respect to the characterized TetR- and AraC-family transcriptional factors, respectively. SchA4 and SchA21 consist of the conserved DNA-binding domain of the TetR regulators, while SchA16 has the C-terminal conserved HTH DNA-binding domain. Asterisk (*) indicates positions which have a single, fully conserved residue. Colon (:) indicates conservation between groups of strongly similar properties. Period (.) indicates conservation between groups of weekly similar properties. GenBank accession numbers are: WP_010981587 (SAV_151), WP_052413891 (TcmR), WP_011028825 (SCO3201), WP_108908224 (TdrK), CAH10095 (SchA4), CAA60451 (RapY), CCC55902 (ClmR1), AAD13556 (LanK), WP_030240800 (Au1R), WP_053125906 (AlpW), WP_053125912 (AlpZ), Q9ZN78 (ArpA), WP_010982014 (SAV_576), AAK06798 (SimR), CAL99365 (SACE_0012), NP_733542 (SCO1712), WP_004193135 (TetR), BAG20734 (SgAtrA), EFE75762 (SrAtrA), CBA11576 (SlgR1), AFU52889 (GouR), CAH10121 (SchA21), WP_069862081 (GdmRIII), WP_009951472 (SACE_7301), EDY64415 (SSDG_03033), WP_006124065 (DepR1), WP_003968987 (AdpA), AAP42877 (NanR4), CAH10107 (SchA16), CAA60472 (RapG), and BAC68452 (SAV_742).

Characterization of SchA4 as a repressor and enhanced production of Sch47554 by disrupting *schA4*

Based on the multiple amino acid sequence alignment and analysis of the conserved domains, SchA4 was predicted to be a TetR family transcriptional factor. To characterize the role of *schA4*, we first disrupted this gene on the genome of *Streptomyces* sp. SCC-2136. A single crossover approach was used (Fig. 9A). Apramycin

resistant exconjugants were obtained from intergenic conjugation between *Streptomyces* sp. SCC-2136 and *E. coli* ET12567/pRY19. The correct mutant was verified by PCR using vector- (RV-M and M13-47) and genome-specific primers (Fig. 9A). As illustrated in Fig. 9B, the 1.50 kb and 1.55 kb PCR products were amplified from the genome of *Streptomyces* sp. SCC-2136/Δ*schA4*, whereas the wild type genome did not yield these fragments. This indicated that the *schA4* gene was successfully disrupted.

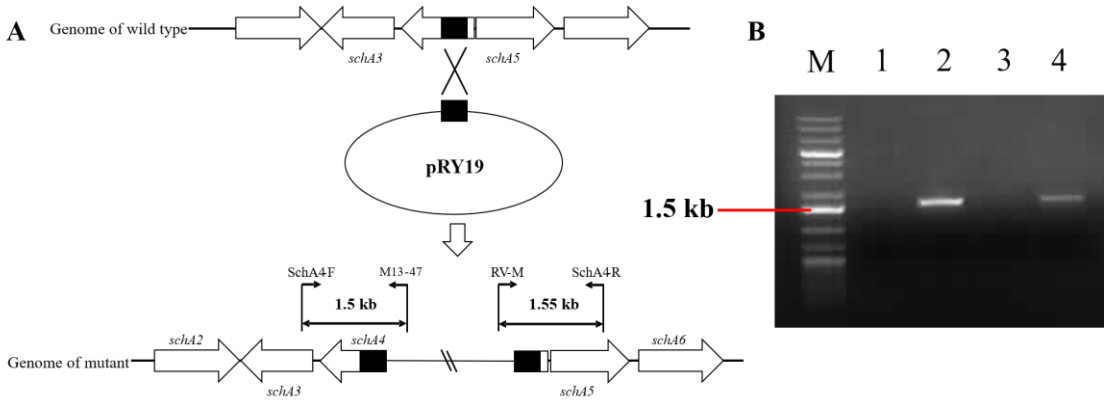

FIG. 9. Illustration of the single crossover recombination strategy for disruption for *schA4* (A) and PCR confirmation of gene disruption (B). M: DNA ladder. 1: Genomic DNA of wild type *Streptomyces* sp. SCC-2136 as template with the forward check primer and M13-47 universal primer. 2: Genomic DNA of *Streptomyces* sp. SCC-2136 mutant strains with the forward check primer and M13-47 universal primer. 3: Genomic DNA of wild type *Streptomyces* sp. SCC-2136 as template with the RV-M universal primer and reverse check primer. 4: Genomic DNA of *Streptomyces* sp. SCC-2136 mutant strains with the RV-M universal primer and reverse check primer.

Upon confirmation of gene inactivation, the positive exconjugants were grown on YM plates supplemented with apramycin for product analysis. As shown in Fig. 10A, *Streptomyces* sp. SCC-2136/Δ*schA4* showed the same product profile as the wild type. The major peak was isolated and characterized as Sch47554 by a comparison of its MS and NMR data (Figs. C1 and C2) with those reported. However, the titer of Sch47554 in *Streptomyces* sp. SCC-2136/Δ*schA4* reached 27.94 mg/L, which is significantly higher than the wild type (6.72 mg/L) (Fig. 10B). This result suggests that SchA4 acts as a repressor, similar to most regulatory proteins in the TetR family (17,18,54-56). For instance, SAV576 negatively controls the biosynthesis of avermectin in *S. avermetilis* (18). Similarly, AlpZ is another TetR family transcriptional factor with a repressor role in the biosynthesis of angucyclinone antibiotic alpomycin in *S. ambofaciens* (17).

To ensure that the above result was not due to a polar effect caused by insertion of the disruption plasmid into the gene cluster (Fig. 9A), we next overexpressed *schA4* in *Streptomyces* sp. SCC-2136. Overexpression approach has been used to investigate the function of regulatory proteins. For instance, Yoo et al. overexpressed three regulatory genes (*rapY, rapR* and *rapS*) using the pSET152 integrative expression plasmid to understand the function of regulatory elements in rapamycin biosynthesis (56). Kuščer et al. also exploited a pSET152-based integrative expression system to overexpress regulatory proteins in rapamycin-producing wild type strain, which led to an increase in the titer of rapamycin (24). Thus, we used the pSET152 integrative expression system to overexpress the corresponding regulatory genes. First, the *schA4* gene was ligated into pSET152 to yield pOKF10, which was subsequently introduced into *Streptomyces* sp. SCC-2136 through intergenic conjugation. The resulting strain was grown on YM plates

supplemented with apramycin for product analysis. As shown in Fig. 11A, *Streptomyces* sp. SCC-2136/OE-*schA4* produced the same metabolites as the wild type. The titer of Sch47554 in this strain was determined to be 2.42 mg/L (Fig. 11B), which is much lower than the wild type. This result further supports that SchA4 is a repressor for the biosynthetic pathway of Sch47554. Enhanced expression of SchA4 would have down-regulated the pathway, resulting in a lower production titer of the target compound.

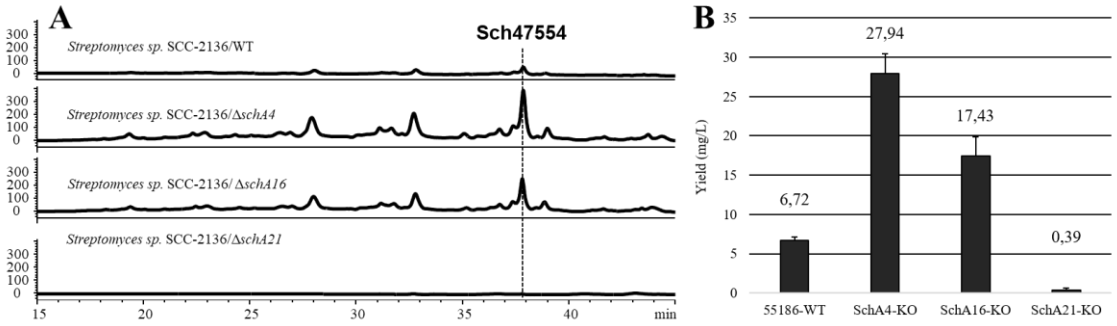

FIG. 10. Effects of disruption of *schA4*, *schA16* and *schA21* on the production of Sch47554. (A) HPLC analysis (420 nm) of the extracts of *Streptomyces* sp. SCC-2136 wild type and mutant strains for *schA4*, *schA16* and *schA21*. The peak at 37.9 min is the antifungal compound of Sch47554. (B) A comparison of the titers of Sch47554 in wild type *Streptomyces* sp. SCC-2136 with those in the mutants with disrupted *schA4*, *schA16* or *schA21*. 55186-WT: *Streptomyces* sp. SCC-2138 wild type strain, SchA4-KO: *Streptomyces* sp. SCC-2136/Δs*chA4*, SchA16-KO: *Streptomyces* sp. SCC-2136/Δ*schA16*, and SchA21-KO: *Streptomyces* sp. SCC-2136/Δ*schA21*. The average titers are shown at the top of the bars.

Identification of SchA16 as an AraC-family repressor and enhanced production of Sch47554 by disrupting *schA16*

The multiple amino acid sequence alignment predicted that SchA16 is an AraC family transcriptional factor. Similar to other characterized AraC family regulators, SchA16 has 15 amino acid consensuses for the conserved DNA-binding domain. To understand its role in the biosynthesis of Sch47554, we used a similar approach to disrupt this gene. The correct exconjugant was selected by apramycin resistance and verified using PCR. Two fragments, 1.50 kb and 1.40 kb, respectively, were amplified from the genome of *Streptomyces* sp. SCC-2136/Δ*schA16* using genome- and vector-specific primers, while the wild type gave no PCR products using the same primers (Fig. C3A). Production of Sch47554 by this mutant was confirmed by HPLC (Fig. 10A) and the titer of this compound was determined to be 17.43 mg/L (Fig. 10B). This represents a nearly 200% increase compared to the wild type strain, suggesting that SchA16 plays a role of repressor in the biosynthesis of Sch47554.

We then amplified the *schA16* gene and ligated it into pSET152 to yield the overexpression plasmid pOKF11. This plasmid was introduced into *Streptomyces* sp. SCC-2136 to overexpress SchA16. Although this engineered strain produced Sch47554 (Fig. 11A), the titer of this compound was only 1.78 mg/L (Fig. 11B), which is much lower than the wild type. This result was consistent with that from the disruption of *schA16*. In general, the AraC family regulators are considered as activators with some exceptions (11). For instance, NanR4 and RapG act as activator in the biosynthesis of nanchangmycin and rapamycin, respectively (24,26). However, SAV742 is a repressor in the biosynthesis of avermectin in *S. avermitilis* and its deletion increased the yield of

avermectin (25), same as what we observed in the disruption of *schA16*. Similar to the TetR family regulators, the AraC family regulators could exhibit both positive and negative roles in the secondary metabolite production. Our results support that SchA16 is a repressor in the biosynthetic pathway of Sch47554 and the disruption of this gene can be an effective approach for improving the production of angucyclines in this strain.

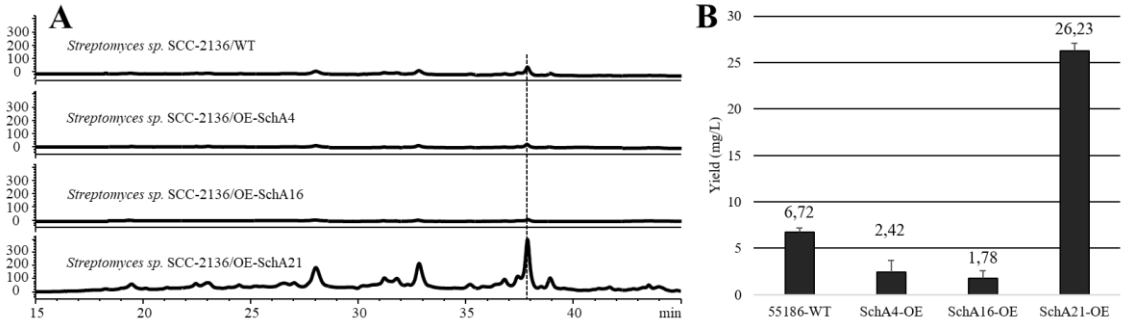

FIG. 11. Effects of overexpression of *schA4*, *schA16* and *schA21* on the production of Sch47554. (A) HPLC analysis (420 nm) of the extracts of *Streptomyces* sp. SCC-2136 wild type and overexpression strains for *schA4*, *schA16* and *schA21*, respectively. (B) A comparison of the titers of Sch47554 in wild type *Streptomyces* sp. SCC-2136 with those in the engineered strains overexpressing *schA4*, *schA16* or *schA21*. 55186-WT: wild type strain; SchA4-OE: *Streptomyces* sp. SCC-2136-SchA4 overexpression strain; SchA16-OE: *Streptomyces* sp. SCC-2136-SchA16 overexpression strain; SchA21-OE: *Streptomyces* sp. SCC-2136-SchA21 overexpression strain. The average titers are shown at the top of the bars.

Characterization of SchA21 as a TetR family activator and enhanced production of Sch47554 by overexpressing *schA21*

BLAST analysis showed that SchA21 also belongs to the TetR family of transcriptional factors. To under its role in the biosynthesis of Sch47554, we disrupted this gene through the single crossover homologous recombination approach shown in Fig. 9A. The genome of the resulting mutant was extracted and subjected to PCR verification. Using the genome- and vector-specific primers, we were able to amplify the 1.40 and 1.25 kb fragments from the genome of *Streptomyces* sp. SCC-2136/Δ*schA21*, but none from the wild type (Fig. C3B), confirming that this strain is the correct mutant. Product analysis showed that this mutant did produce the same products as the wild type (Fig. 10A), but Sch47554 was only produced at a very low level (0.39 mg/L, Fig. 10B), indicating that disruption of SchA21 has significantly repressed the biosynthetic pathway of Sch47554. This gene was then ligated into pSET152 to yield the overexpression plasmid pOKF12. Overexpression of this gene in *Streptomyces* sp. SCC-2136 led to significantly enhanced production of Sch47554, with a titer of 26.23 mg/L, as shown in Figs. 11A and 11B. The disruption and overexpression strains for SchA4 and SchA21 led to the opposite results even though both regulatory proteins are in the same family. The titer of Sch47554 increased significantly in the knockout strain for SchA4 by approximately 4-fold, whereas it was dramatically decreased when *schA21* was disrupted (Fig. 10B). The overexpression strains for these TetR family transcriptional factors produced consistent results with the gene-disrupted strains. Therefore, it can be concluded that SchA21 serves as an activator of the Sch47554 biosynthetic pathway. Although most known TetR family transcriptional factors are repressors, there are some

TetR family transcriptional regulators that act as activators in the biosynthesis of secondary metabolites. As an example, SAV3818 cloned from high avermectin-producing *S. avermitilis* ATCC31780 was overexpressed in the low avermectin-producing *S. avermitilis* ATCC 31267, showing a stimulatory effect in the low avermectin-producing strain. In addition, the overexpression of SAV3818 stimulated actinorhodin production in both *S. coelicolor* M145 and *S. lividans* TK21, implying that this TetR-family transcriptional regulator might be a global up-regulator acting in antibiotic production in *Streptomyces* species (20). Another example is GdmRIII, which not only plays a positive regulatory role in the biosynthesis of geldanamycin, but also plays a negative role in elaiophylin biosynthesis in *S. autolyticus* CGMCC0516 (22). We successfully increased the titer of Sch47554 through the overexpression of the TetR family activator, SchA21.

CONCLUSIONS

In conclusion, the roles of three putative regulatory genes in the *sch* biosynthetic gene cluster were investigated for enhanced production of a pharmaceutically important angucycline. Gene disruption of *schA4* and *schA16* led to a significant increase in the production of angucyclines. In contrast, disruption of *schA21* dramatically decreased the yield of Sch47554. The overexpression strains produced opposite results. Therefore, SchA4 and SchA16 were identified as repressors, while SchA21 acts as an activator. Our results demonstrated that both disruption of pathway repressors and overexpression of activators are effective ways to improve the production of target compounds in the producing strains. This work provides an initial understanding of regulatory elements in

the biosynthesis of Sch47554, which will facilitate further engineering of this producing strain for the efficient production of these medicinally important angucyclines.

ACKNOWLEDGMENTS

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CHAPTER IV DISCOVERY AND ENGINEERING OF AN ENDOPHYTIC *PSEUDOMONAS* STRAIN FROM *TAXUS CHINENSIS* FOR EFFICIENT PRODUCTION OF ZEAXANTHIN DIGLUCOSIDE[‡]

Abstract

Endophytic microorganisms are a rich source of bioactive natural products. They are considered as promising biofertilizers and biocontrol agents due to their growth-promoting interactions with the host plants and their bioactive secondary metabolites that can help manage the plant pathogens. In this study, I isolated an endophytic bacterium from the leaves of *Taxus chinensis*, which was identified as *Pseudomonas* sp. 102515 based on the 16S rRNA gene sequence. Analysis of its secondary metabolites revealed that this endophytic strain produces zeaxanthin diglucoside as a major product, a promising antioxidant natural product that belongs to the family of carotenoids. A carotenoid (*Pscrt*) biosynthetic gene cluster was amplified from this strain, and the functions of PsCrtI and PsCrtY in the biosynthesis of zeaxanthin diglucoside were confirmed in *Escherichia coli* BL21(DE3). The entire *Pscrt* biosynthetic gene cluster was successfully reconstituted in *E. coli* BL21(DE3) and *Pseudomonas putida* KT2440. The engineered strain of *P. putida* KT2440 produced zeaxanthin diglucoside at 144 ± 4 mg/L in SOC medium supplemented with 0.5% glycerol at 23 °C, while the titer of zeaxanthin diglucoside in *E. coli* BL21(DE3) was only 2 mg/L. The production of zeaxanthin diglucoside in *Pseudomonas* sp. 102515 was improved through the optimization of

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fermentation conditions such as medium, cultivation temperature and cultivation time. The highest titer under the optimized conditions reached 206 mg/L. The engineered strain of *P. putida* KT2440 produced zeaxanthin diglucoside at 121 mg/L in SOC medium supplemented with 0.5% glycerol at 18 °C, while the yield of zeaxanthin diglucoside in *E. coli* BL21(DE3) was only 2 mg/L. To further enhance the production, I introduced an expression plasmid harboring the *Pscrt* biosynthetic gene cluster into *Pseudomonas* sp. 102515. The titer in this engineered strain reached 380 mg/L, 85% higher than the wild type. Through PCR, I also discovered the presence of a turnerbactin biosynthetic gene cluster in *Pseudomonas* sp. 102515. Turnerbactin was reported to be involved in nitrogen fixation, suggesting that this endophytic strain might have a role of promoting growth of the host plant. Therefore, in addition to producing zeaxanthin diglucoside, *Pseudomonas* sp. 102515 might also be utilized as a plant-promoting strain for agricultural applications.

INTRODUCTION

Nature provides a huge repertoire of bioactive molecules from various living organisms. As such, natural products are a major source of new drugs. Microorganisms are known to produce many pharmaceuticals such as lovastatin (anti-cholesterol), penicillin (antibacterial) and vancomycin (antibacterial). There is an estimated more than 99 per cent of microbial species left undiscovered in the world (1). Therefore, microorganisms remain an underexplored source of bioactive natural products. In particular, endophytes hold a huge potential for the discovery of natural products with pharmaceutical importance (2). Endophytes, microorganisms (bacteria and fungi) that live in the tissues of living plants without causing any apparent disease symptoms in the

host, are potential sources of novel natural products with applications in medicine, agriculture, and industry (3,4). They have been isolated from many important medicinal plants, weeds, and ornamental and fruit trees (2). Endophytes enter plants through the roots and the aerial portions of plants, such as leaves, flowers, stems and cotyledons (5). Upon entering the host, they reside within cells or the intercellular spaces or the vascular system (6). Some of the endophytes can exhibit plant growth-promoting effects and protect plants from biotic and abiotic stresses under different environmental conditions. Thus, they are considered as endosymbiotic microorganisms with potential agricultural applications such as biofertilizers and biocontrol agents (6-9).

Another important aspect is that the endophytes are known to produce a wide variety of natural products. For example, ecomycins produced by *Pseudomonas viridiflava*, are a family of novel lipopeptides and consist of some unusual amino acids including homoserine and β-hydroxy aspartic acid (10). Another example is pseudomycins, which represent a group of peptide antifungal compounds isolated from the plant-associated bacterium *Pseudomonas syringae* (11). New microbial species can sometimes lead to the rediscovery of known natural products as happened in the example of camptothecin, a potent antineoplastic agent. Camptothecin was originally isolated from the wood of *Camptotheca acuminate*. Interestingly, several endophytes such as *Entrophospora infrequens* and *Nodulisporium* sp. were reported to produce camptothecin (12), representing an alternative and potential mean for the production of this pharmaceutically important molecules.

Carotenoids are isoprenoid pigments that are seen throughout the nature. They are produced by all known phototrophic organism and some non-phototrophic fungi, bacteria

and archaea (13). Carotenoids exhibit diverse biological functions in different organisms that either produce or consume carotenoids. They serve as accessory pigments in the light harvesting center of phototrophic organisms (14). The primary roles of carotenoids in non-phototrophic organisms are membrane stability and the relief of oxidative stress (15). Additionally, carotenoids have been used in various applications, ranging from food colorants and feed supplements to nutritional and cosmetics purposes (16). This family of natural products has shown a variety of biological activities, such as antioxidant, anticancer, and anti-inflammatory properties. They are also used as precursors for the synthesis of vitamin A and as nutritional factors for the prevention of chronic diseases (17,18). Due to the negative impacts of synthetic coloring agents on human health (e.g., toxicity, hyperallergenicity, and carcinogenicity) and the increase in the consumer demand for natural and health-promoting food ingredients, the utilization of carotenoids as colorants and supplements is rising in the food, cosmetics, nutraceutical and pharmaceutical industries, with an expected global market value of $1.8 billion in 2019 (19,20).

Even though more than 750 different carotenoids were isolated from nature, all carotenoids share a linear, conjugated chromophore backbone and a common early biosynthetic pathway (Fig. 12). Carotenoid biosynthesis typically begins with the isomerization of isopenthyl diphosphate (IPP) from the mevalonate pathway to yield dimethylallyl diphosphate (DMAPP), catalyzed by the IPP isomerase (Idi). Geranyl diphosphate (GPP) is synthesized through the head-to-tail condensation of IPP and DMAPP. Addition of IPP to GPP generates farnesyl pyrophosphate (FPP) formation and a further IPP molecule yields geranylgeranyl diphosphate (GGPP) by geranylgeranyl

diphosphate synthase (CrtE). Phytoene synthase (CrtB) catalyzes the head-to-head condensation of two GGPPs to phytoene. Phytoene desaturase (CrtI) extends the double bond conjugation of phytoene to yield lycopene. Terminal β-cyclization catalyzed by lycopene β-cyclase (CrtY) results in the formation of β-carotene, which is subsequently hydroxylated by β-carotene hydroxylase (CrtZ), yielding zeaxanthin. Zeaxanthin can be further modified by different tailoring enzymes to yield a variety of carotenoids. For example, it can be glycosylated by a glycosyltransferase (CrtX) to generate zeaxanthin diglucoside (21-24).

