Biosynthetic Studies of the α-Amylase Inhibitor Trestatin in *Streptomyces dimorphogenes*

By

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Abstract

The prevalence of obesity and diabetes in the United States and globally has been rising in the last few decades and it continues to climb. One of the causes for this increase is the over consumption of sugars and/or carbohydrates. Among medications available to treat obesity and diabetes are the α-glucosidase (α-amylase) inhibitors. This class of medication decreases carbohydrate hydrolysis in the intestines, leading to reduced glucose absorption. Trestatins are a group of α-amylase inhibitors produced by the soil bacterium *Streptomyces dimorphogenes*. Despite their high pharmaceutical potential, little is known about their modes of formation in nature. Therefore, the main objective of this study is to identify the genes involved in trestatin biosynthesis and to understand their roles in the pathway. To confirm the production of trestatin in *S. dimorphogenes*, the bacterium was grown on R2A agar plates and cultured in a liquid production medium for 4.5 days. The culture broth was passed through an ion-exchange [Dowex 50Wx2 (H^+ form)] column and the lyophilized crude product was analyzed by mass spectrometry. To create mutant strains of *S. dimorphogenes*, two plasmids, pTMAD003 and pTMAD004, were constructed using Gibson Assembly and subsequently transferred into *S. dimorphogenes* through conjugation. However, the conjugation experiments have so far been unsuccessful. Alternative methods for gene transfer and inactivation are currently being investigated.

Introduction

Two of the current major problems related to health in the U.S. are obesity and diabetes. According to a recent study by the Center for Disease Control and Prevention (CDC), more than 36.5% of U.S. adults are obese. The State of Colorado has the lowest obese adult population at 20.2%, with 9.3% of its population diabetic, whereas Mississippi has the highest rate of diabetes at 14.7 %. In order to help fight these problems, efforts to discover and develop new antidiabetic drugs, particularly those of natural origin, are needed.

The trestatin complex (Fig. 1), which contains trestatins A, B, and C as major components, was discovered by Hoffmann-La Roche Ltd. in the early 1980s from a culture broth of the soil bacterium *Streptomyces dimorphogenes*. These natural products showed potent inhibitory activity against various α-amylases,[1,2] enzymes that digest carbohydrates to glucose. α-Amylase inhibitors have been known to have good anti-diabetic properties.[3] Therefore, the trestatins may be developed as useful therapeutic agents for the treatment of diabetes and obesity.

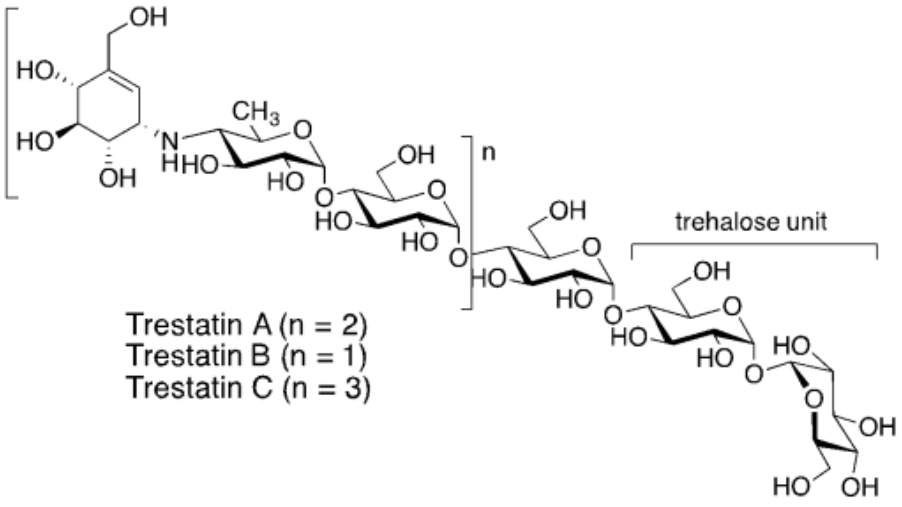

Figure 1. Chemical structures of trestatins

α-Amylase inhibitors are also known as starch blockers. Starches are complex carbohydrates consisting of a large number of glucose units joined by glycosidic bonds. They are used by most green plants as an energy storage. Starches need to be broken down into simple sugars, usually by the digestive enzyme amylase, to be absorbed by the body. Amylase is found mainly in saliva and pancreatic fluid; so digestion of starch starts from the moment we put starch rich food in our mouth. Inhibiting α-amylase will decrease the breakdown of starch in our body and lead to a decrease in sugar intake in the body.

Trestatins may aid in weight loss because they block the breakdown of starch into simple absorbable sugar molecules. When glucose is present in our body, our cells absorb it to make high energy molecules, like ATP or acetyl-CoA, to power the cell. If the cell acquires too much glucose, there will be large production of acetyl-CoA, which can be converted to fatty acids through a process called lipogenesis and stored as fat in the body, which can lead to obesity.

Trestatins can also reduce the rise in blood sugar levels of people with diabetes. Diabetes is a disease in which our blood sugar levels are significantly higher than that of a healthy person. Glucose comes from the breakdown of foods we consume, and insulin is a hormone that helps the glucose get absorbed into our cells. If insulin is not produced or properly used by the body, our blood sugar level will increase dramatically. Inhibiting α-amylases could result in controlling the increase of blood sugar level after a meal.

Despite their significant pharmaceutical prospect, little is known about trestatins' mode of formation in nature. Therefore, we embarked on an investigation of the biosynthesis of the trestatins in *S. dimorphogenes* ATCC 31484. We have obtained a draft genome sequence of *S. dimorphogenes* and identified the putative biosynthetic gene cluster of trestatins. Further, we will confirm the role of this cluster in trestatin biosynthesis. This will set the stage for further in-

depth biosynthetic studies and metabolic engineering. The main objective of this study is to identify the function of a number of genes involved in trestatin biosynthesis, particularly those encode glycosyltransferases.

MATERIALS AND METHODS

Culturing *Streptomyces dimorphogenes*

Medium 2 (25 mL) was prepared using materials shown in Table 1 and sterilized at 121°C for 30 min. This medium was used for both seed and production cultures. Medium 2 was inoculated with *S. dimorphogenes* and incubated on a rotary shaker (220 rpm) at 28°C for 72 h. The culture (5 mL) was transferred into 500 mL Erlenmeyer flask containing medium 2 (100 mL) and incubated on a rotary shaker (220 rpm) at 28 °C for 4.5 days. The fermentation broth was then acidified with 1 M oxalic acid to pH 4 and centrifuged. The supernatant was filtered and subjected to Dowex 50Wx2 (H^+ form). The column was washed with water and eluted with 1 N NH_4OH, and the eluent was lyophilized to give extracts containing trestatins.

Table 1. Ingredients of Medium 2

Materials	In 100 mL water
Glucose	2.0 g
Soytone	2.0 g
Potato starch	2.0 g
Yeast extract	0.25 g
NaCl	0.25 g
$ZnSO_4 \cdot 7H_2O$	5.0 mg
$CuSO_4 \cdot 5H_2O$	0.5 mg
$MnCl_2 \cdot 4H_2O$	0.5 mg
$CaCO_3$	0.32 g

Bacterial strains, plasmids, and primers

Escherichia coli DH10B and ET12567 (pUZ8002) were used as hosts for cloning and conjugation, respectively. *S. dimorphogenes* ATCC 31484 was used as a recipient in conjugation. The plasmids pTMAD003 and pTMAD004 were constructed from pTMN002 using DNA Assembly. The primers used for PCR amplification of DNA fragments are shown in Table 3.