In this study, my primary goal was to isolate an endophytic bacterium with the purpose of discovering new natural products and/or finding a new effective mean to produce known natural products. I isolated an endophytic *Pseudomonas* strain, named *Pseudomonas* sp. 102515, from the leaves of the yew tree. This bacterium was found to produce zeaxanthin diglucoside as the major product, and a complete carotenoid (*Pscrt*) biosynthetic gene cluster was discovered in *Pseudomonas* sp. 102515. Two biosynthetic genes from this gene cluster, *PscrtI and PscrtY*, were functionally characterized. The entire carotenoid (*Pscrt*) biosynthetic gene cluster was amplified from the endophytic strain and successfully expressed in two heterologous hosts, *E. coli* BL21 (DE3) and *Pseudomonas putida* KT2440. Additionally, the production of zeaxanthin diglucoside in *Pseudomonas* sp. 102515 was significantly improved by optimizing the fermentation conditions, with a titer of 206 ± 6 mg/L. Furthermore, when a plasmid harboring the entire *Pscrt* gene cluster was introduced into *Pseudomonas* sp. 102515, the titer of zeaxanthin diglucoside reached 380 ± 12 mg/L, 85% higher than the wild type strain. This engineered strain represents a promising host for the production of zeaxanthin

diglucoside.

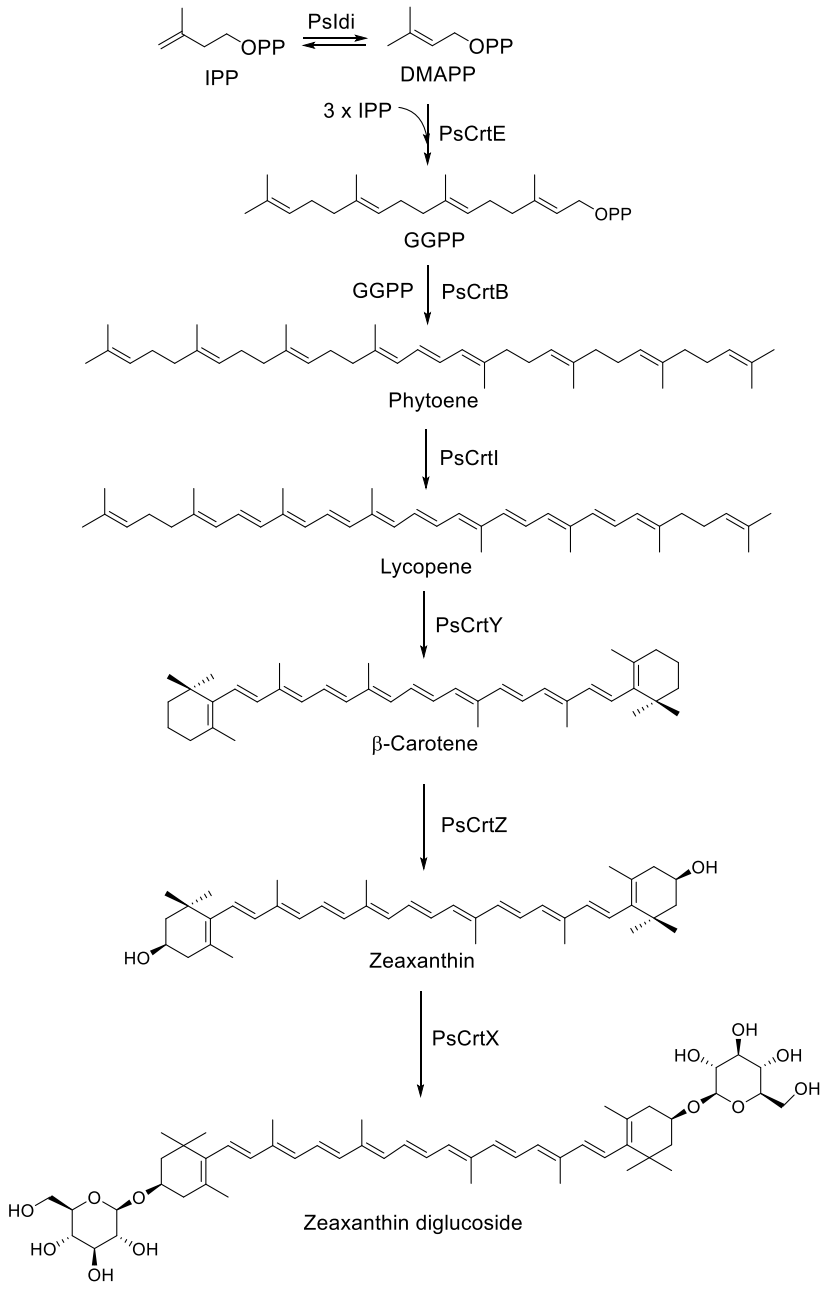

FIG. 12. Proposed biosynthetic pathway of zeaxanthin diglucoside in *Pseudomonas* sp. 102515. The involved enzymes include isopentenyl-diphosphate delta-isomerase (PsIdi), geranylgeranyl pyrophosphate synthetase (PsCrtE), phytoene synthase

(PsCrtB), lycopene synthase (PSCrtI), lycopene cyclase (PsCrtY), β-carotene hydroxylase (PsCrtZ), and zeaxanthin glucosyltransferase (PsCrtX).

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

E. coli XL1-Blue, which was routinely grown in LB (Luria-Bertani) medium at 37 ºC, was used for general genetic manipulations. *Pseudomonas* sp. 102515 was grown in LB overnight for genomic DNA (gDNA) extraction. LB, SOB, SOC, 2×YT, TB, 2×TB and superbroth media (Recipes for media are in Table D1) were tested for the production of zeaxanthin diglucoside by *Pseudomonas* sp. 102515. *E. coli* BL21(DE3) and *P. putida* KT2440 were utilized for heterologous expression and co-expression studies. All wild type and engineered bacterial strains are listed in Table 4. Chloramphenicol (25 μg/mL), ampicillin (50 μg/mL) and kanamycin (50 and 30 μg/mL) were supplemented when appropriate. The concentrations of kanamycin for engineered *E. coli* and *Pseudomonas* strains are 50 and 30 μg/mL, respectively.

Isolation of endophytic bacteria from *Taxus chinensis*

The leaves were collected from a cultivated shrub of *T. chinensis* on the Logan campus of Utah State University (Latitude: 41.7424, Longitude: -111.8080). A voucher specimen is deposited at the Intermountain herbarium, accession number UTC00282046. The collected leaves were thoroughly washed in running water. The disinfection and isolation were performed according to de Oliveira Costa et al. with minor modifications (25). Briefly, the leaves were disinfected superficially through dipping into 70% ethanol

for 2 minutes three times, followed by rinsing with 70% ethanol three times. Then, the leaves were dipped into sterile distilled water for a few minutes and rinsed with sterile distilled water. This process was repeated three times. To confirm the disinfection protocol, aliquots of the sterile water used in the final rinse were plated in LB plates at 28 ºC for 7 days and the plates were examined for the presence or absence of any microbial colonies.

The disinfected leaves were grounded with 6 mL of an aqueous NaCl solution (0.85 %) using a sterile mortar and pestle. The tissue extract was subsequently incubated at 28 ºC for 3 hours to allow the complete release of endophytic microorganisms from the host tissue. For the isolation of endophytic bacteria, the tissue extract was diluted with the NaCl solution and plated on LB plates with different dilutions. The plates were incubated at 28 ºC for around a week. Promising colonies were selected and streaked on fresh LB plates for the isolated bacteria.

TABLE 4 Strains used in Chapter 4.

Strain	**Description**	**Source**
E. coli XL1-Blue	endA1 gyrA96(nal^{R}) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB^{+} lacI^{q} Δ(lacZ)M15] hsdR17(r_{K}^{-} m_{K}^{+})	Agilent Technologies
E. coli BL21(DE3)	BF^{-} ompT gal dcm lon hsdS_{B}($r_{B}^{-}m_{B}^{-}$) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB^{+}]$_{K-12}$(λ^{S})	Novagen
*Pseudomona*s sp. 102515	Isolated endophytic strain from *Taxus chinensis*	This study
Pseudomonas putida KT2440	Heterologous host for expression of the *Pscrt* biosynthetic gene cluster	(81)

E. coli BL21(DE3)/ pAC-PHYTipi	*E. coli* BL21(DE3) expressing the phytoene biosynthetic genes from pAC-PHYTipi (phytoene producing)	This study
E. coli BL21(DE3)/ pAC-PHYTipi+pOKF89	*E. coli* BL21(DE3) expressing the phytoene biosynthetic genes from pAC-PHYTipi with *PscrtI* (lycopene producing)	This study
E. coli BL21(DE3)/ pAC-LYCipi	*E. coli* BL21(DE3) expressing the lycopene biosynthetic genes from pAC-LYCipi (lycopene producing)	This study
E. coli BL21(DE3)/ pAC-LYCipi+pOKF91	*E. coli* BL21(DE3) expressing the lycopene biosynthetic genes from pAC-LYCipi with *PscrtY* (β-carotene producing)	This study
E. coli BL21(DE3)/ pAC-BETAipi	*E. coli* BL21(DE3) expressing the β-carotene biosynthetic genes from pAC-BETAipi (β-carotene producing)	This study
E. coli BL21(DE3)/ pAC-ZEAXipi+pOKF72	*E. coli* BL21(DE3) expressing the β-carotene biosynthetic genes from pAC-ZEAXipi with CrtX (zeaxanthin diglucoside producing)	This study
E. coli BL21(DE3)/ pAC-EHER	*E. coli* BL21(DE3) expressing the zeaxanthin diglucoside biosynthetic genes from pAC-EHER (zeaxanthin diglucoside producing)	This study
E. coli BL21(DE3)/pOKF184	*E. coli* BL21(DE3) expressing the *Pscrt* biosynthetic gene cluster from *Pseudomonas* sp. 102515	This study
Pseudomonas putida KT2440/pOKF192	*Pseudomonas putida* KT2440 expressing the *Pscrt* biosynthetic gene cluster from *Pseudomonas* sp. 102515	This study
Pseudomonas sp. 102515/pOKF192	Engineered *Pseudomonas* sp. 102515 strain with an additional *Pscrt* biosynthetic gene cluster in pMIS1-mva	This study

General genetic manipulation and PCR

Standard molecular biology protocols were performed as previously described (26). Genomic DNA of *Pseudomonas* sp. 102515 was extracted with a ZR Fungal/Bacterial DNA Miniprep Kit (Irvine, CA, USA). Plasmid DNA extraction from *E. coli* cells was performed using a Thermo Scientific GeneJET Plasmid Miniprep Kit (Logan, UT, USA). PCR reactions were performed with an Arktik™ Thermal Cycler (Thermo Scientific) using Phusion DNA polymerase (Thermo Scientific, Logan, UT, USA). Primers were ordered from Thermo Scientific and dissolved in TE buffer to the final concentration of 100 ng/mL.

Identification and phylogenetic analysis of endophytic bacterium

The partial 16S rRNA gene fragment was PCR amplified from the gDNA of *Pseudomonas* sp. 102515 using the 16S universal primers of GGCTACCTTGTTACGACTTC and AGTTTGATCCTGGCTCAG (27). The PCR product was sent out for sequencing by Sanger's method. The 16S rRNA gene sequence was deposited into NCBI Genbank database under the accession number of MK610450 (Sequence D1). BLAST analysis of this 16S rDNA sequence was performed using the 16S ribosomal RNA sequences database on NCBI. Using the 16S rRNA sequences of relative bacteria to our isolated endophytic bacterium from BLAST analysis, I created phylogenetic tree by the neighbor-joining method using an online platform (https://itol.embl.de/) (28). Both BLASTn analysis and phylogenetic tree indicated the bacterial strains closely related to our strain. For physiological characteristics, scanning electron microscopy images were taken after performing the fixation of sample (29).

Amplification and annotation of the *Pscrt* biosynthetic gene cluster

The colonies of *Pseudomonas* sp. 102515 on LB agar plates showed a yellow color. LC-MS analysis of the methanol extract of the cells confirmed that zeaxanthin diglucoside was produced. To discover the carotenoid (*Pscrt*) biosynthetic gene cluster in *Pseudomonas* sp. 102515, I first analyzed the genome of *Pseudomonas psychrotolerans* (GenBank accession number: NZ_CP018758), which is the closest relative of our strain based on the phylogenetic analysis. I used an online genome analysis platform, AntiSMASH, and found a complete *crt* biosynthetic gene cluster in *P. psychrotolerans* responsible for the biosynthesis of zeaxanthin diglucoside. I designed two sets of primers (primers 3-6, Table D2) based on the DNA sequence of this *crt* gene cluster. Briefly, the whole *Pscrt* gene cluster was divided into two fragments, which were PCR amplified from the gDNA of *Pseudomonas* sp. 102515 using the primers (primers 3 and 4 for fragment A, and primers 5 and 6 for fragment B) listed in Table D2. Fragments A and B were first ligated into pJET1.2, yielding pOKF163 and pOKF166 (Table 5), respectively. These two plasmids were sent out for DNA sequencing to obtain the whole DNA sequence of the *Pscrt* gene cluster using the walking primers (primes 7-16) listed in Table D2. The whole DNA sequence of *Pscrt* biosynthetic gene cluster from *Pseudomonas* sp. 102515 was deposited into GenBank under the accession number of MK613929 (Sequence D2).

Construction of plasmids for functional characterization of PsCrtI and PsCrtY

I first constructed a pET28a(+)-based expression plasmid for the carotenoid

biosynthetic genes in *E. coli*. *crtX* (glycosyltransferase) was PCR amplified using the primers 17 and 18 (Table D2) from pAC-EHER, which was a gift from Francis X Cunningham Jr (Addgene plasmid # 53262). PCR product was directly ligated into the cloning vector, pJET1.2, yielding pOKF55 (Table 5), which was sent out for DNA sequencing using Sanger's method (Eton Bioscience, San Diego, CA, USA). *crtX* was excised with NcoI and HindIII from pOKF55 and ligated into pET28a(+) between the same sites to yield pOKF63 (Table 5). pOKF63 and pAC-ZEAXipi (Addgene plasmid # 53287) were introduced into *E. coli* BL21(DE3) for co-expression. The engineered strain was grown in LB medium with 50 μg/mL kanamycin and 25 μg/mL chloramphenicol, and induced with 200 μM of IPTG for product analysis.

To replace the T7 promoter in pET28a(+) with a strong constitutive promoter, J23119, I redesigned the forward primer for *crtX* by including the J23119 promoter and B0034 ribosome binding site before the start codon (Table D2). I re-amplified *crtX* with J23119 and B0034 from pAC-EHER using this new forward primer and previous reverse primer (primers 19 and 20). The new PCR product was ligated into pJET1.2 (pOKF69, Table 5) and the sequence was confirmed by sequencing. Subsequently, *crtX* with J23119 and B0034 was excised from pOKF69 using BglII and HindIII, and then ligated into pET28a(+) between the same sites to yield pOKF72 (Table 5). The coexpression of pOKF72 and pAC-ZEAXipi (Addgene plasmid # 53287) in *E. coli* BL21(DE3) did produce zeaxanthin diglucoside, which was confirmed by HPLC through the comparison of retention time and UV-spectra with *E. coli* BL2(DE3) harboring pAC-EHER. *PscrtI* and *PscrtY* were PCR amplified from the genomic DNA of *Pseudomonas* sp. 102515 using the primers (primers 21 and 22 for *PscrtI*, and primers 23 and 24 for *PscrtY*) listed

in Table D2 and were subsequently ligated into pJET1.2 to yield pOKF85 and pOKF88 (Table 5), respectively. Upon confirmation of sequences by Sanger's method, *PscrtI* and *PscrtY* were transferred into pET28a(+) with J23119 and B0034 using NdeI and HindIII sites to yield pOKF89 and pOKF91 (Table 5), respectively. PsCrtI (pOKF89) and PsCrtY (pOKF91) were separately co-expressed with pAC-PHYTipi (Addgene plasmid #: 53283) and pAC-LYCipi (Addgene plasmid #: 53279) in *E. coli* BL21(DE3), respectively.

TABLE 5 Plasmids used in Chapter 4.

Plasmid	**Description**	**Source**
pJET1.2	Cloning vector	Fermentas
pET28a(+)	*E. coli* expression plasmid, pBR322	Novagen
pACYC184	*E. coli* expression plasmid, p15A	ATCC 37033
pAC-PHYTipi	Phytoene producing plasmid	Addgene plasmid #: 53283
pAC-LYCipi	Lycopene producing plasmid	Addgene plasmid #: 53279
pAC-BETAipi	β-carotene producing plasmid	Addgene plasmid #: 53277
pAC-ZEAXipi	Zeaxanthin producing plasmid	Addgene plasmid # 53287
pAC-EHER	Zeaxanthin diglucoside producing plasmid	Addgene plasmid #: 53262
pMIS1-mva	Expression plasmid for *Pseudomonas putida* KT2440 that contains the mevalonic acid (MVA) pathway genes	(78)
pOKF55	*crtX* gene cloned from pAC-EHER in pJET1.2	This study

pOKF63	*crtX* gene from pOKF55 in pET28a(+)	This study
pOKF69	*crtX* gene with the J23119 constitutive promoter and B0034 ribosome binding site in pJET1.2	This study
pOKF72	*crtX* gene with the J23119 constitutive promoter and B0034 ribosome binding site in pET28a(+)	This study
pOKF85	*PscrtI* gene cloned from *Pseudomonas* sp. 102515 in pJET1.2	This study
pOKF88	*PscrtY* gene cloned from *Pseudomonas* sp. 102515 in pJET1.2	This study
pOKF89	*PscrtI* gene with the J23119 constitutive promoter and B0034 ribosome binding site in pET28a(+)	This study
pOKF91	*PscrtY* gene with the J23119 constitutive promoter and B0034 ribosome binding site in pET28a(+)	This study
pOKF163	The *Pscrt* biosynthetic gene cluster fragment A in pJET1.2	This study
pOKF166	The *Pscrt* biosynthetic gene cluster fragment B in pJET1.2	This study
pOKF169	The *Pscrt* biosynthetic gene cluster in pJET1.2	This study
pOKF173	The *Pscrt* biosynthetic gene cluster in pET28a(+)	This study
pOKF184	The *Pscrt* biosynthetic gene cluster in pACYC184	This study
pOKF187	*tnbA* gene cloned from *Pseudomonas* sp. 102515 in pJET1.2	This study
pOKF192	The *Pscrt* biosynthetic gene cluster in pMIS1-mva	This study

Heterologous expression of the *Pscrt* gene cluster from *Pseudomonas* sp. 102515 in *E. coli* BL21(DE3) and *P. putida* KT2440

For the heterologous expression of the *Pscrt* gene cluster from the endophytic

strain, I first excised fragment A from pOKF163 using NheI and MfeI and ligated it into pOKF166 that contains fragment B between the same sites to yield pOKF169 (Table 5), which harbors the entire *Pscrt* gene cluster in pJET1.2. Due to a high copy number of pJET1.2, I then ligated the *Pscrt* gene cluster excised from pOKF169 using the SpeI and HindIII sites into pET28a(+) digested with NheI and HindIII (pOKF173, Table 5). Since pET28a(+) has a relatively higher copy number, I decided to also ligate the *Pscrt* gene cluster from pOKF169 into pACYC184 between the XbaI and HindIII sites to yield pOKF184 (Table 5). The corresponding expression plasmids including pOKF173 and 184, were transferred into *E. coli* BL21(DE3) for heterologous expression of the *Pscrt* biosynthetic gene cluster.

I also constructed an expression plasmid for expression of the *Pscrt* gene cluster in *P. putida* KT2440. The pMIS1-MVA-GES plasmid was generously provided by Jens Schrader and Josef Altenbuchner at Dechema-Forschungsinstitut and University of Stuttgart, respectively (78). The whole *Pscrt* gene cluster was excised from pOKF169 using the SpeI and PmeI sites and ligated into the pMIS1-MVA-GES plasmid digested with AvrII and PmeI to yield pOKF192 (Table 5). pOKF192 was introduced into *P. putida* KT2440 by electroporation as described in the literature (30). Briefly, *P. putida* KT2440 was grown until the OD_{600} reached 0.4 at 28 °C. The cells were immediately placed on ice and harvested by centrifugation. Then, the cells were washed three times with 300 mM sterile sucrose solution and re-suspended in 100 µL of 300 mM sucrose solution. The cells were mixed with pOKF192 and the mixture was transferred into a pre-chilled electroporation cuvette. After 10 min of incubation on ice, the following settings

were used for electroporation: set voltage - 2.5 kV (12.5 kV/cm); capacitor - 25 μF. After electroporation, 900 μL of LB broth was immediately added to the cuvette. The mixture was then transferred into a culture tube, which was incubated at 28 °C with shaking (250 rpm) for 2 h. Finally, the cells were plated on LB agar plates with 30 μg/mL kanamycin and incubated at 28 °C. The transformants were picked and grown in LB with kanamycin for product analysis. The same electroporation protocol was followed to introduce pOKF192 into *Pseudomonas* sp. 102515.

Discovery of a turnerbactin biosynthetic gene cluster in *Pseudomonas* sp. 102515 by PCR

To find out whether *Pseudomonas* sp. 102515 contains a turnerbactin biosynthetic gene cluster, the *tnbA* gene was cloned from the genomic DNA of *Pseudomonas* sp. 102515 using primers 25 and 26 listed in Table D2. The PCR product was ligated into the pJET1.2 cloning vector to yield pOKF187 (Table 5), which was subsequently sent out for sequencing (Sequence D3).

Optimization of carotenoid production and extraction and HPLC-MS analysis of zeaxanthin diglucoside

For *Pseudomonas* sp. 102515, different culture media were tested to increase the yield of zeaxanthin diglucoside. Upon the determination of a suitable medium, I investigated the effect of glycerol supplementation as an additional carbon source. Cultivation time and temperature were also tested for an improved production of carotenoids. I also analyzed the yield of zeaxanthin diglucoside in heterologous expression systems.