Table 2. Plasmids used in this study

Plasmids	Other names	Relevant genotype/comments	Reference
pJTU12578+ aac(3)IV	pTMN002	pJTU1278+ derivative containing a 1 kb *aac(3)IV* apramycin resistance cassette from pOJ446	(Ito et al., 2009)
pTMN002-A1-A2	pTMAD003	Addition of 3 kb fragment *trsA1* + *trsA2* from *S. dimorphogenes* between HindIII and XbaI sites	This study
pTMN002-B5-B7	pTMAD004	Addition of 3 kb fragment *trsB5* + *trsB7* from *S. dimorphogenes* between HindIII and XbaI sites	This study

Table 3. Primers used in this study

Primers	**Name**	**Sequence**
pTMN002 amplification primers	pTMN002 FWD	AAGCTTATCGATACCGTCGACCTCGA
	pTMN002 REV	TCTAGAGCGGCCGCCACCG
trsB4 inactivation primers (Fragments *trsA1* and *trsA2* amplification)	A1 FWD	CGGTGGCGGCCGCTCTAGA**GTGATCGACGACGA CGCACT**
	A1 REV	TCGAACACCCCGGGGAATTC**GAACGCCTTGGAG AGGTTCC**
	A2 FWD	TCTCCAAGGCGTTCGAATTC**CCCGGGGTGTTCGA GGGCCC**
	A2 REV	TCGAGGTCGACGGTATCGATAAGCTT**TCATGCGA GGGTTCCTTCCGTTG**
trsB6 inactivation primers (Fragments *trsB5* and *trsB7* amplification)	B5 FWD	CGGTGGCGGCCGCTCTAGA**CGCTACCTGGAGAA GCTGGACG**
	B5 REV	GCCATCGCCCGCAGGAATTC**GTACTCCCGGCACA GGTTCCAC**
	B7 FWD	TGTGCCGGGAGTACGAATTC**CTGCGGGCGATGG CGGAC**
	B7 REV	TCGAGGTCGACGGTATCGATAAGCTT**GACGTAGG TGCCCTCCGACGG**

DNA assembly and colony screening

The plasmid pTMN002 was digested with HindIII and XbaI and ligated with fragment 1 (*trsA1* or *trsB5*) and fragment 2 (*trsA2* or *trsB7*) using HiFi DNA Assembly. The assembly reaction contained 50 ng of inserts and 100 ng of pTMN002 mixed with 5 µL of DNA Assembly master mix. The reaction took place in the thermocycler at 50 °C. After 15 min, 2 µL was taken and

diluted in 8 μL of water. Two μL of this dilution product was used in transformation. After overnight incubation of the transformants, colonies were screened for the desired clones.

Competent cell preparation

LB medium was inoculated with small amount of *E. coli* DH10B cells and incubated at 37 ˚C overnight with vigorous shaking. The *E. coli* DH10B cells were subcultured to OD_{600} 0.4. After subculturing, cells were collected by centrifugation and re-suspended in 10 mL cold 0.1 M $CaCl_2$. The suspension was incubated on ice for 30 min and centrifuged. Supernatant was decanted and cells were re-suspended in 5 mL cold 0.1 M $CaCl_2$/15% glycerol. Cells were stored at -80 °C.

Transformation

Competent cells were thawed on ice for approximately 20 min. Agar plates containing 50 μg/mL apramycin were pre-warmed at 37 °C. DNA Assembly product (2 μL) was added to competent cells in a micro centrifuge tube. The mixture was gently mixed by flicking the bottom of the tube. The competent cells were incubated on ice for 15 min. The transformation tube was heat shocked by placing it in 42 °C water bath for 45 secs. After 2 min ice incubation Luria-Bertani (LB) medium (450 μL) was added and the cells were incubated in a 37 °C shaking incubator for 45 min. Cells were plated on LB agar plates containing apramycin (50 μg/mL). Plates were incubated at 37 °C overnight.

Plasmid DNA extraction

LB medium (5 mL) containing apramycin (50 μg/mL) were inoculated with a single bacterial colony. The culture was incubated at 37 ˚C overnight with vigorous shaking. Bacteria were pelleted from the culture (1 mL) at room temperature. Resuspension buffer, lysis buffer and neutralization buffer were added to the cells and centrifuged at 12,000 rpm for 10 min at room temperature. Supernatant was recovered and ethanol precipitated.