The wild type and engineered strains were typically cultured in 250-mL flasks containing 50 mL of medium. The cultures were centrifuged at 3,500 rpm for 8 min to harvest the cells and the cell pellets were suspended in 50 mL of methanol. The cell suspension in methanol was sonicated for 5 min to extract carotenoids. After centrifugation at 3,500 rpm for 8 min, the resulting extract was dried *in vacuo*, and the residues were redissolved in a DMSO-methanol mixture (10% v/v) for HPLC-MS analysis with a gradient mobile phase of acetonitrile-water from 50 % to 90 % over 45 min at 1 mL/min. Low-resolution ESI-MS spectra were obtained on an Agilent 6130 LC-MS to confirm the molecular weights of carotenoids. For co-expression experiments, I used a different HPLC condition for better separation of phytoene, lycopene and β-carotene. For this HPLC method, I used an isocratic elution system of methanol-tetrahydrofuran (6/4, v/v) over 45 min at 1 mL/min.

Zeaxanthin diglucoside was subsequently purified by HPLC with an Agilent Eclipse XDB-C18 column (5 μm, 250 mm × 4.6 mm) for the standard curve preparation. The standard curve was prepared with methanol as the solvent using UV spectrophotometer at 456 nm to calculate the yields in the optimization experiments. All crude extracts before drying *in vacuo* were directly measured using spectrophotometer to calculate the yield values. All samples were done in three replicates.

RESULTS

Identification and phylogenetic analysis of endophytic bacteria

I isolated an endophytic bacterium from the leaves of *Taxus chinensis*, whose colonies showed a bright yellow color. To identify this strain, I amplified the 16S rRNA

gene fragment by PCR using a set of universal primers. The gene sequence of this fragment was subjected to BLAST analysis, with the 16S ribosomal RNA sequences (bacteria and archaea) as the reference database. The BLASTn analysis revealed that there are three *Pseudomonas* strains with 99 % identity and coverage. Some other *Pseudomonas* strains (*P. stutzeri*, *P. indoloxydans*, *P. luteola,* etc.) also have 99 % coverage but are 96 % identical to our endophytic isolate. Using an online platform (https://itol.embl.de/), I constructed a phylogenetic tree using the neighbor-joining method (Fig. 13A). The phylogenetic tree indicated that *P. psychrotolerans* is the closest relative to our isolate. *P. oryzihabitans* and *P. oleovorans* are also closely related to this endophytic strain. I also looked into the physiology of our isolate under SEM (Fig. 13B) and confirmed that it is a rod-shaped bacterium. Additionally, it is a non-spore-forming and a yellow-pigment-producing bacterium. The closest relative bacterium, *P. psychrotolerans*, was also reported as a yellow-pigmented bacterium (31). All these physiological characteristics and genetic analysis (phylogenetic relationships) indicated that our isolate is a *Pseudomonas* strain, which was named as *Pseudomonas* sp. 102515.

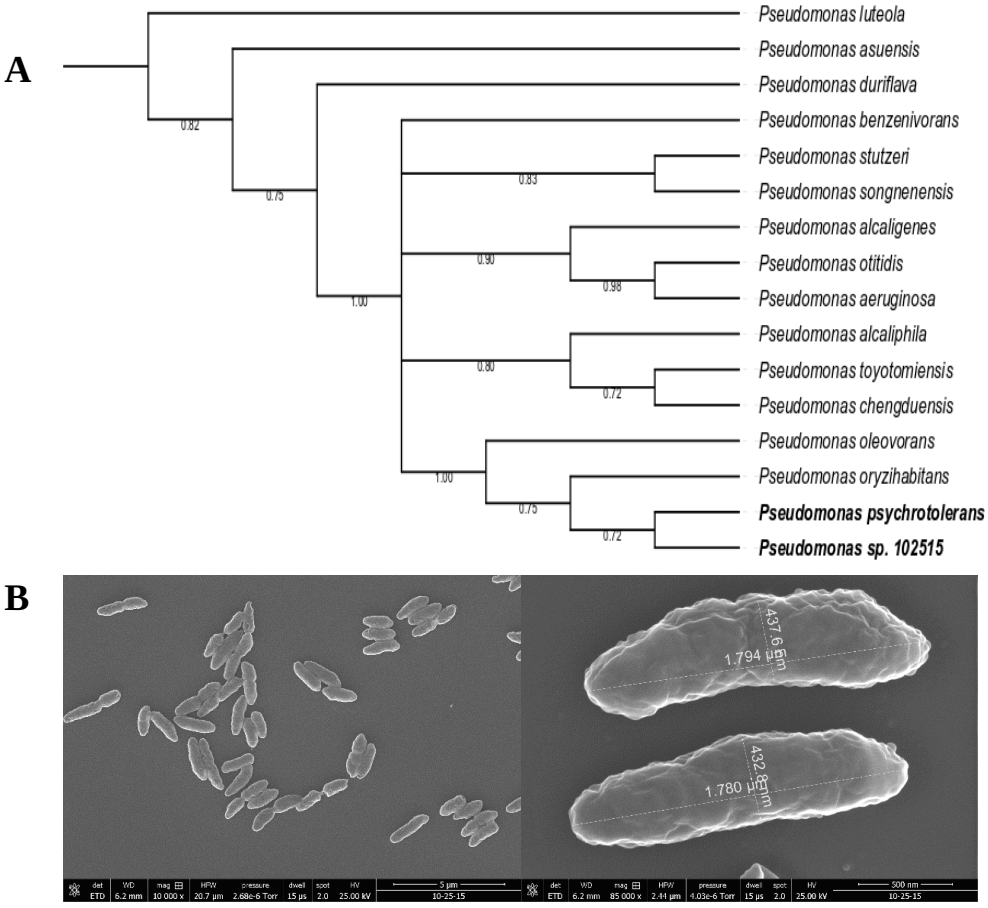

FIG. 13. Identification of *Pseudomonas* sp. 102515. (A) A phylogenetic tree constructed based on the 16S rRNA sequences of *Pseudomonas* strains from BLASTn analysis. It estimates the relationship between strain 102515 and other *Pseudomonas* strains that shared the highest 16S rRNA gene sequence similarities. Sequences of reference species were obtained from GenBank. *P. luteola* (NR_114215), *P. asuensis* (NR_136445), *P. duriflava* (NR_044390), *P. benzenivorans* (NR_116904), *P.stutzeri* (NR_041715), *P. songnenensis* (NR_148295), *P. alcaligenes* (NR_113646), *P. otitidis* (NR_043289), *P. aeruginosa* (NR_117678), *P. alcaliphila* (NR_114072), *P. toyotomiensis* (NR_112808), *P. chengduensis* (NR_125523), *P.oleovorans* (NR_043423),

P. oryzihabitans (NR_114041), *P. psychrotolerans* (NR_042191.1). (B) SEM electron micrograph of *Pseudomonas* sp. 102515. The bacterium was fixed with 2 % glutaraldehyde in 0.1 % HEPES buffer overnight. The samples were subjected to alcohol series dehydration (50-100 % ethanol) and then chemically dried using hexamethyldisilazane.

Amplification and analysis of a zeaxanthin diglucoside biosynthetic gene cluster from *Pseudomonas* sp. 102515

As described above, *P. psychrotolerans* is the closest relative strain to *Pseudomonas* sp. 102515. The genome of this strain is available at NCBI under the accession number, NZ_CP018758. I analyzed the genome of *P. psychrotolerans* with the help of AntiSMASH online genome analysis platform. Four biosynthetic gene clusters were identified. The first one is a nonribosomal peptide synthetase (NRPS)-type gene cluster with a 50 % similarity to the known taiwachelin biosynthetic gene cluster. Even though this gene cluster was identified as a NRPS type gene cluster, it includes the turnerbactin (*tnb*) biosynthetic gene cluster. Turnerbactin was reported to have an important role in the plant-microbe interactions (32). In order to find out whether a *tnb* gene cluster is present in *Pseudomonas* sp. 102515, I amplified the *tnbA* gene from its genomic DNA using a pair of primers designed based on the genome of *P. psychrotolerans*. The sequence of *tnbA* was provided in the supplementary information (Sequence D3). The second gene cluster is an arylpolyene (APE)-type biosynthetic gene cluster from which 40% of genes show similarity to the genes from the characterized APE biosynthetic gene cluster. APE gene clusters are widely distributed in gram-negative

bacteria, including *Pseudomonas* strains (33). In addition to a siderophore biosynthetic gene cluster, genome analysis revealed that *P. psychrotolerans* has a carotenoid (*crt*) biosynthetic gene cluster. The genes from this *crt* gene cluster is similar to those found in the *crt* gene cluster from *Pantoea agglomerans*. It contains *crtE*, *idi*, *crtX*, *crtY*, *crtI*, *crtB* and *crtZ* genes (34).

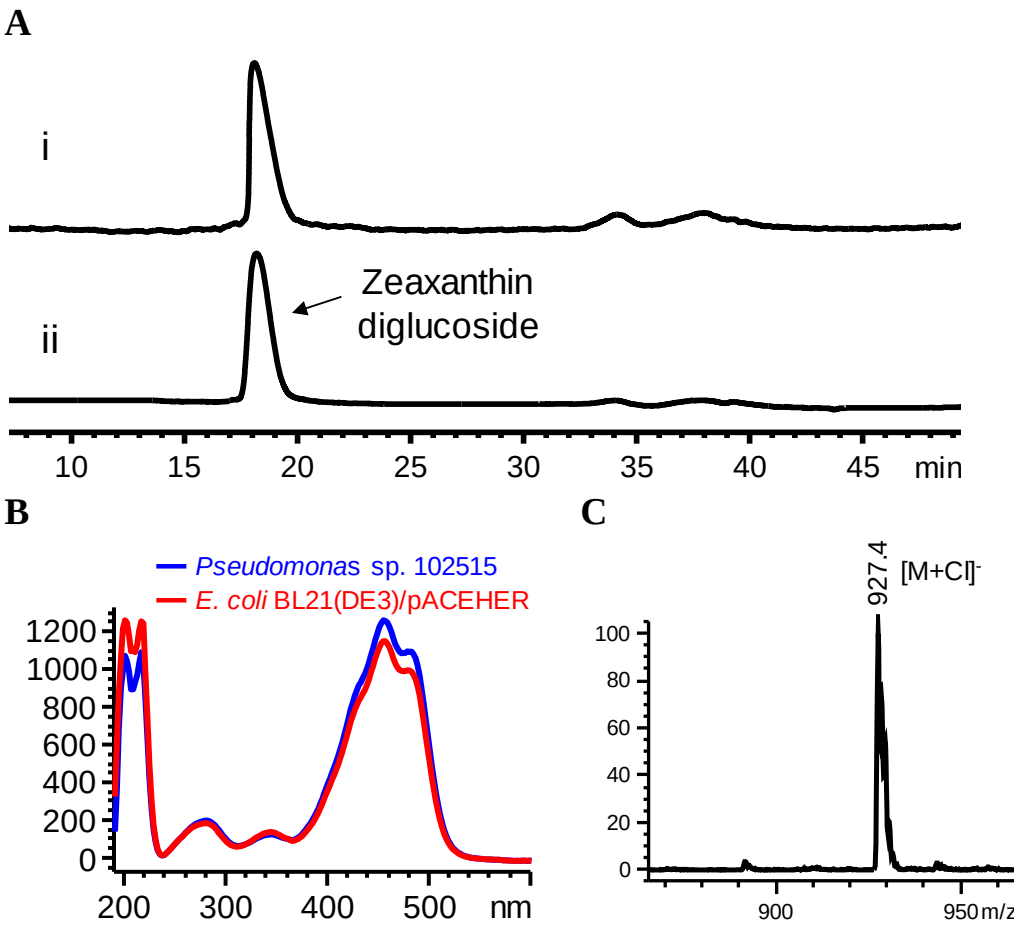

FIG. 14. Production of zeaxanthin diglucoside by *Pseudomonas* sp. 102515. (A) HPLC analysis (460 nm) of zeaxanthin diglucoside by *Pseudomonas* sp. 102515. (i) *Pseudomonas* sp. 102515; (ii) *E. coli* BL21(DE3)/pAC-EHER. (B) Comparison of the UV spectra of the major product of *Pseudomonas* sp. 102515 and *E. coli*

BL21(DE3)/pAC-EHER. (C) ESI-MS(-) spectrum of the major product of *Pseudomonas* sp. 102515.

To find out what yellow compound *Pseudomonas* sp. 102515 produced, I extracted the cells with methanol/chloroform (2:1, v/v) and analyzed the products by LC-MS. The extract of *Pseudomonas* sp. 102515 showed a major peak at 18.2 min (Fig. 14A). Based on its UV and MS spectra (Figs. 14B and 14C), this compound was characterized as zeaxanthin diglucoside. Furthermore, this peak has the same retention time and UV spectrum as zeaxanthin diglucoside produced by *E. coli* BL21(DE3)/pAC-EHER (35). Therefore, I confirmed that the yellow pigment from this endophyte is zeaxanthin diglucoside.

TABLE 6 List of genes from the *Pscrt* biosynthetic gene cluster in *Pseudomonas* sp. 102515.

Gene	Size (aa)	Function
orf1	314	YegS-like lipid kinase
PscrtZ	174	Beta-carotene hydroxylase
orf2	176	Gluconate 2-dehydrogenase subunit
PscrtB	311	15-cis-phytoene synthase
PscrtI	500	Phytoene desaturase
PscrtY	397	Lycopene cyclase
PscrtX	431	Zeaxanthin glycosyltransferase
Psidi	349	Isopentenyl-diphospahte delta-isomerase
PscrtE	293	Geranylgeranyl diphosphate synthase

Based on the sequence of the *crt* gene cluster in *P. psychrotolerans*, I designed two sets of primers and successfully amplified a carotenoid (*Pscrt*) biosynthetic gene cluster from *Pseudomonas* sp. 102515. The open reading frames (ORFs) in this gene

cluster were analyzed and annotated (Table 6), and based on the predicted functions of these genes, I propose that this gene cluster is responsible for the biosynthesis of zeaxanthin diglucoside.

Functional analysis of two carotenoid biosynthetic genes from the isolated strain

To confirm the functions of the carotenoid biosynthetic genes from *Pseudomonas* sp. 102515, it is necessary to develop an effective expression system for carotenoid biosynthetic enzymes in *E. coli*. I first ligated the *crtX* (zeaxanthin glucosyltransferase) gene from pAC-EHER into pET28a(+) to yield pOKF63. However, co-expression of pOKF63 and zeaxanthin-producing pAC-ZEAXipi in *E. coli* BL21(DE3) did not produce zeaxanthin diglucoside upon induction with 200 μM of IPTG. I next replaced the T7 promoter in pET28a(+) with a strong constitutive promoter J23119 and the B0034 ribosome binding site (pOKF72). The coexpression of pOKF72 and pAC-ZEAXipi in *E. coli* BL21(DE3) did produce zeaxanthin diglucoside, which was confirmed by HPLC through the comparison of retention time and UV spectra of the authentic sample obtained from *E. coli* BL21(DE3) harboring pAC-EHER (data not shown). I next used this system to test the functions of two selected biosynthetic genes in the *Pscrt* gene cluster. PsCrtI and PsCrtY were chosen as their functions can be easily observed by the color change in the products. Co-expression of the phytoene-producing plasmid pAC-PHYTipi with PsCrtI in *E. coli* BL21(DE3) led to the production of lycopene. I observed the color change from colorless cell pellets (*E. coli* BL21(DE3)/pAC-PHYTipi) to red cell pellets as seen in Fig. 15A, indicating that a red compound was produced. For further

confirmation, I compared the extract of this engineered strain with those of the negative (pAC-PHYTipi) and positive (pAC-LYCipi) controls by HPLC (Fig. 15A), which clearly revealed that lycopene was formed. This was supported by the UV spectra (Fig. 15B). This result confirmed that PsCrtI is a phytoene desaturase that converts phytoene to lycopene (Fig. 12). Similarly, I also confirmed the function of PsCrtY as a lycopene cyclase in *E. coli* BL21(DE3) by co-expression pAC-LYCipi with PsCrtY. I observed the color change from red to yellow due to the conversion of lycopene into β-carotene by PsCrtY (Fig. 16A). Production of β-carotene was further confirmed by its retention (Fig. 16A) time and UV spectrum (Fig. 16B).

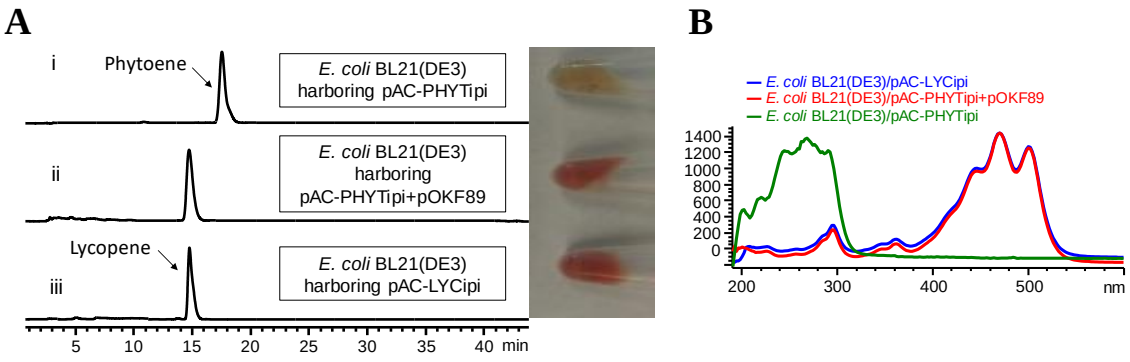

FIG. 15. Functional identification of PsCrtI as a phytoene desaturase. (A) HPLC analysis of lycopene production through co-expression of PsCrtI with the phytoene biosynthetic enzymes. (i) *E. coli* BL21(DE3)/pAC-PHYTipi (negative control) producing phytoene (retention time: 17.5 min, 280 nm), (ii) *E. coli* BL21(DE3)/pAC-PHYTipi+pOKF89 (pAC-PHYTipi+PsCrtI) producing lycopene (retention time: 14.8 min, 460 nm), (iii) *E. coli* BL21(DE3)/pAC-LYCipi (positive control) producing lycopene (retention time: 14.8 min, 460 nm). Color change of harvested cells due to the

co-expression of PsCrtI with pAC-PHYTipi in *E. coli* BL21(DE3). (B) A comparison of the UV spectra of lycopene produced by *E. coli* BL21(DE3)/pAC-PHYTipi+pOKF89 with lycopene produced by the positive control and phytoene produced by the negative control.

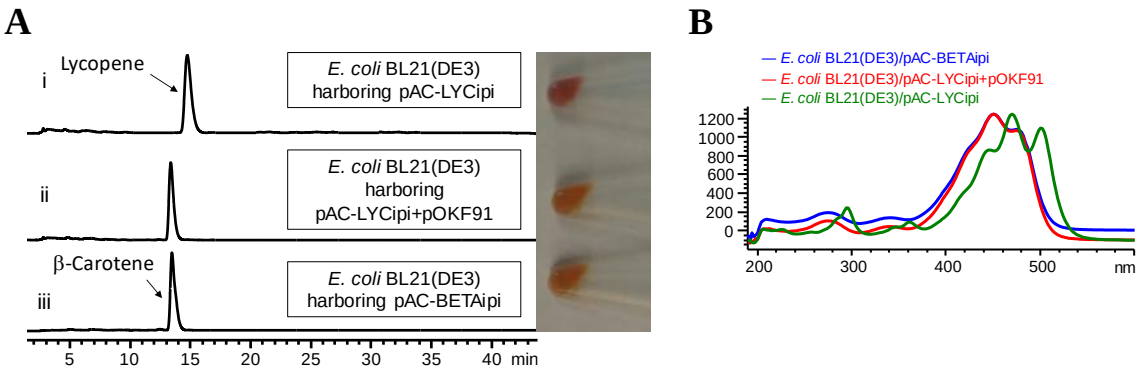

FIG. 16. Functional identification of PsCrtY as a lycopene cyclase. (A) HPLC analysis (460 nm) of β-carotene production through co-expression of PsCrtY with the lycopene biosynthetic enzymes. (i) *E. coli* BL21(DE3)/pAC-LYCipi (negative control) producing lycopene (retention time: 14.8 min), (ii) *E. coli* BL21(DE3)/pAC-LYCipi+pOKF91 (pAC-LYCipi+PsCrtY) producing β-carotene (retention time: 13.3 min), (iii) *E. coli* BL21(DE3)/pAC-BETAipi (positive control) producing β-carotene (retention time: 13.3 min). Color change of harvested cells due to the co-expression of PsCrtY with pAC-LYCipi in *E. coli* BL21(DE3) is shown on the right. (B) A comparison of the UV spectra of β-carotene produced by *E. coli* BL21(DE3)/pAC-LYCipi+pOKF91 with β-carotene produced by the positive control and lycopene produced by the negative control.

Heterologous expression of the *Pscrt* gene cluster in *E.coli* BL21(DE3) and *Pseudomonas putida* KT2440

Identification of PsCrtI and PsCrtY further suggested that this gene cluster is responsible for the biosynthesis of zeaxanthin diglucoside in *Pseudomonas* sp. 102515. Instead of characterizing each of the remaining genes in this gene cluster, I attempted to express the entire gene cluster in a heterologous host. I first transferred the whole *Pscrt* gene cluster into the pET28a(+) expression vector. Heterologous expression of the whole *Pscrt* gene cluster using the pET28a(+) expression system did not produce detectable amounts of zeaxanthin diglucoside. Then, I cloned the *Pscrt* gene cluster into a pACYC184 based expression system that has been previously used for the heterologous production of carotenoids in *E. coli* (35). Expression of the *Pscrt* gene cluster in *E. coli* BL21(DE3) led to the production of zeaxanthin diglucoside (Fig. 17), which confirmed that this gene cluster is indeed responsible for the biosynthesis of this glycosylated carotenoid. However, the yield of zeaxanthin diglucoside was very low in *E. coli* BL21(DE3) as the cells only showed a slight yellow color. I figured that *P. putida* KT2440 might be a better host since it belongs to the genus of *Pseudomonas*. To this end, I cloned the *Pscrt* biosynthetic gene cluster into the pMIS1-mva vector, and expressed the resulting plasmid (pOKF192) in *P. putida* KT2440. Expression of this plasmid in *P. putida* KT2440 was deemed successful by observing the bright yellow color of the cells, and the formation of zeaxanthin diglucoside was verified by HPLC analysis (Fig. 17).

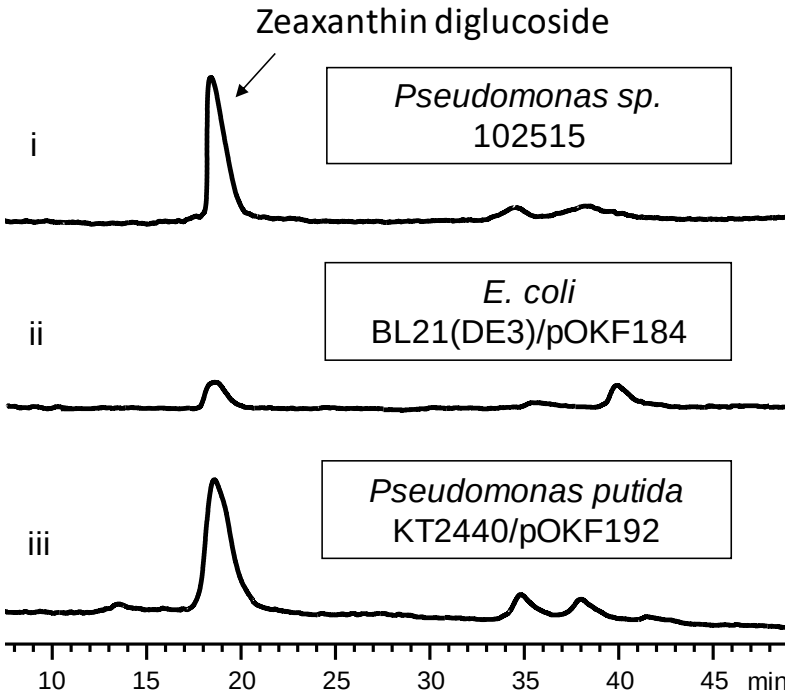

FIG. 17. Heterologous reconstitution of the zeaxanthin diglucoside biosynthetic pathway in *E. coli* BL21(DE3) and *Pseudomonas putida* KT2440. Shown are HPLC traces (460 nm) of the extracts of *Pseudomonas* sp. 102515 (i), *E. coli* BL21(DE3)/pOKF184 (ii), and *P. putida* KT2440/pOKF192 (iii).

Enhanced production of zeaxanthin diglucoside in *Pseudomonas* sp. 102515

For yield optimization studies, I used a UV spectrophotometer to measure the amount of zeaxanthin diglucoside in the extracts at 456 nm, which is the maximum wavelength for zeaxanthin diglucoside as applied in the literature (36). Using the standard curve prepared with purified zeaxanthin diglucoside (Fig. D1), I calculated the yields from the crude extracts of each sample. Seven different media with 3 replicates were tested for the production of zeaxanthin diglucoside by *Pseudomonas* sp. 102515 in

culture tubes. The LB, SOC and superbroth media showed better yields compared to the other media (Fig. D2). I then tried the LB, SOC and superbroth media in 50-mL flasks, resulting in a further improved production of zeaxanthin diglucoside. As shown in Fig. 18A, SOC exhibited a relatively better yield (98 ± 7 mg/L).

Glycerol was reported to enhance the yield of carotenoids (37). I thus tested the effects of glycerol on the production of zeaxanthin diglucoside in flasks at different concentrations (0.5%, 1% and 2% final concentration). The supplementation of 0.5% glycerol (final concentration) into the SOC medium increased the yield of zeaxanthin diglucoside to 127 ± 1.5 mg/ L after 3 days at 28 °C, representing a 30 % increase in the yield (Fig. 18B). I next tested how the cultivation time and temperature (18 °C, 23 °C, 28 °C and 37 °C) affect the yield of zeaxanthin diglucoside in the SOC medium supplemented with 0.5% glycerol. The cultivation temperature of 37 °C resulted in extremely low yields (data not shown). Among the tested temperatures, 18 °C worked best and showed a yield of 206 ± 6 mg/L in the SOC medium after 5 days of cultivation (Fig. 18C). Under these conditions, I also tested the yields of zeaxanthin diglucoside in the engineered strains of *E. coli* BL21(DE3) and *P. putida* KT2440. Consistent with the cell colors, *E. coli* BL21(DE3) only produced 2 mg/L zeaxanthin diglucoside, while *P. putida* KT2440/pOKF192 generated the product in a much higher yield (121 ± 6 mg/L). However, since this yield is still lower than the wild type (206 mg/L), the latter is a better starting strain for further improvements. I successfully engineered *Pseudomonas* sp. 102515 by introducing pOKF192 into this strain, which allows this endophyte to have another copy of the *Pscrt* gene cluster. Expression of this plasmid further improved the

yield to 380 ± 12 mg/L after 5 days of incubation at 18 °C (Figs. 18C and 18D).

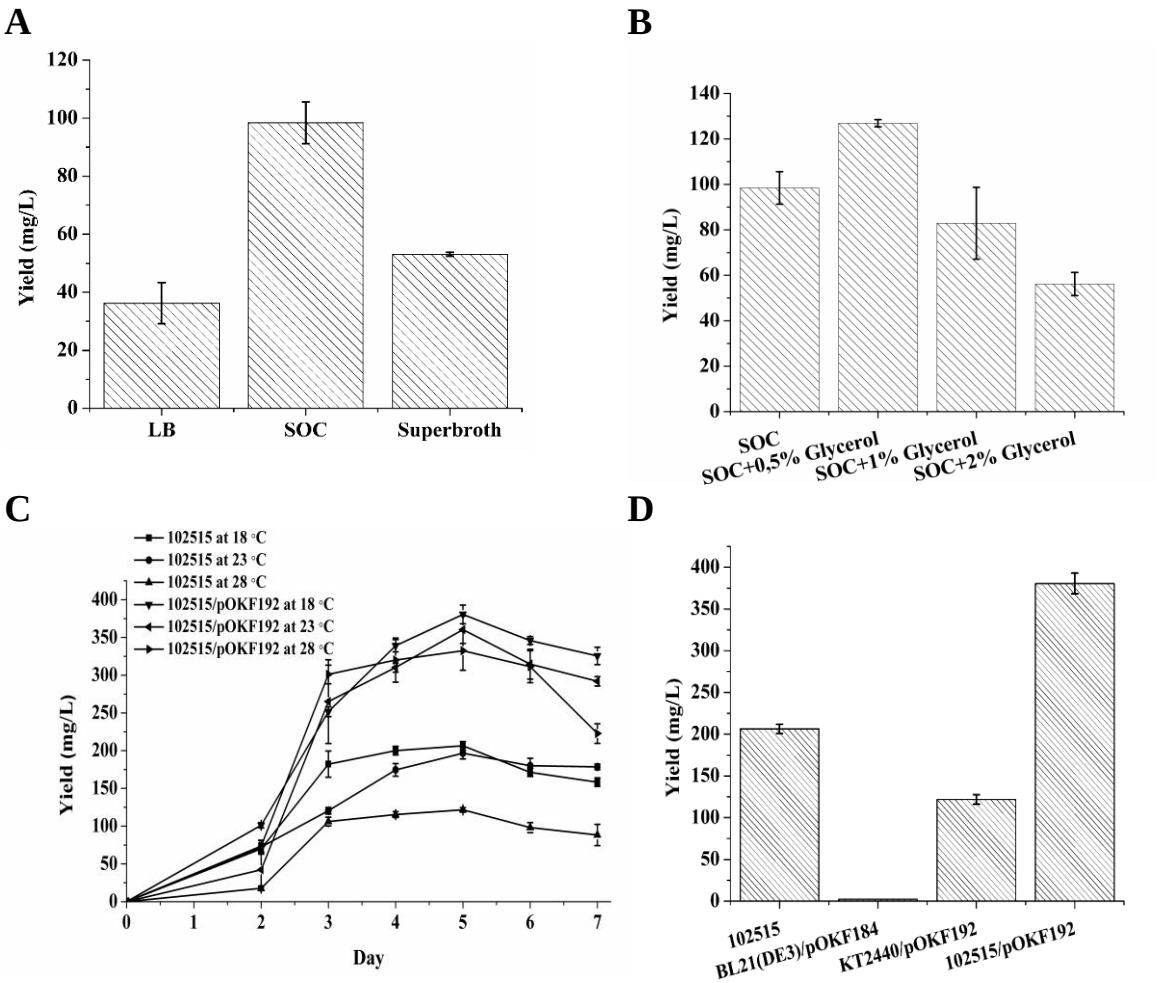

FIG. 18. Optimization of zeaxanthin diglucoside production in *Pseudomonas* sp. 102515. (A) The effect of culture media on the production of zeaxanthin diglucoside. (B) The effect of glycerol supplementation on the production of zeaxanthin diglucoside. (C) The effects of cultivation temperature and time on the production of zeaxanthin diglucoside by *Pseudomonas* sp. 102515 and *Pseudomonas* sp. 102515/pOKFF192. Cells were grown in 50 ml of SOC medium supplemented with 0.5% glycerol. (D) A comparison of the production yield of zeaxanthin diglucoside by *Pseudomonas* sp.

102515 and three engineered strains.

DISCUSSION

Carotenoids with a huge market value play a significant role in maintaining good health and preventing human diseases including cardiovascular diseases, cancer and other chronic diseases due to their significant biological activities (38). Carotenoids are considered to protect cells from the damaging effects of reactive oxygen species (ROS), which are formed by normal metabolic activities and lifestyle factors such as diet, smoking and exercise. Carotenoids might potentially diminish the destructive effects of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH) (39,40). In specific, $^1O_2$ as a product in both biochemical and photochemical systems is reported to be responsible for the cell destruction caused by light and certain photosensitizers. The number of conjugated double bands in the carotenoids is a significant factor for the $^1O_2$ quenching activity (41,42). Carotenoids such as lycopene, lutein, β-carotene, astaxanthin, and zeaxanthin are quite common in nature, however, glycosylated carotenoids such as dihydroxylycopene diglucoside, adonixanthin diglucoside and zeaxanthin diglucoside are very rare (43-46). Particularly, glycosylated carotenoids can be a potent antioxidant agent with protective properties against photooxidative damages from ROS and visible light (47,48). For instance, a novel glycosylated carotenoid, caloxanthin 3′-β-D-glucoside, was reported to have a potent $^1O_2$ quenching activity with an IC_{50} of 19 μM (49). Moreover, Tatsuzawa et al. reported that the viability of zeaxanthin diglucoside producing *E. coli* cells is higher in an $^1O_2$

generation mixture compared to the cells that produce other carotenoids such as zeaxanthin, astaxanthin, β-carotene, and canthaxanthin (40). Glycosylated carotenoids are also reported to stabilize the membrane through integrating within the lipid membrane due to their polar functional groups (47,50).

In this study, I isolated an endophytic bacterium *Pseudomonas* sp. 102515 from the leaves of *Taxus chinensis*, which was found to produce zeaxanthin diglucoside as a major metabolite. BLAST and phylogenetic analyses (Fig. 13) revealed that this endophytic isolate is a *Pseudomonas* strain. *Pseudomonas* species have been isolated from diverse sources including marine, freshwater, animals and plants (51-54). Certain *Pseudomonas* strains such as fluorescent *Pseudomonads*, are predominantly found in the rhizosphere and have been reported to move from rhizosphere to aerial plant tissue as in the case of *P. aeurofaciens* (55). *Pseudomonas* species were also reported as endophytes in the literature (56,57). For instance, *P. stutzeri* A15 is an endophytic nitrogen-fixing bacterium isolated from paddy rice (58). Another endophyte is *P. fluorescens* with beneficial interactions with plants (59). Plant-endophyte interactions have not been fully understood. However, many endophytes not only showed beneficial effects on their hosts, but also play a significant role in plant physiology. For example, some endophytic bacteria were reported to provide phytohormones, low-molecular compounds or enzymes to the host plants that led to the enhanced plant growth (60-62). Endophytic bacteria also provide an alternative way to manage plant pathogens as a promising biocontrol agent through various ways such as releasing antimicrobial substances, producing siderophores, and inducing the systemic resistance to pathogens (63-65). For instance, biological control of *P. syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit, was

achieved by using an endophytic bacterium isolated from a medicinal plant (66).

To better understand *Pseudomonas* sp. 102515, I looked into the closely related strains, *P. psychrotolerans* as well as *P. oryzihabitans*. Both strains were reported as yellow-pigmented gram-negative bacteria and isolated from different sources including clinical samples, cupper coins, diseased rice and rice seeds (67-71). An endophytic *P. oryzihabitans* strain isolated from *Hibiscus rosasinensis* was reported (72). *P. psychrotolerans* was isolated from copper coins, diseased rice and rice seeds as an endosymbiotic bacterium in two different studies (67,71). The later endophytic bacterium was reported to enhance the plant growth due to potential nitrogen fixing characteristics of the turnerbactin (*tnb*) biosynthetic gene cluster, which is responsible for the biosynthesis of turnerbactin, a tricatecholate siderophore. Plants infected with *P. psychrotolerans* PRS08-11306 showed enhanced growth (32,71). In this work, I also detected the *tnb* gene cluster and confirmed the existence of this gene cluster in *Pseudomonas* sp. 102515 by amplifying one of the key genes, *tnbA*. Thus, this endophyte also has a potential to be utilized as plant growth-promoting bacterium for agricultural applications.

In addition to the *tnb* gene cluster, I found a complete carotenoid (*Pscrt*) biosynthetic gene cluster in *Pseudomonas* sp. 102515, which contains a series of carotenoid biosynthetic genes (Table 6**)**. However, there are some differences in the organization of the genes between the *Pscrt* gene cluster and other reported ones (Fig. 19). One obvious difference is the additional non-carotenoid gene (*orf2*) encoding for gluconate 2-dehydrogenase, which has not been reported in a carotenoid gene cluster to our knowledge. The role of this gene remains to be characterized. Typically, all genes in

a *crt* biosynthetic gene cluster except *crtZ* have the same direction and are controlled by the same promoter in many γ-proteobacteria (34). However, the *PscrtE* gene has a different direction compared to most of the genes in the *Pscrt* gene cluster and is controlled by its own promoter (Fig. 19). The similar organization of *crtE* gene in the gene cluster was also reported in *Pseudomonas* sp. strain Akiakane isolated from the excrement of autumn darker, yet the *crtZ* gene in that gene cluster is located differently than ours and overlapped with the sequences of *crtX* and *crtY* as shown in Fig. 19 (54).

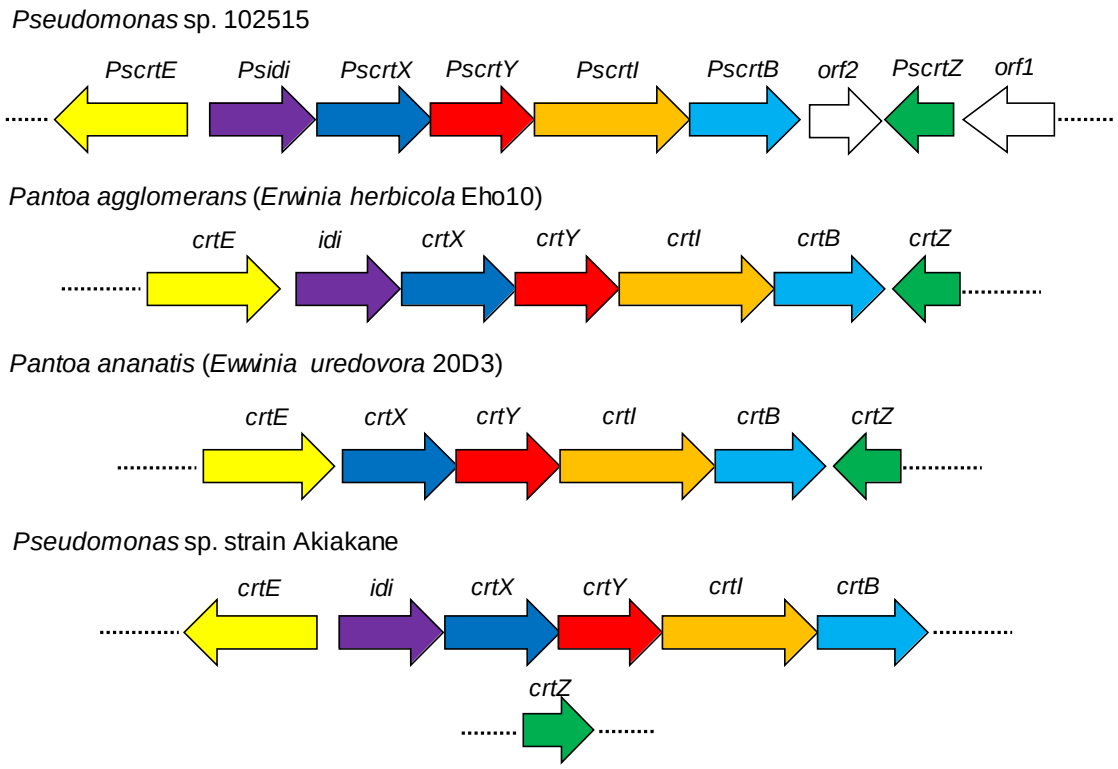

FIG. 19. Organization of carotenoid biosynthetic gene clusters in the γ-proteobacteria strains. NCBI accession numbers: M87280 for *Pantoa agglomerans*, CP001875 for *Pantoa ananatis*, and LC317091 and LC317092 for *Pseudomonas* sp. strain Akiakane.

I analyzed the functions of two *Pscrt* genes from *Pseudomonas* sp. 102515 through heterologous expression of these genes with corresponding carotenoid-producing plasmids in *E. coli* BL21(DE3) (73,74). PsCrtI was confirmed to be a phytoene desaturase and PsCrtY is a lycopene cyclase. The function of the *Pscrt* gene cluster was characterized by expression of the entire biosynthetic gene cluster in two heterologous hosts, *E. coli* BL21(DE3) and *P. putida* KT2440, both yielding zeaxanthin diglucoside (Fig. 17). This heterologous expression strategy not only allows the functional confirmation of this gene cluster, but also provides an alternative way to produce zeaxanthin diglucoside. However, the yield of zeaxanthin diglucosde in *E. coli* was very low compared to *Pseudomonas* sp. 102515, likely due to the low efficiency of the native promoter in the this host. Sometimes a native promoters might not work well in a heterologous host. For instance, the *phaC* genes from *P. putida* were expressed in *E. coli* under the control of a native promoter or an external promoter. They were only expressed in *E. coli* with the external promoter (75). In another study, the levansucrase genes from *P. syringae* pv. *glycinea* PG4180 and *P. syringae* pv. *phaseolicola* NCPPB 1321 were cloned with the native promoters, yet the expression of levansucrase was only achieved upon the use of the *lac* promoter (76). Thus, heterologous expression of the *Pscrt* biosynthetic gene cluster from *Pseudomonas* sp. 102515 needs to be conducted using external promoters, in particular constitutive promoters, which might potentially enhance the production of zeaxanthin diglucoside in *E. coli*. Another approach is to choose another host microorganism. Since our isolate is a *Pseudomonas* strain, I proposed that the *Pscrt* gene cluster might be expressed better in *P. putida* KT2440 than *E. coli*. *P. putida* KT2440 was previously used as a heterologous host for the production of natural

products including zeaxanthin (77,78). Our results confirmed that expression of the *Pscrt* gene cluster in *P. putida* KT2440 resulted in a much higher yield of zeaxanthin diglucoside than in *E. coli* (Fig. 18D).

A common strategy to improve the yield is the optimization of culture conditions (79). I used LB medium for the isolation of *Pseudomonas* sp. 102515 and the initial product analysis of this endophyte. However, the nutrients in different media can affect the production of zeaxanthin diglucoside. A screening of 7 different media (Table D1) in culture tubes showed that LB, superbroth and SOC media had better yields ranging from 13 to 15 mg/L (Fig. D2). I chose SOC medium for further optimization studies due to its higher titer in flasks (Fig. 18A) and found that 0.5 % glycerol supplementation further increased the yield to 127 ± 2 mg/L (Fig. 18B), which is consistent with a previous work on the effect of glycerol on the production of carotenoids (37). The effects of cultivation temperature and time were also examined. I originally used 28 °C as cultivation temperature as reported in the literature for the production of carotenoids (35,73,74,77). However, a comparison of four different temperatures revealed that 18 °C was the best cultivation temperature for the production of zeaxanthin diglucoside in *Pseudomonas* sp. 102515. The yield reached 145 ± 5 mg/L in 50 mL of SOC medium. I then cultivated this endophyte in SOC medium supplemented with 0.5 % glycerol (final concentration) at 18 °C for a week to find out the best fermentation time. Based on the time course analysis, the yield reached the highest (206 ± 6 mg/L) after 5 days of cultivation. However, low cultivation temperature is an economic burden for industrial production although it often favors the expression of enzymes (80). Therefore, I cultivated *Pseudomonas* sp. 102515 in 50 mL of SOC medium supplemented with 0.5 % glycerol at 23 °C for a week (Fig.

18C). After 5 days of cultivation, I obtained a titer of 197 ± 8 mg/L that is slightly lower than but comparable to the titer at 18 °C. Thus, 23 °C can be used for industrial production of zeaxanthin diglucoside with *Pseudomonas* sp. 102515. To further improve the yield, I introduced pOKF192 carrying the *Pscrt* gene cluster into *Pseudomonas* sp. 102515. With the additional copy of the *Pscrt* gene cluster, engineered *Pseudomonas* sp. 102515/pOKF192 produced zeaxanthin diglucoside at 380 ± 12 mg/L at 18 °C after 5 days of cultivation, which is 85 % higher than the parent strain (Figs. 18C and 18D). Similar to *Pseudomonas* sp. 102515, this engineered strain at 23 °C led to a slightly lower titer than but comparable to the titer at 18 °C (Fig. 18C). To our knowledge, this study showed the highest yield of zeaxanthin diglucoside in bacteria.

In conclusion, I isolated a carotenoid-producing endophytic *Pseudomonas* strain from the yew tree, which offers an alternative way to produce zeaxanthin diglucoside. A carotenoid biosynthetic gene cluster was identified in this endophyte, from which the functions of two *Pscrt* genes were confirmed in *E. coli* BL21(DE3). I also cloned the whole *Pscrt* gene cluster and successfully expressed it in two heterologous hosts, *E. coli* BL21(DE3) and *P. putida* KT2440. PCR analysis also showed that a turnerbactin biosynthetic gene cluster exists in the isolated strain, which will render our isolate a promising plant-growth promoting bacteria for agricultural applications. Additionally, I enhanced the yield of zeaxanthin diglucoside in the engineered endophytic isolate to 380 ± 12 mg/L by optimizing the culture conditions and introducing an additional copy of the *Pscrt* biosynthetic gene cluster.