Conjugation using *Streptomyces dimorphogenes* mycelia

Bacterial conjugation is a genetic exchange mechanism that requires direct contact between cells. *E. coli* ET12567/pUZ8002 containing pTMAD003 or pTMAD004 were cultured in LB medium at 37 °C, with shaking at 220 rpm, supplemented with chloramphenicol, kanamycin, and apramycin. Liquid SM medium (containing 1% glucose, 0.4% yeast extract, 0.4% peptone, 0.4% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4$, pH 7.0) was used for *S. dimorphogenes* culturing to collect mycelia. 2×YT medium and mannitol soya flour (MS) medium containing 10-20 mM of $MgCl_2$ were used for conjugation.

An *E. coli* ET12567/pUZ8002/pTMAD004 overnight culture (100 μL) was added to 5 mL of LB (containing 25 mg/L kanamycin, 25 mg/L chloramphenicol and 50 mg/L apramycin), and incubated at 37 °C with shaking at 220 rpm for 4 h to OD600 0.5. To remove the antibiotics, the cells were collected at 4000 rpm and washed twice with an equal volume of LB (containing 10 mM $MgCl_2$) without antibiotics. *E. coli* cells were re-suspended in 0.5 mL of LB (containing 10 mM $MgCl_2$), and used as the donor cells. *S. dimorphogenes* mycelia were prepared from 5 mL of Day 3 liquid cultures. The mycelia were collected at 4000 rpm and washed with an equal

volume of 10% of glycerol once and 2×YT medium twice. Finally, the mycelia were re-suspended in 0.5 mL of 2×YT medium and used as the recipient.

E. coli cells were transferred into the re-suspended mycelia and vortexed briefly to achieve homogeneity. The mixture was collected by centrifugation at 4000 rpm, and 600 μL of supernatant was removed. The residue was spread on the MS plate (containing 1-20 mM $MgCl_2$). The plate was let dry in a laminar flow hood, and then incubated for 17 h in a 30 °C incubator with plates inverted. One mL of mixed antibiotics (0.5 mg nalidixic acid and 1 mg apramycin) was evenly overlaid on plates and the surface was let dry. The plates were incubated at 30 °C.

Conjugation using *Streptomyces dimorphogenes* spores

To achieve quicker sporulation of *S. dimorphogenes,* cells were grown on ISP medium 4 BACTO agar plates. Sporulation on this medium was observed in 6 days. Spores were collected using standard methods and stored at -80 °C. Frozen spores were thawed on ice and added to 500μL 2xYT medium. The mix was incubated at 50 °C for 10 min. Donor *E. coli* cells were added to the pre-germinated spores and the cells were centrifuged for 2 min and the majority of the supernatant was decanted. The pellet was resuspended in the remaining fluid so that the final volume is 100 μL and spread on MS plates.

Antibiotic overlay

After overnight incubation, the conjugation plates were overlaid with antibiotics to select for successful exconjugants. Nalidixic Acid and apramycin were used to overlay the plates. After

this step, the plates were left at 30 °C for 3-4 days. Plates were examined after 4 days, but there was no sign of exconjugants. Therefore, the incubation time was extended to 10 to 15 days.

Results

Confirmation of trestatin production in *Streptomyces dimorphogenes*

Although previous studies have shown that trestatins inhibit the activities of various α-amylases, their modes of formation in nature remain unclear.[2,1] Therefore, we embarked on an investigation of the biosynthesis of trestatins and the importance of individual genes within the trestatin biosynthetic gene cluster. Before proceeding to our genetic and biochemical studies, we confirmed the ability of our *S. dimorphogenes* strain to produce trestatins by analyzing the secondary metabolites in its fermentation broth.

The supernatant from the fermentation broth of *S. dimorphogenes* loaded onto Dowex 50Wx2 (H^+ form) column. The fractions were then lyophilized and analyzed by mass spectrometry. As shown in Fig. 2, the peak at *m/z* 970.2 indicates the presence of trestatin B, confirming that the strain is able to produce trestatins.