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CHAPTER V SUMMARY AND ENGINEERING VALUE

Summary of my dissertation research and future work

My dissertation is focused on exploring the potential of bacteria for the biosynthesis of natural products with the purposes of creating novel natural product derivatives and of improving the production of pharmaceutically important natural products. In Chapter 2, I have investigated the glycosylation steps in the biosynthesis of two antifungal angucyclines from *Streptomyces* sp. SCC-2136, Sch47554 and Sch47555. Two different strategies were exploited for the characterization of three glycosyltransferases responsible for the glycosylation process. Co-expression of the aglycone and sugar biosynthetic genes with *schS7* in *S. lividans* K4 led to the production of a novel C-glycosylated rabelomycin derivative, confirming that SchS7 is the dedicated C-glycosyltransferase. For *schS9* and *schS10*, a gene disruption strategy was used to understand their functions in the glycosylation steps. Gene inactivation studies revealed that these glycosylation steps take place in a sequential manner in which SchS9 first attaches either a L-aculose or L-amicetose moiety to the 4'-OH of the C-glycosylated aglycone, and then SchS10 transfers a L-aculose moiety to the 3-OH of the angucycline core. In this research, I isolated two novel derivatives of angucycline and gained new insights into the glycosylation pathways in the biosynthesis of Sch47554 and Sch47555. In the future work, we can add additional glycosyltransferase genes cloned from similar biosynthetic gene clusters such as the landomycins and urdamycin biosynthetic gene clusters into our heterologous expression cassette to extend the sugar chain of the angucyclines (1-3). This will not only further diversify the chemical repertoire of

angucyclines, but also potentially enhance the biological activities of these angucyclines as reported in the literature (4).

In Chapter 3, I worked on the same biosynthetic gene cluster for improved production of the angucyclines. The *sch* gene cluster includes several putative regulatory genes. *schA4* and *schA21* were predicted as the TetR family transcriptional regulators, whereas *schA16* showed a significant similarity to the AraC family transcriptional regulators. I disrupted all of these regulatory genes in *Streptomyces* sp. SCC-2136. Disruption of *schA4* and *schA16* led to a significant increase in the production of Sch47554, while the titer was dramatically decreased in *Streptomyces* sp. SCC-2136/Δ*schA21*. I also overexpressed them to further confirm their functional roles in the biosynthesis of angucyclines. Overexpression strains produced consistent results with the gene disruption strains. The highest titer of Sch47554 was achieved in *Streptomyces* sp. SCC-2136/Δ*schA4* (27.94 mg/L), which is significantly higher than the wild type. Based on these results, I was able to characterize the functions of these three regulatory elements. SchA4 and SchA16 are repressors, while SchA21 acts as an activator. This work provided an initial understanding of functional roles of regulatory elements in the biosynthesis of Sch47554. Several efficient producing strains of Sch47554 were constructed by disrupting or overexpressing particular regulatory genes, which can be further engineered for industrial production of this medicinally important molecule. In the future work, a combination of the gene disruption with overexpression approaches might further enhance the titer of Sch47554. For instance, targeted gene deletion of *schA4* through double crossover recombination will result in a strain without any antibiotic resistance genes, which can be used to overexpress SchA21. This could potentially lead

to a far better yield of Sch47554.

In Chapter 4, I isolated an endophytic bacterium from the leaves of *Taxus chinensis*. BLAST and phylogenetic analyses of the 16S rRNA sequence revealed that it is a *Pseudomonas* strain, named *Pseudomonas* sp. 102515. Based on the genome of a closely related *Pseudomonas* strain, a carotenoid (*Pscrt*) biosynthetic gene cluster was amplified from this endophyte. The functions of PsCrtI and PsCrtY were respectively identified as phytoene desaturase and lycopene cyclase in the biosynthesis of zeaxanthin diglucoside. The *Pscrt* biosynthetic gene cluster was successfully reconstituted in *E. coli* BL21(DE3) and *Pseudomonas putida* KT2440. The engineered strain of *P. putida* KT2440 produced zeaxanthin diglucoside at 144 ± 4 mg/L in the SOC medium supplemented with 0.5% glycerol at 23 °C, while the titer of zeaxanthin diglucoside in *E. coli* BL21(DE3) was very low. The production of zeaxanthin diglucoside in *Pseudomonas* sp. 102515 was improved through the optimization of fermentation conditions. The highest titer under the optimized conditions reached 206 ± 6 mg/L. To further enhance the production, I introduced an expression plasmid that harbors the *Pscrt* biosynthetic gene cluster into *Pseudomonas* sp. 102515, yielding an efficient producing strain of zeaxanthin diglucoside. The titer in this engineered strain reached 380 ± 12 mg/L, 85% higher than the wild type. Through PCR, I also discovered the presence of a turnerbactin biosynthetic gene cluster in *Pseudomonas* sp. 102515. Turnerbactin was reported to be involved in nitrogen fixation, which can promote the growth of host plants. Therefore, in addition to producing zeaxanthin diglucoside, *Pseudomonas* sp. 102515 might also be utilized as a plant-promoting strain for agricultural applications. In the

future work, the fermentation of engineered *Pseudomonas* sp. 102515 harboring pOKF192 in the bioreactor can be conducted to further enhance the titer of zeaxanthin diglucoside. The optimized fermentation conditions in the bioreactor such as dissolved oxygen, agitation speed and pH may lead to a further improved titer.

Engineering value

Engineered production of angucycline derivatives

The heterologous expression approach helps researchers understand and engineer the biosynthetic pathways for creating structural diversity in natural products (5,6). In Chapter 2, I heterologously expressed some of the genes from the *sch* biosynthetic gene cluster, which yielded a novel angucycline derivative, C-glycosylated rabelomycin. I also disrupted two tailoring enzymes in the wild type strain, resulting in the isolation of another novel derivative of angucycline. These two strategies offer an effective platform to generate novel angucycline derivatives, which is quite useful for new drug development in the pharmaceutical industry. This study also provides new insights into the glycosylation steps in the biosynthesis of angucyclines, which can then be used to further engineer this biosynthetic pathway for generation of "unnatural" natural products.

Engineered production of angucyclines in *Streptomyces* sp. SCC-2136

Streptomyces sp. SCC-2136 produces two angucyclines, Sch47554 and Sch47555, which exhibit antifungal activity against various yeasts (*Candida albicans, C. tropicalis* and *C. stellatoidea*) and dermatophytes (*Trichophyton mentagrophytes, T. rubrum, T. tonsurans* and *Microsporum canis*) (7). Additionally, Sch47554 and Sch47555 may

possess potent antitumor and antibacterial activities, as well as enzyme inhibitory and agonistic activities due to their structural similarity to other reported angucycline metabolites (8). Therefore, in Chapter 3, I attempted to enhance the titer of these compounds by strain engineering. In particular, the manipulation of regulatory genes through overexpression of activator genes and/or deletion of repressor genes has been successfully performed for improved production of natural products (9). I exploited these two strategies to create overexpression and gene disruption strains for three regulatory genes in the *sch* biosynthetic gene cluster. Two of the engineered strains showed significantly increased titers of Sch47554. The highest titer of Sch47554 was achieved in *Streptomyces* sp. SCC-2136/Δ*schA4* (27.94 mg/L), which is much higher than the wild type (6.72 mg/L). The overexpression of SchA21 in *Streptomyces* sp. SCC-2136 also improved the yield substantially to 26.23 mg/L. This work thus provides a more efficient way to produce this pharmaceutically important molecule (Sch47554).

Engineered production of zeaxanthin diglucoside in *Pseudomonas* sp. 102515

Carotenoids play a significant role in maintaining good health and preventing human diseases including cardiovascular diseases, cancer and other chronic diseases. These natural products possess a variety of biological activities such as antioxidant, anticancer, and anti-inflammatory properties. They are also an important dietary source of vitamin A (10,11). Therefore, carotenoids have a huge market value. It was reported that the viability of zeaxanthin diglucoside producing *E. coli* cells is higher in the $^1O_2$ generation mixture compared to the cells that produce other carotenoids such as zeaxanthin, astaxanthin, β-carotene, and canthaxanthin (12). In Chapter 4, I isolated an

endophytic bacterium from the leaves of *Taxus chinensis*, which provides an alternative effective mean for the production of this promising antioxidant agent. I optimized the fermentation conditions of *Pseudomonas* sp. 102515 such as temperature, cultivation time, medium and glycerol supplementation. I achieved the highest yield of zeaxanthin diglucoside at 206 ± 6 mg/L in the SOC medium with glycerol supplementation after 5 days of cultivation at 18 °C. With the introduction of an additional copy of the entire *Pscrt* gene cluster into *Pseudomonas* sp. 102515, I further increased the titer to 380 ± 12 mg/L. This demonstrates that our strain is a promising microorganism for the production of pharmaceutically important antioxidant agent, zeaxanthin diglucoside. In addition, this isolate also has potential to be utilized as a plant growth-promoting bacterium for agricultural applications due to its turnerbactin biosynthetic capability, which enhances the nitrogen fixation capacity of host plants.

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APPENDICES

APPENDIX A

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APPENDIX B

SUPPORTING INFORMATION for CHAPTER 2

TABLE B1. Primers used in Chapter 2.

Primer Name	Primer Sequence
schP6schP7schP8-Forward-PacI	5'-AATTAATTAAGGAGGAGCCAC CACTGGACCGCACTGTGA-3'
schP6schP7schP8-Reverse-NheI	5'-AAGCTAGCTCAGCTGGACCGGACGGGCA-3'
schP4-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATAT GACCGTCCGTGAGGTCGA-3'
schP4-Reverse-NheI	5'-AAGCTAGCTCAGCGCCGCGCTTCGGCGT-3'
schP5-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATATGA CGACTGCCACGTCCGG-3'
schP5-Reverse-NheI	5'-AAGCTAGCTCAGAAATTGCCGAGGCCGC-3'
schP9-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATAT GCACAGCACGCTGATCGT-3'
schP9-Reverse-NheI	5'-AAGCTAGCTCACAGTGCGGTCCAGTGGT-3'
schP10-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATATG GATGCTTCGGTCATAGT-3'
schP10-Reverse-NheI	5'-AAGCTAGCTCAGGACTTCGGGCCCCGCG-3'
schS2-Forward-SpeI	5'-AAACTCGTGGAGGAGCCCATAT GCTGTCCCACACCCTTG-3'
schS1-Reverse-NheI	5'-AAGCTAGCTCAGGCCGACGGCTCGGAGA-3'
schS3-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATA TGAGCGACCCCAAGGAAACG-3'
schS3-Reverse-NheI	5'-AAGCTAGCTCAGCCGCGCGCGGCCACGAA-3'
schS6-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATATG AAGGCGCTTGTGCTGGCA-3'
schS4-Reverse-NheI	5'-AAGCTAGCTCATCCTGTCCTTTCGTCGA-3'
schS7-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATGTG AGGATCCTCTTCGTCACC-3'
schS7-Reverse-NheI	5'-AAGCTAGCTCAGCGGAGCTGTTCCAGTGC-3'
schS8-Forward-SpeI	5'-AAACTAGTGGAGGAGCCCATATG GTCATTCCCGGTACATA-3'
schS8-Reverse-NheI	5'-AAGCTAGCTCACAGTGTTCCTGCCTTGGT-3'
schS9-SC-KO-XbaI-Forward	5'-AATCTAGAAGGAGGAACGGCCGTTGCAG-3'
schS9-SC-KO-HindIII-Reverse	5'-AAAAGCTTTGGTGGACCATCGCGTCGCA-3'
schS10-SC-KO-XbaI-Forward	5'-AATCTAGATCCGTCGCTGGGCTTCGA-3'
schS10-SC-KO-HindIII-Reverse	5'-AAAAGCTTCGTGGTGGATGATCGCCGAA-3'
schS9-check-Forward	5'-GCATGTTCTCGCTGTACTAC-3'
schS9-check-Reverse	5'-TGATGAACTCCCGGATCG-3'
schS10-check-Forward	5'-GTGGTCAGCACGTCATGA-3'
schS10-check-Reverse	5'-CGACACCGAGTAGTTGATC-3'

RV-M	5'-GAGCGGATAACAATTTCACACAGG-3'
M13-47	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'

```
SimB7(385aa)       mrilfvaagslatafalapvataarnaghavimagnddvipaitgvglpgvsvssltiqd
LanGT2(373aa)      mkilfvasgspatvfalaplataarnaghdvfmgavedmvpyiasagipalsiapssirr
SaqGT5(383aa)      mkvlfvaggspatvfalaplatavrnaghqafmaatddmmpvvagaglpgvpvtplpirh
SchS7(378aa)       mrilfvtagspatvfalaplataarnaghqvvmaanadmmphitgsglagiptthrpire
UrdGT2(365aa)      ------------mfalaplataarnaghqvvmaanqdmgpvvtgvglpavattdlpirh
                               *****:***.***** ..*..  *: * ::. *: .:  :   *:

SimB7(385aa)       fy-tdragrpveiptvpqeqilftgrwfgrmaaawldplrelarawkpdvvvggtmsyaa
LanGT2(373aa)      yatmdregnpvrmpetpeeeldfaghwfgrmaagsmdalrevtanwrpdlvvggsmsfaa
SaqGT5(383aa)      fittdragrpltipadpaeearftgrwfarmavasmpplhelaagwrpdvvvggtmsyaa
SchS7(378aa)       fitqdrqgnpesipsdpveqalftgrwfarmgaeslprmlefarswrpdlvvggtmcyva
UrdGT2(365aa)      fittdregrpeaipsdpvaqarftgrwfarmaasslprmldfsrawrpdlivggtmsyva
                   :   ** *.*  :*  *  :  *:*:**.**..  :  : :.:  *:**::***:*.::*

SimB7(385aa)       gllaaelgvryvrhawdaieatgidvgavdvlepeltrlgldrlpvpelfidvcppsllp
LanGT2(373aa)      aliaaelgvpyvrqawdtgdawrtdpaasdelrpelralgldrlpdpalfvdicppslrp
SaqGT5(383aa)      pllaarlgvpfvrqawdaveatgvhpgadeelqpelrelgldrlpepdlfvdicppslrp
SchS7(378aa)       pllaahlgiphvrqawdaieadgihpgadtelapelaelgldrlpepelfvdicppslrp
UrdGT2(365aa)      pllalhlgvpharqtwdavdadgihpgadaelrpelselglerlpapdlfidicppslrp
                    *:* .**: ..*::**: :*   . .*   * ***  ***:*** * **:*:***** *

SimB7(385aa)       pephpakhtlrmrwipvngqrqlepwtytrterprvclttgsrvahdageqdlprrsyef
LanGT2(373aa)      atappaq---mmrwvpangqrrlepwmytkgnrprilvtsgsrlv--------fakktgf
SaqGT5(383aa)      adagpaq---qmrfvpanaqrslapwmyrrgerprvcvtsgsrvtrgr----tydrnyqf
SchS7(378aa)       anagpaq---mmryipangqrrlepwmysrgerrricvtsgsrvak-d----sydrnfef
UrdGT2(365aa)      anaapar---mmrhvatsrqcplepwmytrdtrqrvlvtsgsrvak-e----sydrnfdf
                       **:    ** : .. *  * ** * :  * *: :*:***:.         :.  *

SimB7(385aa)       lcalsasfkdldvdlvvattdpagkalearvpgvragwvpldvlartcdvivhhgggtts
LanGT2(373aa)      lrglvadmaaldaevviatldevaeelrtelpgvragwvpldvvvptcdvvvhhaggvta
SaqGT5(383aa)      lldmvgklteldvellvaapeevadglraehgglragwipldvvaptcdvlvhhaggvss
SchS7(378aa)       lrgmakdvtawdvelivaapqevadaltaelpglragwvpldviaptcdllvhhaggvst
UrdGT2(365aa)      lrglakdlvrwdvelivaapdtvaealraevpqarvgwtpldvvaptcdllvhhaggvst
                   *  :  ..   *.::::*: : ... * :.    *.** ****:. ***::***.**.::

SimB7(385aa)       ltamaaaapqvavpqgavpnlsaqrlaeygaaislfgsegspevivdactkilgdrsyae
LanGT2(373aa)      ltamnagvpqlivsqggnfveaglrisdfgaaitv--dentpeavekacgelignpsyae
SaqGT5(383aa)      ltalnaalpqllmpkgavlveparriarrgaaialtpeeetteaiitacqqlltepsyra
SchS7(378aa)       ltalnagvpqllipkgavmerpalriaergaaitllpgedtaeriadsceellarpgyre
UrdGT2(365aa)      ltglsagepqllipkgsvleaparrvadygaaiallpgedsteaiadscqelhakdtyar
                   **.: *. **: : :*.     . *::  ****::   * : * :  :* ::     *

SimB7(385aa)       rardlsheiaglalpseavdvvaelv----
LanGT2(373aa)      rarelsaeiaalplpaevvgaleglv----
SaqGT5(383aa)      aaetlsreiaamplpadvvgaleklvqspa
SchS7(378aa)       ragelsaeiatmplpshvvaaleqlr----
UrdGT2(365aa)      raqdlsreisgmplpatvvtaleqla----
                    *  ** **: : **: .* .:  *
```

Fig. B1a. Clustal Omega amino acid sequence alignment of SchS7 with known *C*-

glycosyltranferases including SimB7, LanGT2, SaqGT5, and UrdGT2.

```
LanGT1(390aa)       mrvlmintpvsthltplvplawalraaghevlvlgredvlgaaasaglnavsigdwlkld
SchS9(390aa)        mrvlvictpvpthflplvplvwalrsaghevvvtgqpdvlgavraagltgavvgesfdvd
UrdGT1c(391aa)      mrvlflvtpspthftplvslawalrgaghevvvagqpdvlgavasaglnavsigapfnge
SaqGT4(396aa)       mrvlmmttpvpthfqplvplawalraaghevlvaaqpdvlplvqsaglsavsvgtpfhcd
                    ****.: **  **: *** *.****.*****:* .: ***  . :***... :*  :. :

LanGT1(390aa)       dilverlg-gkrplqswgrpaldqlsnvgslwmpqtlevlpayldlardfgpdlvlsdpi
SchS9(390aa)        gmllrglaeeerplqarprpapevlggygklwmghaksvlghygelvrefgpelilsdpl
UrdGT1c(391aa)      akllaglgpdqrplevrprpapesmggygrvwmtharylvgrymefariygpdlivsdpl
SaqGT4(396aa)       dllyeslpagtrpltvrprpapgmlghygrlwmtharyllpeylslarairpdlivadpl
                      *   *    ***    ***   :.  * :** ::  ::  * .:.*   *:*:::**:

LanGT1(390aa)       efislivgedlgvpvvqqrwgvdpisdqvrgagetwfkdilaergltglprpavlldpcp
SchS9(390aa)        eycslilgarlgvpvvhhrwtvdaisgparrlvrpdfrelceelglpglpdpavlldpcp
UrdGT1c(391aa)      eyssllvggvlgvpvvqqrwgvdlisgparaearpgfgplcerlglaglpdpavlldpcp
SaqGT4(396aa)       eyssllvgavlgvpvahhrwgvdeistparsavrpalagvcdrlgieglpdptvlldpcp
                    *: **::*  *****.::** ** **  .*   .  :  :  . *: *** *:*******

LanGT1(390aa)       pslqlagvtpgtpmryvpfngngrlpewlrrdare----rgtarrvavslggrtlayngv
SchS9(390aa)        pslrlpdsepgtsiryvpysgggevpgwlradrg----pgagrqrvavslgn-tlalhge
UrdGT1c(391aa)      pglqapgaepgspirfvpfngngvvpgwlrepr------sasrprvlvtigg-tlalngv
SaqGT4(396aa)       pslqvpgaepavpmryvpyngrivlppwrheeverrptepagtrrvavsmgg-tlavngv
                    *.*:  .  *.  :*:**:.*   :* * :          .   ** *::*. *** :*

LanGT1(390aa)       plmrnildalgslpdvevlatveeeyrealgevpgnvrvidpvplhlllgschavvhhgg
SchS9(390aa)        pftrdllhalagrpgteilatvperhragigavpehvrlidplplhlfldrcdamvhhgg
UrdGT1c(391aa)      plmrgilrafeelpeveavatvdevfrekvgpvpanvrmvdpvplhlvldgcaavvhhgg
SaqGT4(396aa)       pfvrqvlsafagmpgvsvlatvderyraelgeppknvrlidntpldlflgscdavvhqgg
                    *: * :* *:   * .. :*** * .*  :*  * :**::*  **.*.*. * *:**:**

LanGT1(390aa)       sgtamtasafglpqlvlpqladqfwhgdalarvgaaitiesverqdepepvreaaaallg
SchS9(390aa)        agtamtatafglpqltlpqladhfplgdrlaatgagltfdkaaeqddpqliagaldtlls
UrdGT1c(391aa)      agttmtagafglpqlvlpqladhfghgdrvsevgagislddaesqndshrlavelrrlla
SaqGT4(396aa)       agttmttttfglpqlvlpqlayhfghgdriaavgagiafdtaaeqddpallreslaallf
                    :**:**: :******.***** :*  ** :: .**.:::: .  *::   :      **

LanGT1(390aa)       dpayekaagelraeiaampapsrvvtdleklvaar----
SchS9(390aa)        dpaygaaarrlaaemaampspadvaadleqlarqa----
UrdGT1c(391aa)      epefakaaraladsvrdmpapaqvaadltria-gvvgal
SaqGT4(396aa)       epgyrkaavelqaemartpapdtivadlerrhrgevd--
                    :* :  **  *  .:   *:*  :.:** :
```

Fig. B1b. Clustal Omega amino acid sequence alignment of SchS9 with known *O*-glycosyltranferases including LanGT1, SaqGT4, and UrdGT1c.

```
LanGT4(417aa)       mrvlfavfpaaahvhpivplawalqnaghdvrvaih-----pdavglvteaglaavplga
SchS10(430aa)       mrvlltifpatshlysvvplawalqsaghevvvashsgvvdpgvignigaagltavslgt
UrdGT1a(426aa)      mrvlfsimpatahlypivplawalqasghevrvash-----pdmaatitaagltavsvgr
SaqGT2(443aa)       mrvlfvtfpatahlfpivplawalqaaghevrvashsgvvepdmtatiaatgltpvplgp
                    ****:  :**::*:. :******** :**:* ** *     *.  . :  :**: * :*

LanGT4(417aa)       rhklagvvefnsnldlldsldtlsldteddsarwetqwevmrnvllvyqp---------
SchS10(430aa)       pdelspalgahtgdakpdrpslgfdpnepeesggwrtarsilasmfslyypaper-qggr
UrdGT1a(426aa)      padlgalmrdgasdqcleqitealeigs-ddlnlrnairyytlagvslyfptdpq-raes
SaqGT2(443aa)       gtelaalarvnaaadqtnrptlaldpa---aggdwertrsyviglllslyyppvaadsger
                      .*.      :     :     :.          .         . :* *

LanGT4(417aa)       --vlpdlvdfvqdwkpdlvvwdpfcvpaavaarvggaaqarflwgrdnigwlraksleql
SchS10(430aa)       rpvldnlvdfarawrpdlvlwdplmlaapiaarvsgaaharlvwgmdnvavihdrtkrel
UrdGT1a(426aa)      dravddlvafarswrpdlvlwdplvlaapvaarlsgaaharvlygldyvgwaqqrfteri
SaqGT2(443aa)       wamadelvafarswgpdlvlwdplcppapiaaracgaaharvlwgldnlawmremal---
                         :** *.: * ****:***:   * :***  ***:**.::* * :.  :

LanGT4(417aa)       aargaapaddplvplmsdmlapygleyeeefltgqwtidpmpqgmrlpldlpytgvrrvp
SchS10(430aa)       adprseltehpwlswfgpmlerygleldde mllgqwtldltqsrmrhpldlthipvrrvp
UrdGT1a(426aa)      grraqpltehplaelmrptlrrfgldfdaelavgqwtldllpermrlpvdlrytalrrvp
SaqGT2(443aa)       -rrpegadrdplvewlrplldrygdtfdeevltgqctldlvpermrlpldrryqavrrvp
                             .*    :   *  :*   : *.  ** *:*   . ** *:*  :  :****

LanGT4(417aa)       yngtaalpawvhqrperprvvltlgiggrgrqlfrqsgvsfpevveavagldvelvatvg
SchS10(430aa)       ytgaaalpdwlharperpravltlgvsrrk-ifgkysgfpmreffesvsaldvevvatln
UrdGT1a(426aa)      ytgtttvpqwlydepkrprvcltlgmstrk-flsghgaspiadifglveeldielvatvn
SaqGT2(443aa)       yngagvvpgwlaarphrprvcltlgvstrk-lftehqgfsvaelfalvaeldvelvatln
                    *.*: .:* *:  .*.***. ****:. *   :    .  . :..  *  **:*:***:.

LanGT4(417aa)       aaegkpsarhpdnirlieymplnhllptcsaiihhggggtfaaavahqvpqlvtpmpfw-
SchS10(430aa)       neqlaavgkvpdnvrtveyvplnqvlptssaiihhggggtfaaavahrvpqlvvplvmw-
UrdGT1a(426aa)      saqladveelpdrvrivdylpldlllptcsavvhhggggtfaaavahqvpqlipapgegg
SaqGT2(443aa)       saqlagvdelppnvriveylpln lllptcaavihhggggtyaaavahrlpqlvipvpkw-
                      :     . * .:* ::*:**: :***.:*::*******:******::***:

LanGT4(417aa)       geaataqyvadngaglvidssqftpdalrksltrllddrsfrdgaaalyqemqaapspgd
SchS10(430aa)       demvtaryvadmgaglvadpaaldvdglhkqlvrlledpsfqlgarglyeemlaapaptd
UrdGT1a(426aa)      drvafaryveqrgagltvsrtdysadvfaeqlfrvlhepsfqdgaaglhddmlatpsprd
SaqGT2(443aa)       deevtarhvvergaglrvdep-ftvadvrdgllrllheprfragaeslhrdmlaapspve
                    .. . *::* : ****  .        . . * *:*.:  *: ** .*: :* *:*:* :

LanGT4(417aa)       lvpvleeltarhrr-------------------
SchS10(430aa)       vvpllerltaerr--------------------
UrdGT1a(426aa)      vvpvleklsdryrr-------------------
SaqGT2(443aa)       vvpvlerltarhrrprpegvrsphgqestcays
                    :**:**.*: . *
```

Fig. B1c. Clustal Omega amino acid sequence alignment of SchS10 with known *O*-glycosyltranferases including LanGT4, SaqGT2, and UrdGT1a.

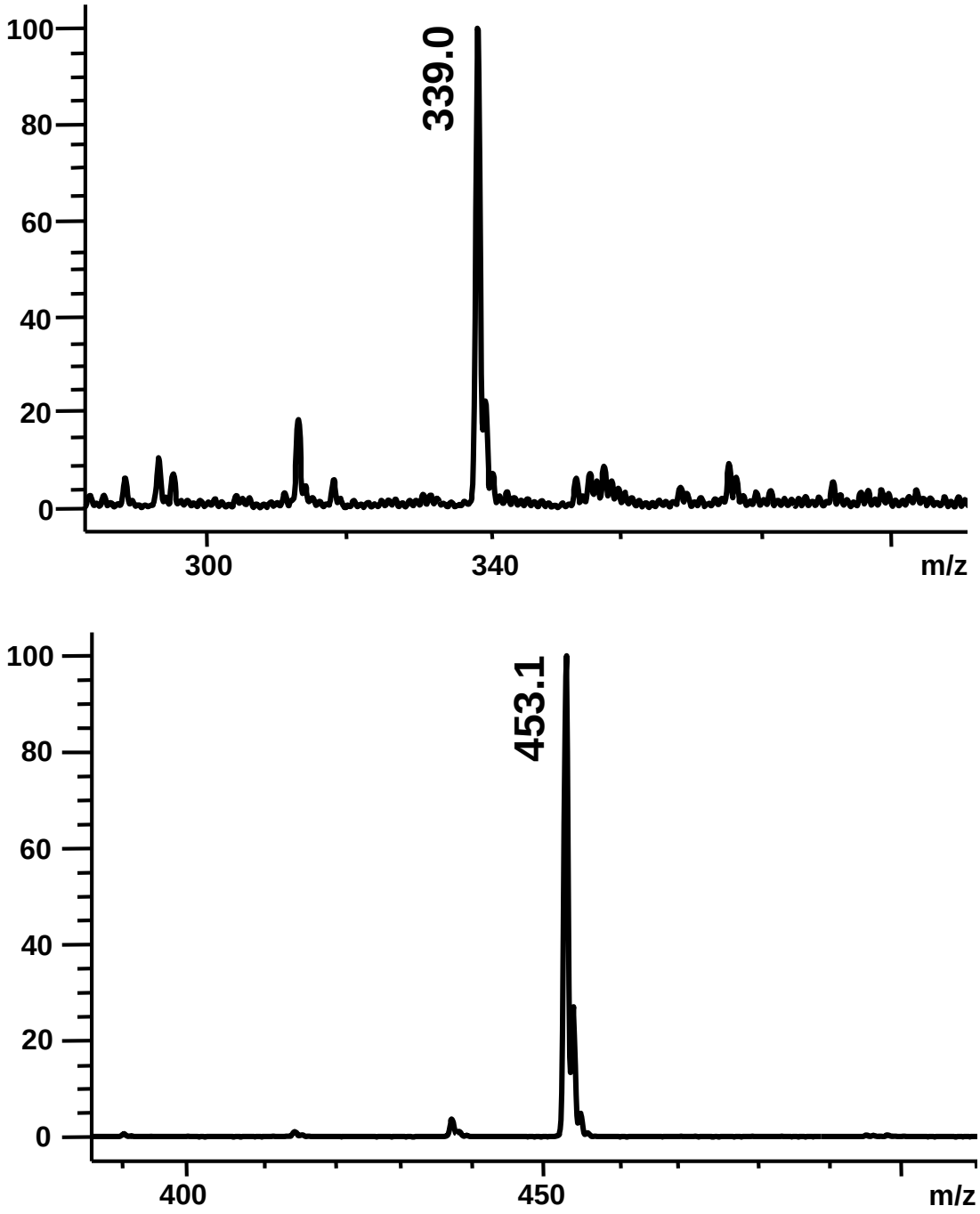


Fig. B2. ESI-MS spectrum of rabelomycin (**1**) with the $[M+H]^+$ ion peak at *m/z* 339.0 and GG31 (**2**) with $[M+H]^+$ at *m/z* 453.1.

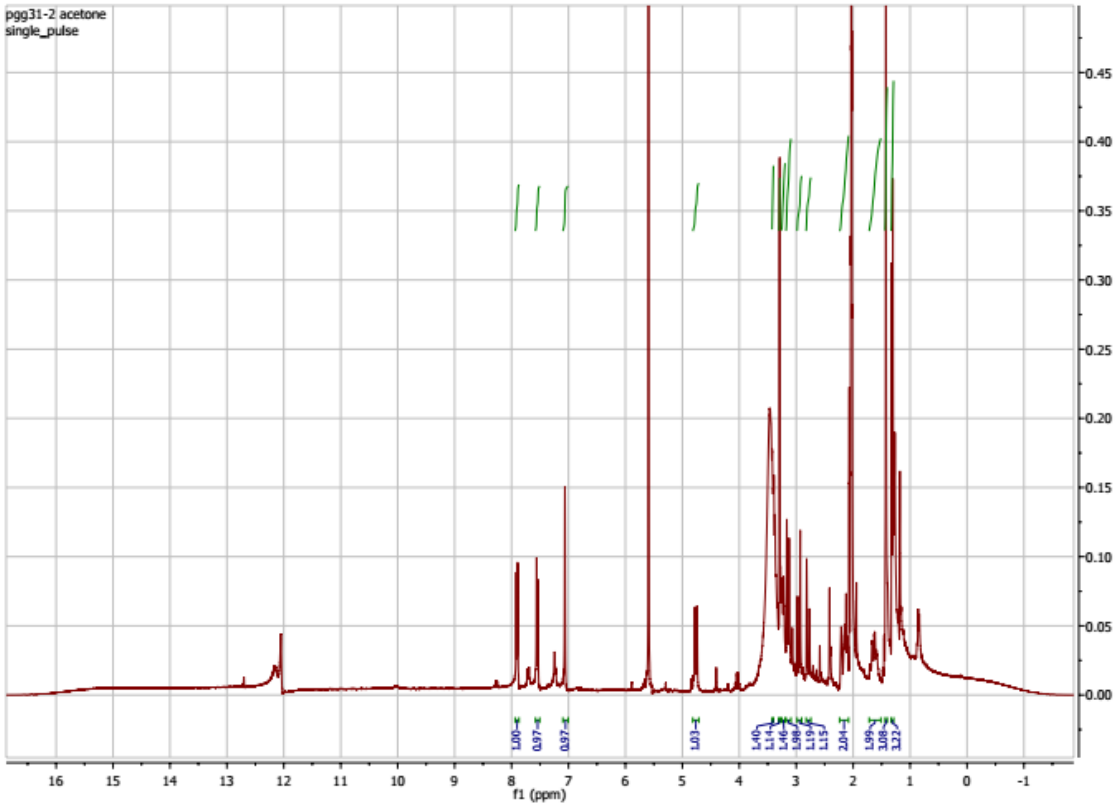


Fig. B3a. ^{1}H NMR for GG31 in $(CD_3)_2CO$, 300 MHz, JEOL ECX-300.

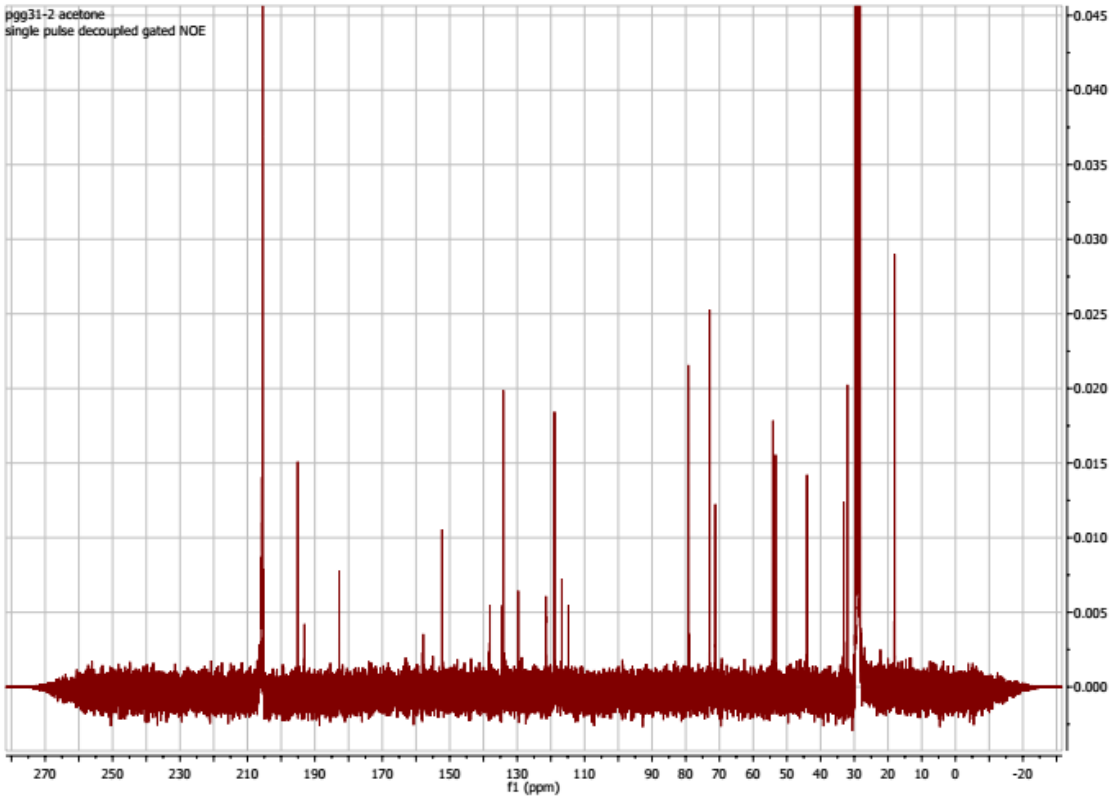


Fig. B3b. ^{13}C NMR for GG31 in $(CD_3)_2CO$, 300 MHz, JEOL ECX-300.

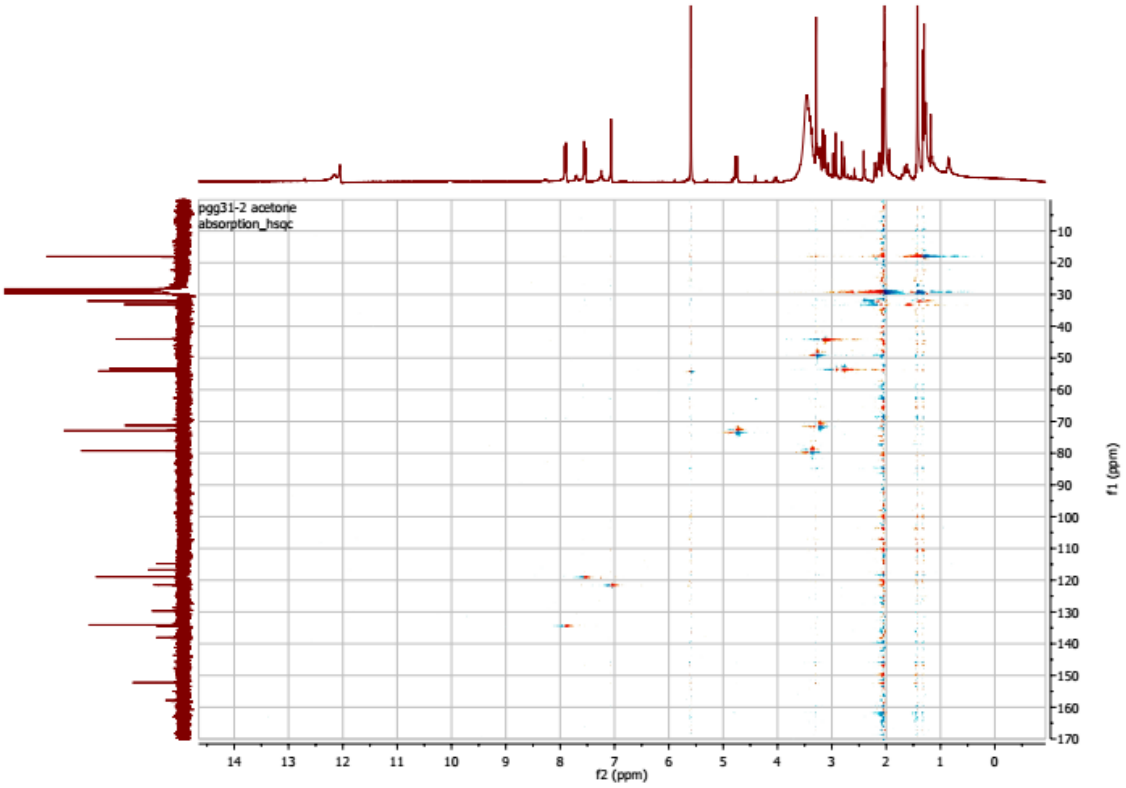


Fig. B3c. HSQC NMR spectrum for GG31 in $(CD_3)_2CO$.

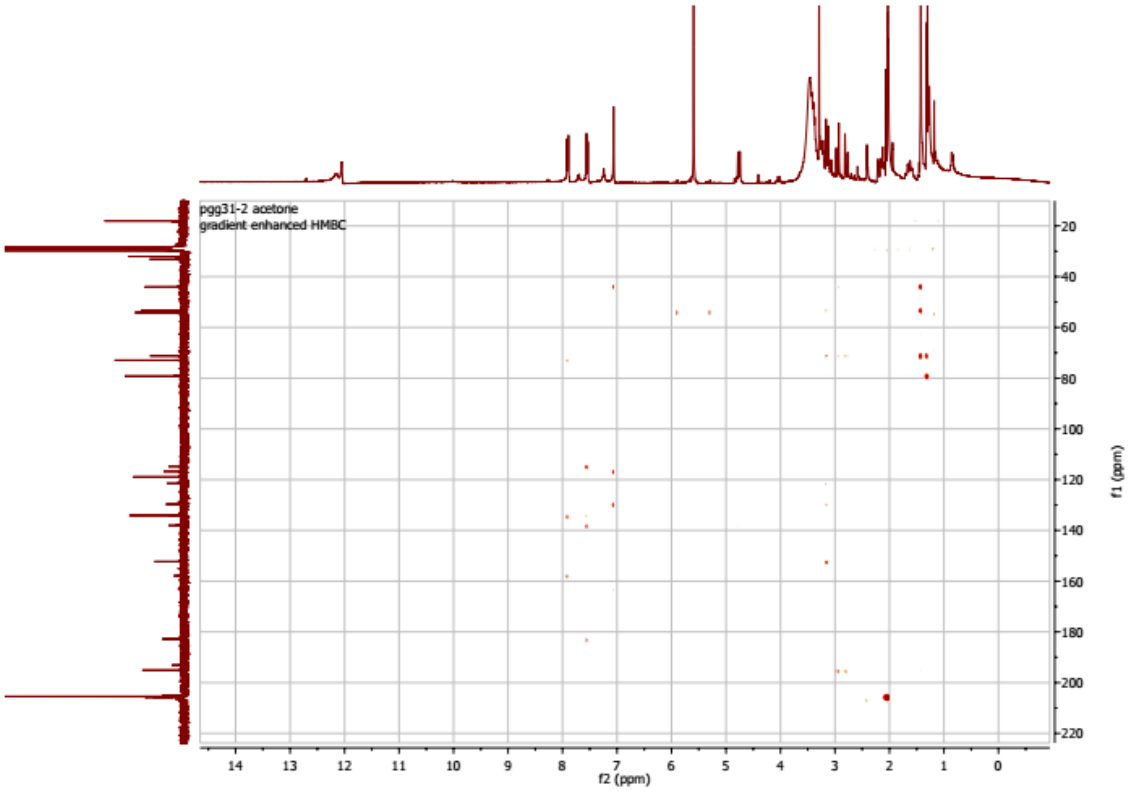
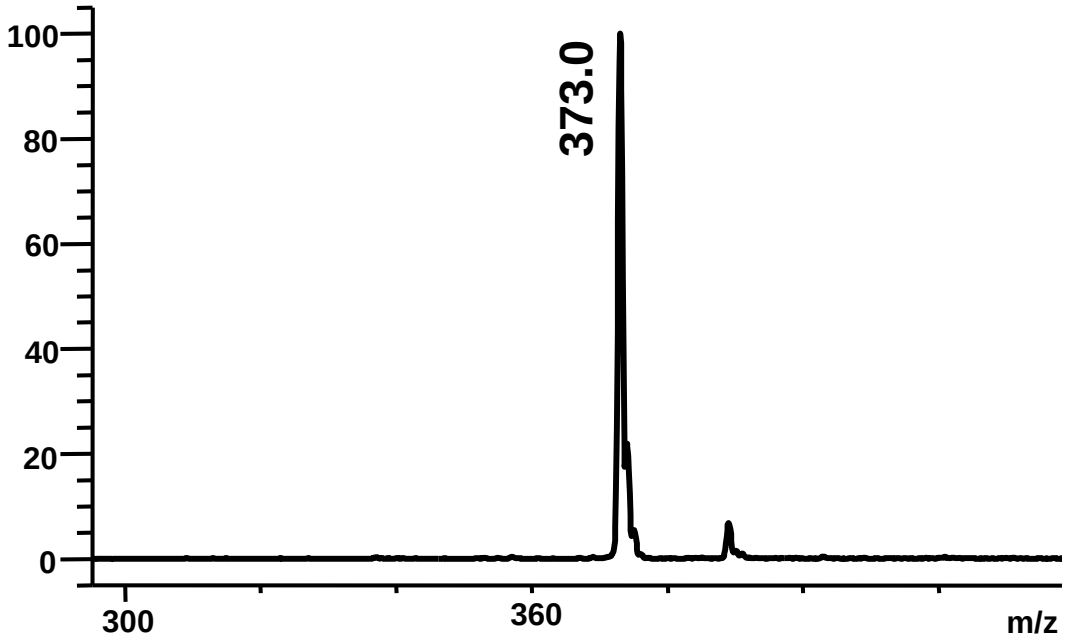
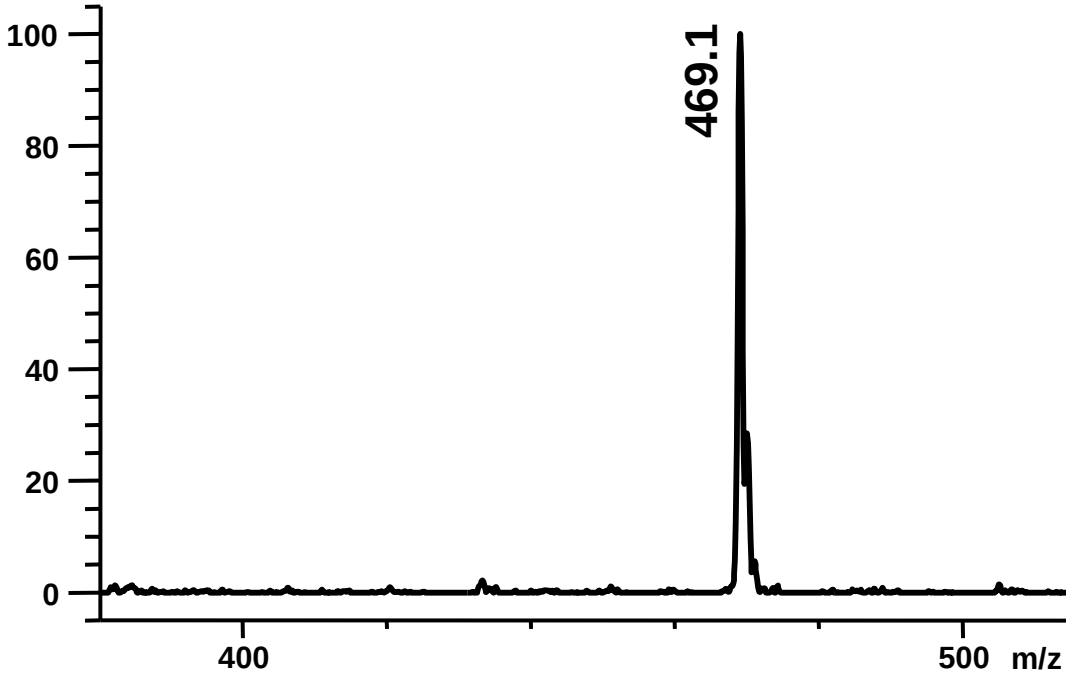


Fig. B3d. HMBC NMR spectrum for GG31 in $(CD_3)_2CO$.