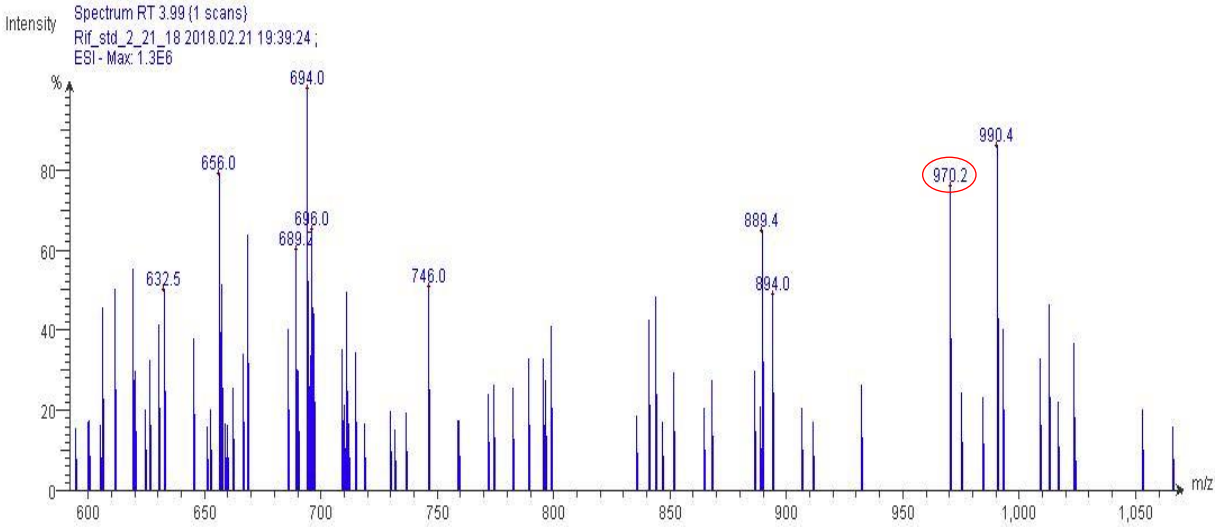

Figure 2. Mass spectrum of a *S. dimophogenes* culture fraction containing trestatin B (*m/z* 970)

Identification and putative functions of genes within the trestatin biosynthetic gene cluster

The putative biosynthetic gene cluster of trestatin (Fig. 3) was identified by obtaining a draft genome sequence of *S. dimorphogenes* and using Basic Local Alignment Search Tool (BLAST) to compare the draft genome sequence with database sequences of other organisms. The genome sequencing was performed by Dr. Takuya Ito at Tokushima Bunri University. Based on alignment results, the putative functions of some of the major genes in the gene cluster are summarized in Table 3. The gene *trsA1* is closely related with *gacU*, a gene that codes for the protein 1-epi-valienol-7-phosphate-1-kinase from the acarbose pathway in *Streptomyces glaucescens*. Similar to trestatin, acarbose is a pseudotetrasaccharide α-glucosidase inhibitor that contains a C_7N-cyclitol moiety.[4] This compound is used for the treatment of patients suffering from type II insulin-independent diabetes and is commercially produced using developed strains of *Actinoplanes*.[5]

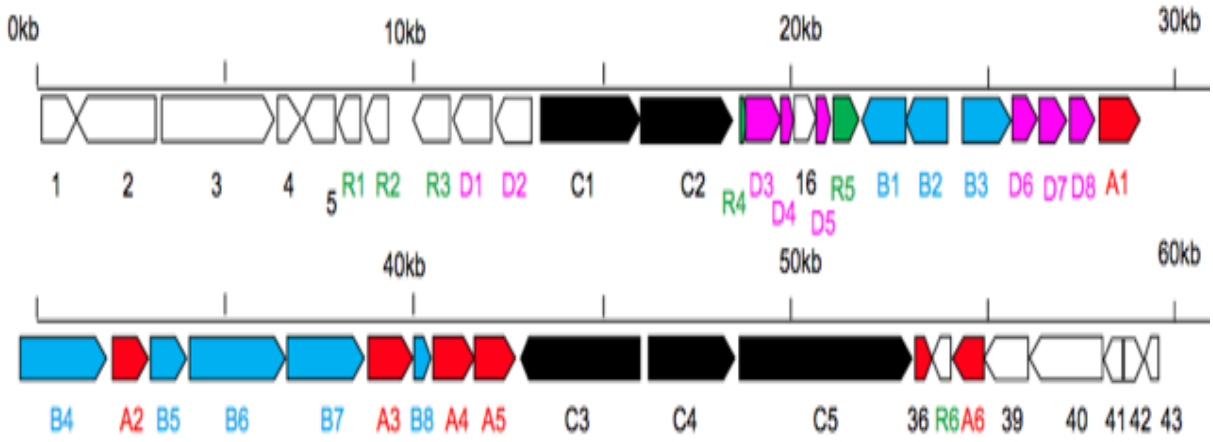

Figure 3. Putative biosynthetic gene cluster of trestatin in *S. dimorphogenes*

Table 3. Some of the major genes from the trestatin biosynthetic gene cluster

Gene	Protein	Putative Function
trsA1	TrsA1	1-epi-valienol-7-phosphate-1-kinase/trehalose synthase
trsB4	TrsB4	glycosyltransferase
trsA2	TrsA2	1-epi-valienol-1,7-bisphosphate-1-adenylyltransferase
trsB6	TrsB6	glycosyltransferase
trsA3	TrsA3	2-*epi*-5-*epi*-valiolone synthase
trsA5	TrsA5	2-epi-5-epi-valiolone dehydratase/epimerase
trsC3	TrsC3	alpha-amylase
trsC4	TrsC4	alpha-amylase

The genes *trsB4* and *trsB6* are both found to resemble genes coding for glycosyltransferases in *Streptomyces* sp. The gene *trsA3* resembles a gene that codes for 2-epi-5-epi-valiolone synthase in *Streptomyces pactum* strain ACT12. This enzyme catalyzes the cyclization of sedoheptulose 7-phosphate to 2-epi-5-epi-valiolone (Fig. 4), a common precursor

for the biosynthesis of C_7N-aminocyclitol-containing natural products, such as trestatins, acarbose, validamycins, acarviostatins, and adiposins (Fig. 5). [6,7]

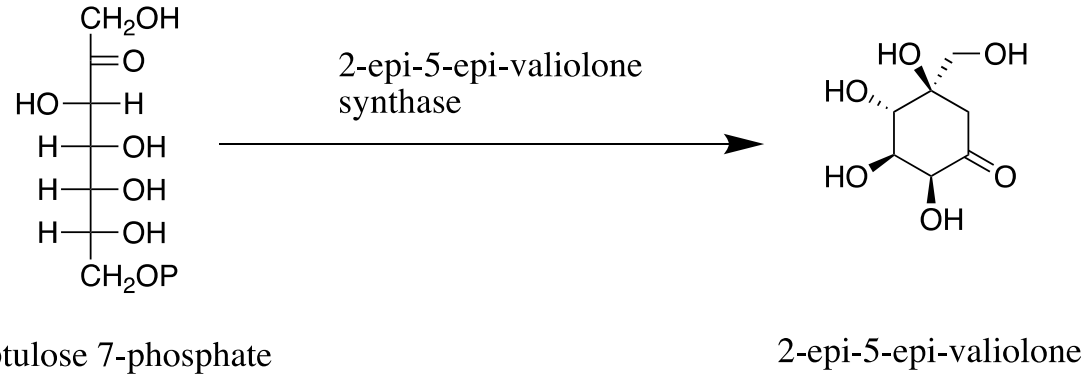

Figure 4. Cyclization of sedoheptulose 7-phosphate to 2-epi-5-epi-valiolone by 2-epi-5-epi-valiolone synthase.