100
80
60
40
20
0
373.0
300
360
m/z


100
80
60
40
20
0
469.1
400
500
m/z

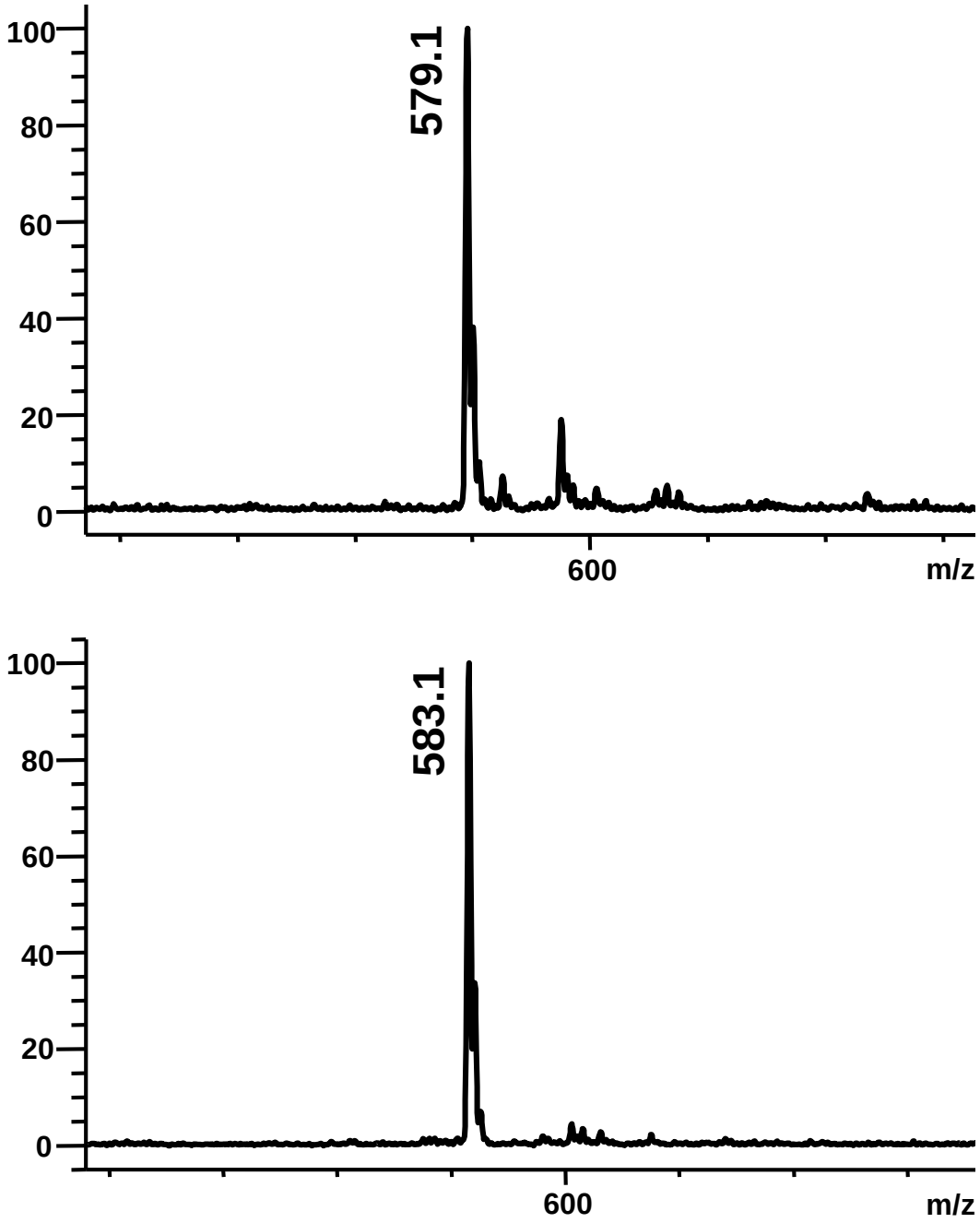


Fig. B4. ESI-MS spectrum of **3** with the $[M-H]^-$ ion peak at *m/z* 373.0, **4** with the $[M-H]^-$ ion peak at *m/z* 469.1, **5** with $[M+H]^-$ ion peak at *m/z* 583.1 and **6** with $[M-H]^-$ ion peak at *m/z* 579.1.

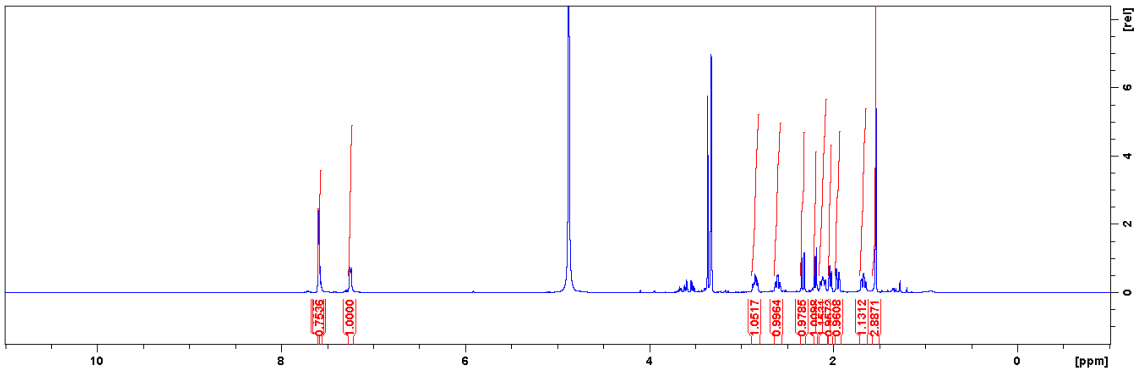


Fig. B5a. ^{1}H NMR for urdamycin X in CD_3OD, 500 MHz, Bruker AvanceIII HD Ascend-500.

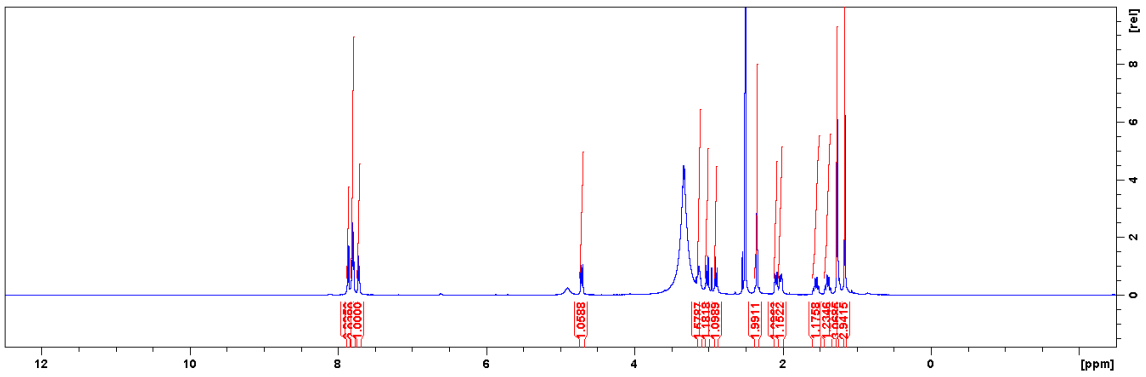


Fig. B5b. ^{1}H NMR for 3'-deoxyaquayamycin in DMSO-d_6, 500 MHz, Bruker AvanceIII HD Ascend-500).

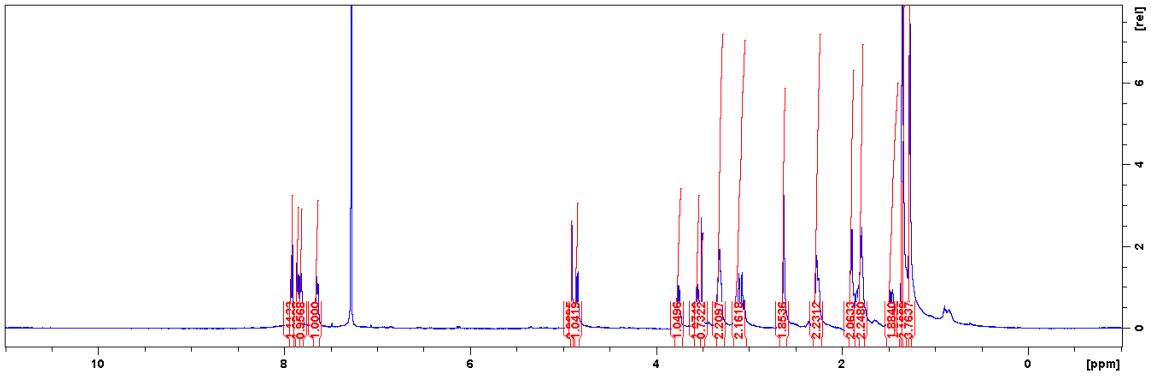


Fig. B5c. ^{1}H NMR for OZK1 in $CDCl_3$, 500 MHz, Bruker AvanceIII HD Ascend-500.

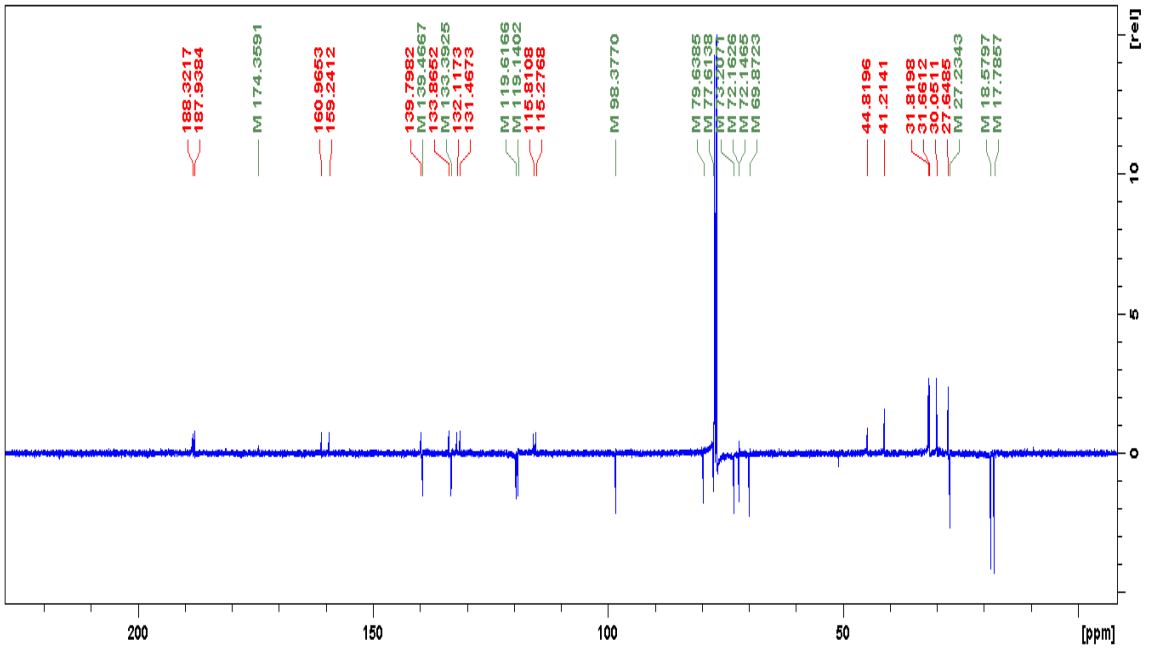


Fig. B5d. ^{13}C NMR for OZK1 in $CDCl_3$, 500 MHz, Bruker AvanceIII HD Ascend-500.

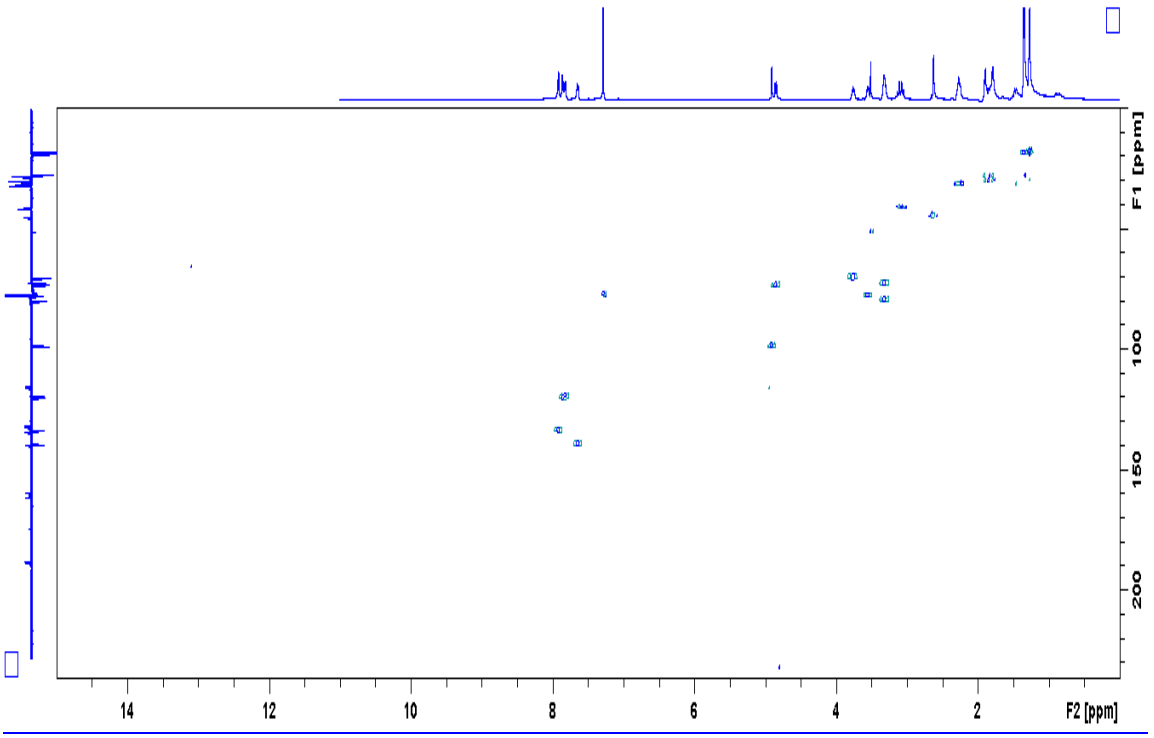


Fig. B5e. HSQC NMR for OZK1 in $CDCl_3$, 500 MHz, Bruker AvanceIII HD Ascend-500.

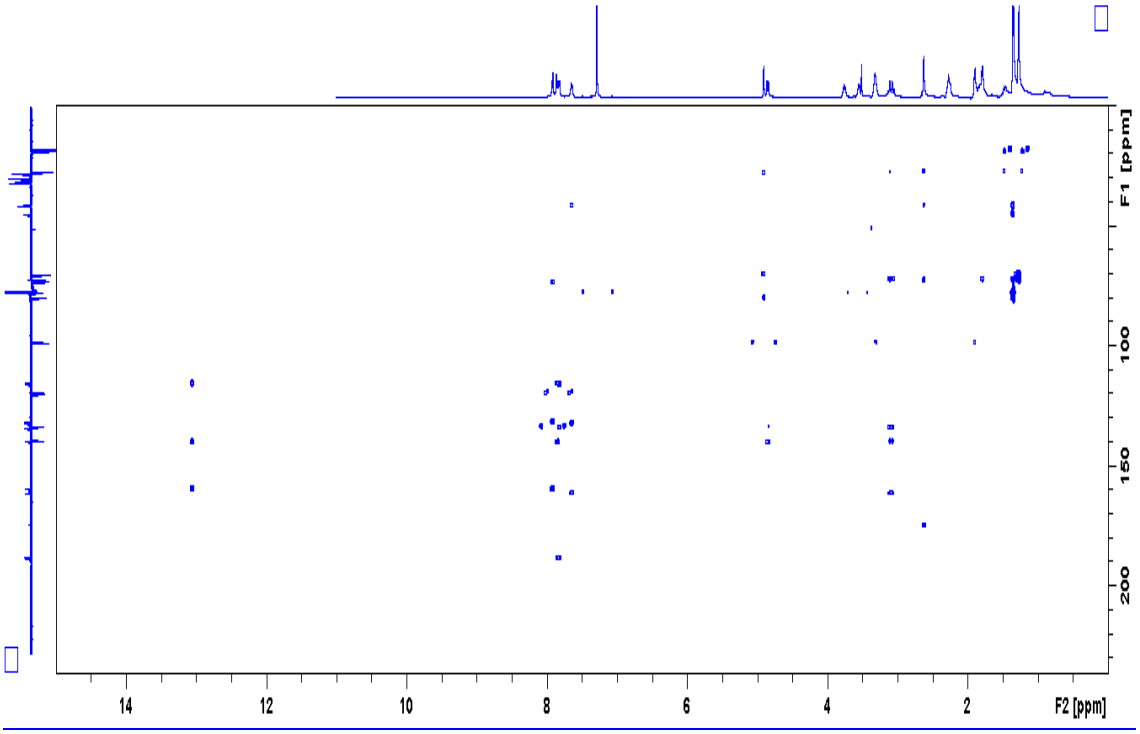


Fig. B5f. HMBC NMR for OZK1 in $CDCl_3$, 500 MHz, Bruker AvanceIII HD Ascend-500.

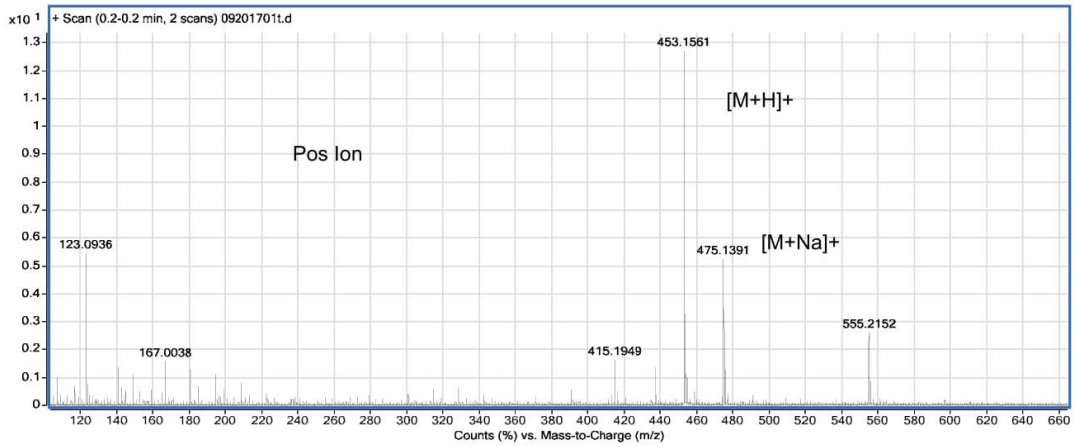


Fig. B6a. High resolution mass spectrum for GG31 with $[M+H]^+$ at *m/z* 453.1561.

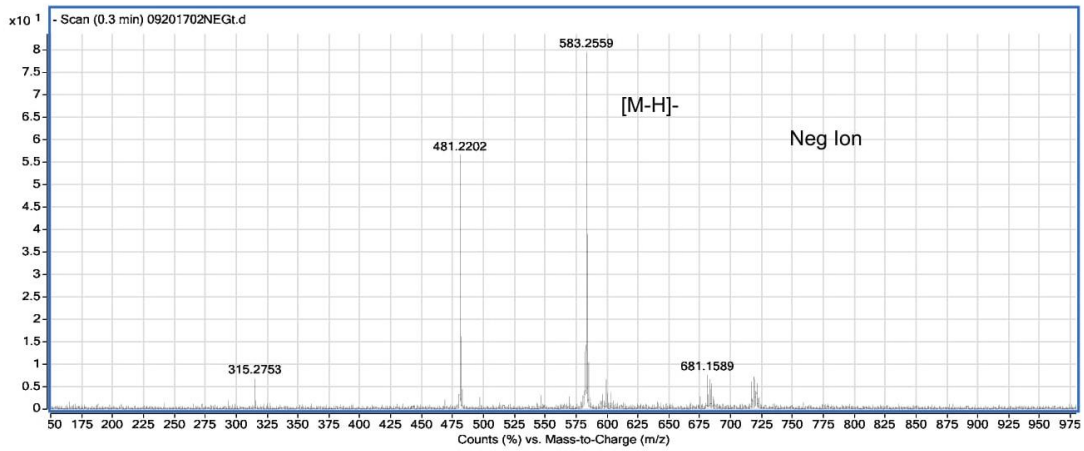


Fig. B6b. High resolution mass spectrum for OZK1 with $[M-H]^-$ at *m/z* 583.2559.