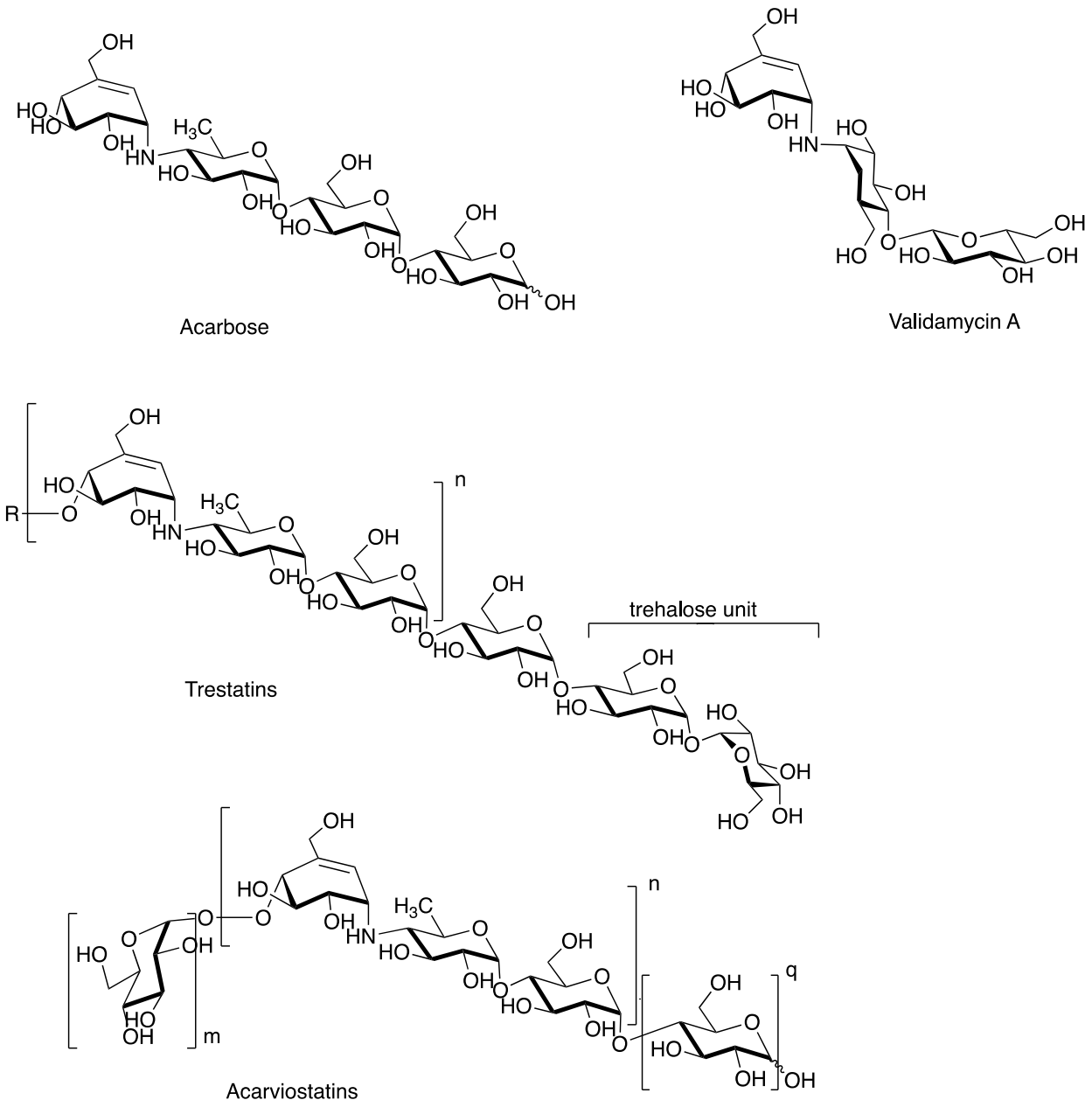

Figure 5. Chemical structures of some microbial derived C_7N-aminocyclitol-containing compounds

In the first part of this project, primary focus was given to the genes *trsB4* and *trsB6*, as one of them may code for a unique enzyme called pseudoglycosyltransferase.

Pseudoglycosyltransferases are proteins that are similar to glycosyltransferases, but instead of catalyzing the transfer of activated sugar donor onto a sugar acceptor, pseudoglycosyltransferases catalyze the transfer of a pseudosugar to an acceptor molecule via a nonglycosidic C-N bond formation.[8] While BLAST analysis showed that both TrsB4 and TrsB6 are highly similar to glycosyltransferases, the presence of a pseudosugar moiety in the structure of trestatin as well as the presence of 2-epi-5-epi-valiolone synthase in the biosynthetic gene cluster suggests that one of these enzymes is responsible for the attachment of the pseudosugar unit to the sugar chain of trestatin.