APPENDIX C

SUPPORTING INFORMATION for CHAPTER 3

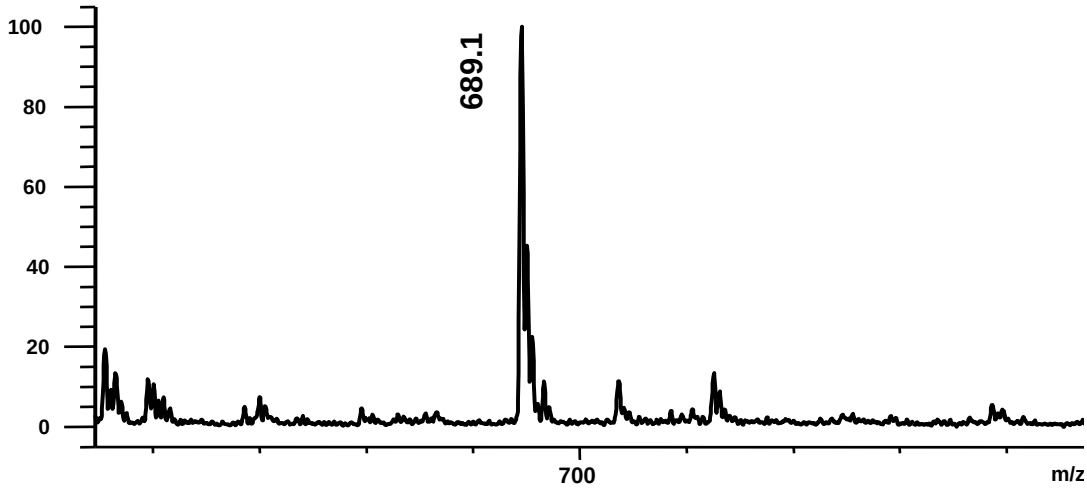


Fig. C1. ESI-MS spectrum of Sch47544 (**1**) with the $[M-H]^-$ ion peak at *m/z* 689.1.

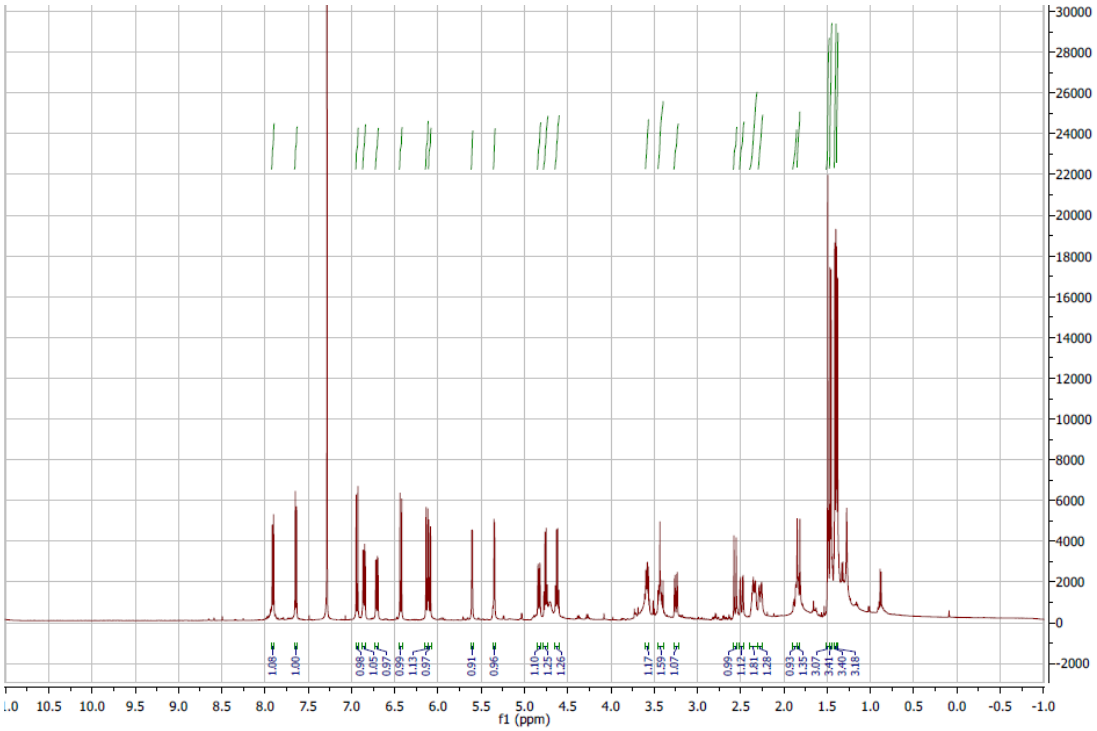


Fig. C2. ^1H NMR for Sch47554 (**1**) in $CDCl_3$ (500 MHz, Bruker AvanceIII HD Ascend-500).

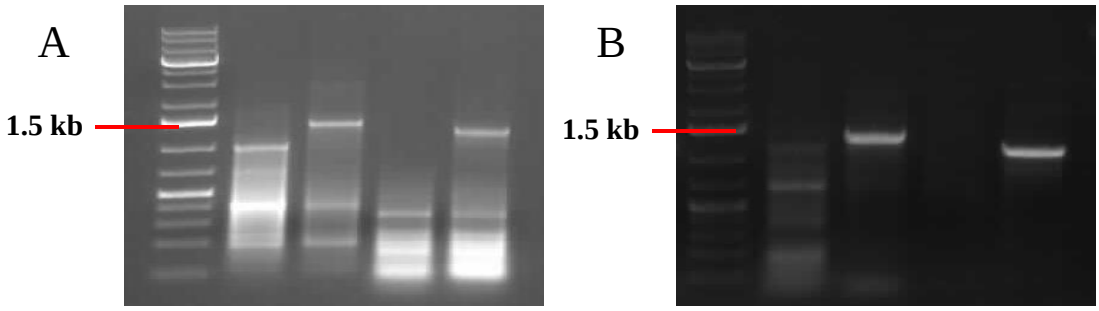


Fig. C3. Confirmation of gene disruption of s*chA16* (**A**) and s*chA21* (**B**) by PCR.

APPENDIX D

SUPPORTING INFORMATION for CHAPTER 4

TABLE D1. Recipes of the media used in Chapter 4.

	LB	**SOB**	**SOC**	**2xYT**	**TB**	**2xTB**	**Superbroth**
Tyrptone (g/L)	10	20	20	16	12	24	32
Yeast extract (g/L)	5	5	5	10	24	42	20
NaCl (g/L)	10	0,58	0,58	5	-	-	5
Glycerol (ml/L)	-	-	-	-	4	15	-
Phosphate buffer (0.17 M KH_2PO_4, 0.72 M K_2HPO_4) (ml/L)	-	-	-	-	100	100	-
KCl (g/L)	-	1,9	1,9	-	-	-	-
$MgCl_2$ (g/L)	-	0,95	0,95	-	-	-	-
$MgSO_4$ (g/L)	-	1,21	1,21	-	-	-	-
Glucose (g/L)	-	-	3,6	-	-	-	-

TABLE D2. Oligonucleotides used in Chapter 4.

Primer Number	Primers	Oligonucleotides
1	16S rRNA universal primer 8F-F1	ggctaccttgttacgacttc
2	16S rRNA universal primer Rn1	agtttgatcctggctcag
3	10-25-15-BGC-A-SpeI-NdeI-fw	aactagtcatatgttggtggagatccgcgaggatcgcacc
4	102515-A-NheI-rev	tggccggcggcctgctagccctgcgcctggcccaaac
5	102515-B-NheI-fw	gccaggcgcagggctagcaggccgccggccaggccg
6	10-25-15-BGC-B-PmeI-HindIII-rev2	aagtttaaacaagctttcaacgaggacgaaagacgatat
7	BGC-A walker primer F1	catgtgctctgtcccaccgccg
8	BGC-A walker primer R1	agggcttcttccgcctgctcaa
9	BGC-A walker primer F2	tccttcggtttcctgctggcgc
10	BGC-A walker primer R2	accaggcccggctggacgagca
11	BGC-B walker primer F1	cagttggcgacaggcctgggcg
12	BGC-B walker primer R1	catcagcagcggacgcagcc
13	BGC-B walker primer F2	cttcgtcttcgcctccttcgg
14	BGC-B walker primer R2	agcgtgctcgatgtcgccggaa
15	Eho10-CrtX-NcoI-SmaI-fw	aaccatggcccgggatgagccattttgccatt
16	Eho10-CrtX-HindIII-rev	aaaagctttcacgatacgctctcactccctgctatggc
17	J23119-B0034-NcoI-CrtX-fw	agatctttgacagctagctcagtcctaggtataatgctagctactaga gaaagaggagaaaccatggatgagccattttgccatt
18	Eho10-CrtX-HindIII-rev	aaaagctttcacgatacgctctcactccctgctatggc
19	10-25-15-CrtI-NdeI-fw	aacatatgatgacccaaggtaacgcgcccaagcgggccat
20	10-25-15-CrtI-HindIII-rev	aaaagctttcatggcttaccgtgctcctcgaggatgacgc
21	10-25-15-CrtY-NdeI-fw	aacatatgatgcgccctgacctgctgatcgtcgggggt
22	10-25-15-CrtY-HindIII-rev	aaaagctttcatggtgcttccttggactgaggggggacgc
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24	Eho10-CrtX-HindIII-rev	aaaagctttcacgatacgctctcactccctgctatggc
25	102515-TnbA-NdeI-fw	aacatatgacgcaattggatttcaccggcaagcg
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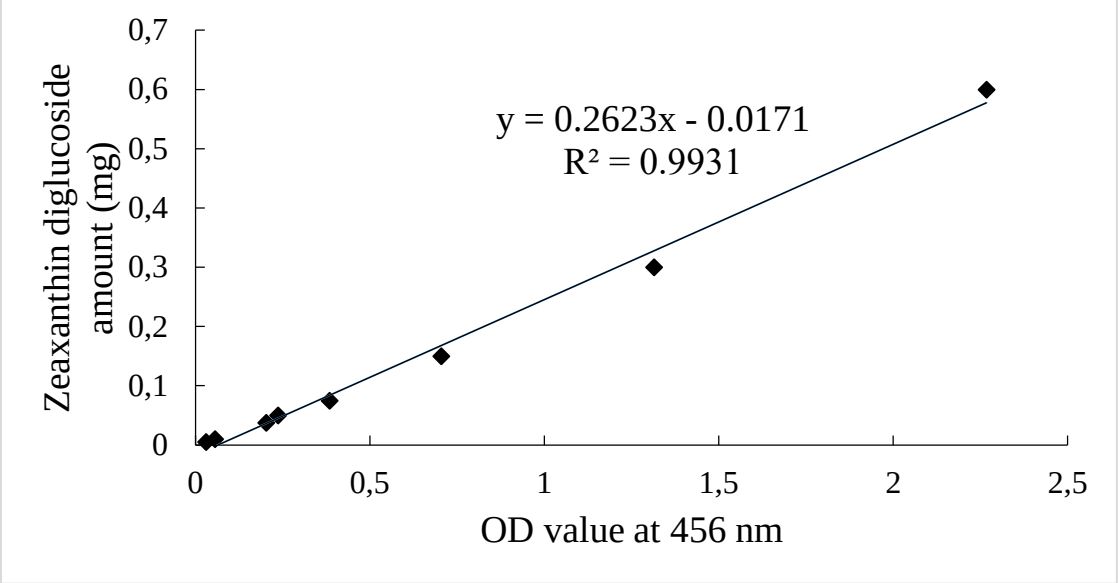

Fig. D1. Standard curve for zeaxanthin diglucoside using UV spectrophotometer at 456 nm.

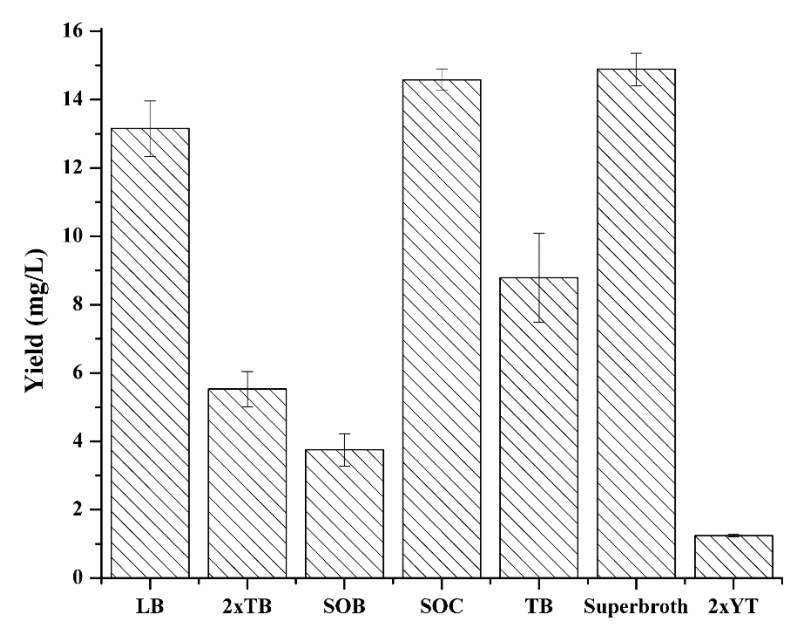

Fig. D2. The effects of different media on the yield of zeaxanthin diglucoside in the culture tubes (5mL).

Sequence D1. *Pseudomonas* sp. strain 102515 16S ribosomal RNA gene, partial sequence

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Sequence D2. *Pseudomonas* sp. strain 102515, whole carotenoid (*Pscrt*) biosynthetic gene cluster

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Sequence D3. *Pseudomonas* sp. strain 102515 *tnbA* DNA sequence

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CURRICULUM VITAE

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RESEARCH INTERESTS

Metabolic engineering, Synthetic biology, Natural product discovery and biosynthesis, Microbial production, Next Generation Sequencing, Metagenomics, Human microbiome,
CRISPR/Cas9 gene editing system

EDUCATION

Ph.D, Biological Engineering May 2019
Utah State University, Logan, UT, USA GPA 3.92
Dissertation: "Exploring the potential of microorganism for natural product biosynthesis" Adviser: Dr. Jixun Zhan

B.S., Chemical Engineering June 2010
Izmir Institute of Technology, Izmir, Turkey GPA 3.06
Graduation Project: "Selective dehydration of fructose to 5-hydromethylfurfural" Adviser: Prof. Dr. Selahattin Yilmaz
Engineering Design Project: "Hydroxytyrosol production from olive trees" (Complete Plant Design), Adviser: Prof. Dr. Mehmet Polat
Freshman Project: "Chemical migration of carcinogens from disposable cups to hot drinks" Adviser: Prof. Dr. Fikret Inal

CURRENT PROJECTS

- Understanding the biosynthesis of natural products in fungi using the heterologous expression approach in *E. coli* and *S. cerevisiae*
- Identification and activation of biosynthetic gene clusters responsible for the production of pharmaceutically important natural products in *Streptomyces* through genome analysis and CRISPR/Cas9 technology
- Chromosomal insertion of exogenous genes for natural product biosynthesis in *E. coli* to eliminate antibiotic use and increase the titer

PUBLICATIONS

Book Chapters:
Fidan, O., Zhan, J.* (**2018**). Reconstitution of Medicinally Important Plant Natural Products in Microorganisms in *Molecular Pharming: Applications, Challenges and Emerging Areas* edited by Allison Kermode, Simon Fraser University and Liwen Jiang, John Wiley & Sons, NJ-USA.

Journals: (# equal contribution, * corresponding author)

Fidan, O., Zhan, J.* (**2019**). Discovery and engineering of an endophytic *Pseudomonas* strain from *Taxus chinensis* for efficient production of zeaxanthin diglucoside. (in preparation)

Yuan, H., Wang, H., **Fidan, O.**, Qin, Y., Xiao, G.,* Zhan, J.* **(2019)** Identification of new glutamate decarboxylases from *Streptomyces* for efficient production of γ-aminobutyric acid in engineered *Escherichia coli*. *Journal of Biological Engineering*, 13, 24.

Fidan, O., Yan, R., Zhu, D., Zhan, J.* **(2019).** Improved production of antifungal angucycline Sch47554 by manipulating three regulatory genes in *Streptomyces* sp. SCC-2136. *Biotechnology and Applied Biochemistry*, 1-10.

Fidan, O.,# Yan, R.,# Gladstone, G., Zhu, D., Zhan, J.* **(2018).** New insights into the glycosylation steps in the biosynthesis of Sch47554 and Sch47555. *ChemBioChem*, 19, 1424-1432.

Fidan, O.,* Zhan, J. **(2015).** Recent advances in engineering yeast for pharmaceutical protein production. *RSC Advances* **5**, 86665–86674.

PRESENTATIONS

Oral Presentations:

Fidan, O., Liang, L., Zhan, J., **(2019).** Bio-halogenation of flavonoids by a fungal flavin-dependent halogenase. 2019 Institute of Biological Engineering (IBE) Annual Conference, St. Louis, MO, April 4-7.

Fidan, O., Zhan, J., **(2018).** The Roles of Three Regulatory Proteins in the Biosynthesis of Angucyclines Sch47554 and Sch47555. 2018 IBE Annual Conference, Norfolk, VA, April 5-7.

Poster Presentations:

Fidan, O., Zhan, J., **(2018).** Isolation of an endophytic carotenoid-producing *Pseudomonas* strain and functional analysis of the carotenoid biosynthetic genes. IBE 2018 Annual Conference, Norfolk, VA, April 5-7.

Fidan, O., Yan, R., Gladstone, G., Zhu, D., Zhan, J., **(2017).** Functional characterization of three glycosyltransferases involved in the biosynthesis of Sch47554 and Sch47555. 2017 IBE Annual Conference, Salt Lake City, UT, March 30- April 1.

Fidan, O., Liu, L., Fronk, T., Britt. D. W. **(2014).** Vapor phase deposition of alkyl- and fluoro-silanes on nanoporous glass. ACS 2014 Colloid & Surface Science Symposium, Philadelphia, PA, June 22-25.

Fidan, O., Liu, L., Britt. D. W. **(2014).** Surface Modification of Nanoporous Glass by Vapor Phase Deposition of Trifunctional Silanes. Research Symposia: Research Week 2014, USU, Logan, UT, April 10.

Fidan, O., Liu, L., Britt. D. W. **(2013).** Controlled porous glass beads in nanofluidics systems for the purpose of energy absorption. NanoUtah - Nanotechnology conference & exhibition, Salt Lake City, UT, October 18.

RESEARCH EXPERIENCE

Department of Biological Engineering, Utah State University, Logan, UT

Graduate Research Assistant in the Metabolic Engineering Lab
May 2015-Present

- Performed recombinant DNA technology: Molecular cloning in *E. coli*, primer design, PCR, gel electrophoresis, DNA isolation, plasmid construction, transformation, protoplast preparation, and DNA sequence analysis
- Worked with recombinant protein: protein expression and purification from *E. coli* and *S. cerevisiae,* SDS-PAGE
- Performed solvent extraction of bioactive compounds from liquid and solid bacterial cultures
- Executed and evaluated analytical experiments: HPLC, Mass Spectrometry, Natural Product Analysis, Purification and Structure identification using NMR
- Conducted Microbiome analysis: Total DNA extraction from mice fecal samples, next-generation sequencing preparation and microbiome analysis using MOTHUR
- Performed genome analysis of several bacterial and fungal strains to identify the responsible gene cluster for natural products
- Exploited CRISPR/Cas9 system for the activation of silent biosynthetic gene clusters in various *Streptomyces* species

Graduate Research Assistant in the Synthetic Biological Engineering Lab Sept. 2014-May 2015

- Adapted an experimental setup for real-time monitoring of recombinant *E. coli* to observe the secretion of GFP-fused PHB bioplastic granules
- Performed the fluorescence microscope monitoring of recombinant *E. coli* for the bioplastic secretion
- Developed a laboratory module on the protein expression and purification using synthetic biological engineering techniques for engineering students

Graduate Research Assistant in the Biomaterials & Surface Chemistry Lab Aug. 2013-Sept. 2014

- Developed an experimental setup for surface modification of nanoporous glass
- Performed the surface modification of nanoporous glass by silanization
- Analyzed surface properties of modified and unmodified nanoporous glass using SEM, XPS, TGA, BET, AFM and Goniometer
- Investigated the effect of different silanes and silanization techniques on surface modification efficiency
- Conducted preliminary Nano Energy Absorption System (NEAS) experiments and improved the experimental setup

OTHER EXPERIENCE

Lab manager and safety officer in the Metabolic Engineering Lab
Utah State University, Logan, UT, July 2017-Present
- Inspected and managed the lab safety and cleaning
- Trained new lab members for the instruments
- Maintained the lab instruments

Teaching Assistant for BENG 5620/6620 Metabolic Engineering
Utah State University, Logan, UT, Fall 2017 and 2018
- Led the undergraduate and graduate students for lab sessions to conduct experiments and obtain hands-on gene cloning skills and genetic engineering techniques.

Chemical Process Management Internship
Ege Vitrifiye Incorporated Company, Izmir, Turkey, Summer 2009
- Worked alongside the engineers to observe all chemical processes from raw materials to out-to-door products
- Performed quality control experiments and necessary calculations

Quality Control Lab Internship
PETKIM Petrochemicals Corporation, Izmir, Turkey, Summer 2008
- Conducted experiments in Gas Chromatography, Polymer and General Chemistry labs
- Observed and performed ISO-9001 quality management system
- Gained experience in running various instruments such as GC, HPLC.

SKILLS

Languages: Turkish (native language), English (fluent)
Computer Skills: MATLAB, SAS, Microsoft Word, Excel, PowerPoint, Mendeley, EndNote, DNAMAN, CLC Sequence Viewer, Mestrenova, MOTHUR, BLAST, AntiSMASH, RAST, Chem Bio Draw Office
Instruments: HPLC-MS, NMR, PCR, RT-PCR, IR

AWARDS & HONORS

Outstanding engineering graduate scholar of 2018, Biological Engineering Department at Utah State University
Scholarship from the Republic of Turkey's Ministry of National Education for pursuing Graduate Education in the US

MEMBERSHIPS

Institute of Biological Engineering Student Membership
American Heart Association Student Membership

WORKSHOPS & TRAINING

Grant Proposal Writing Workshop at USU, 2018
Lab Safety Training at USU, 2013
ESL Language Training-PALS at Rutgers University, 2012-2013
English Language Training at 9 Eylul University, 2012
Entrepreneur Leadership Training, 2010

INSTITUTIONAL SERVICE

Supervised Undergraduate Students:
Barner Wald, Spring 2019
Mike Langston, Fall 2017, Summer & Fall 2018- Engineering Undergraduate Research Program (EURP)
Christopher Ruben, Fall 2018
Izabella VanderToolen, Spring 2017
Greg Jensen, Spring 2016
Jaden Turner, Fall 2015
Supervised K-12 students:
Angela Z. (7th Grade), Fall 2018
Jacqueline B. (High school), 2018-USU Biotech Summer Academy
Jaden M. (High school), 2018-USU Biotech Summer Academy
Owen W. (High school), 2018-USU Biotech Summer Academy