Construction of pTMAD003 and pTMAD004

To sort these genes into the right category, a gene inactivation approach was pursued. For this purpose, two plasmids, pTMAD003 and pTMAD004, were constructed. These plasmids carry homologous sequences necessary for recombination with *S. dimorphogenes* chromosome. In addition to the homologous regions, the plasmids carry selectable antibiotic marker to aid in the identification of exconjugants.

The construction of pTMAD003 and pTMAD004 plasmids was done using DNA (Gibson) Assembly method. The gene fragments from the trestatin gene cluster (Fig. 3) were amplified by PCR and purified by agarose gel. Subsequently, the fragments were joined with pTMN002. The expected plasmid constructs are shown in Fig. 6.

Confirmation of the constructs was done by double and triple digestions of pTMAD003 and pTMAD004 (Fig. 7). Double digestion was done using XbaI and HindIII (Figs. 7A and 7C) and triple digestion was done using XbaI, HindIII and EcoRI (Figs. 7B and 7D). The expected

sizes for the XbaI/HindIII digestions were 10.2-kb for pTMN002 and 3-kb for the insert. For pTMAD003 triple digestion (Fig. 7B, the resultant bands were 10.2-kb for pTMN002, 1.53-kb for the *trsA1* fragment and 1.2-kb for the *trsA2* fragment. For pTMAD004 triple digestion (Fig. 7D), the resultant bands were 10.2-kb for pTMN002, 1.5-kb for both fragments from *trsB5* and *trsB7*.

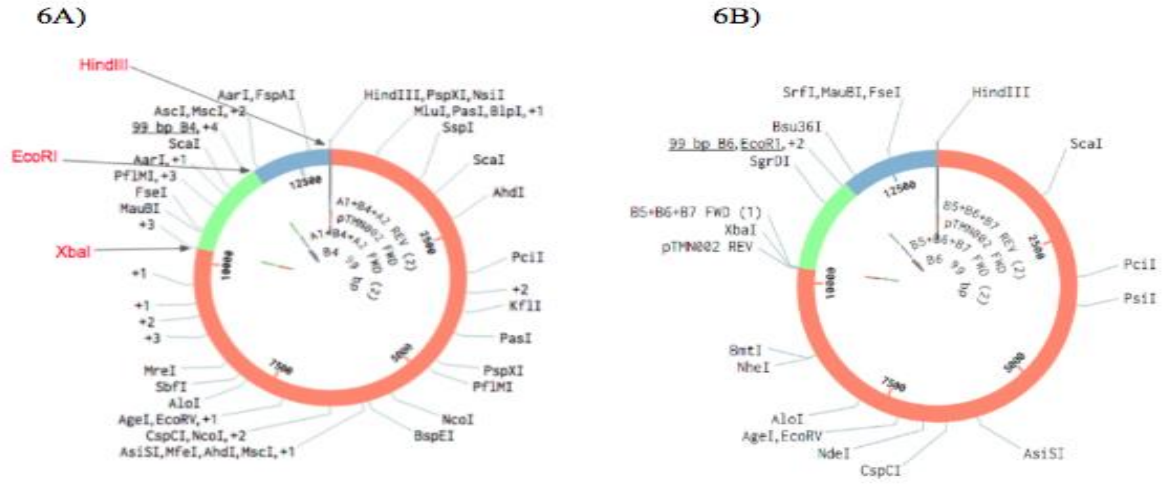

Figure 6. Plasmid maps for pTMAD003 and pTMAD004. (**A**) *trsA1* and *trsA2* fragments in pTMN002. Green is *trsA1* fragment (1.53 kb), blue is *trsA2* fragment (1.24 kb), and orange is pTMN002 (10.23 kb). **(B**) *trsB5* and *trsB7* fragments in pTMN002. Green is *trsB5* fragment (1.51 kb), blue is *trsB7* fragment (1.52 kb) and orange is pTMN002 (10.23 kb).

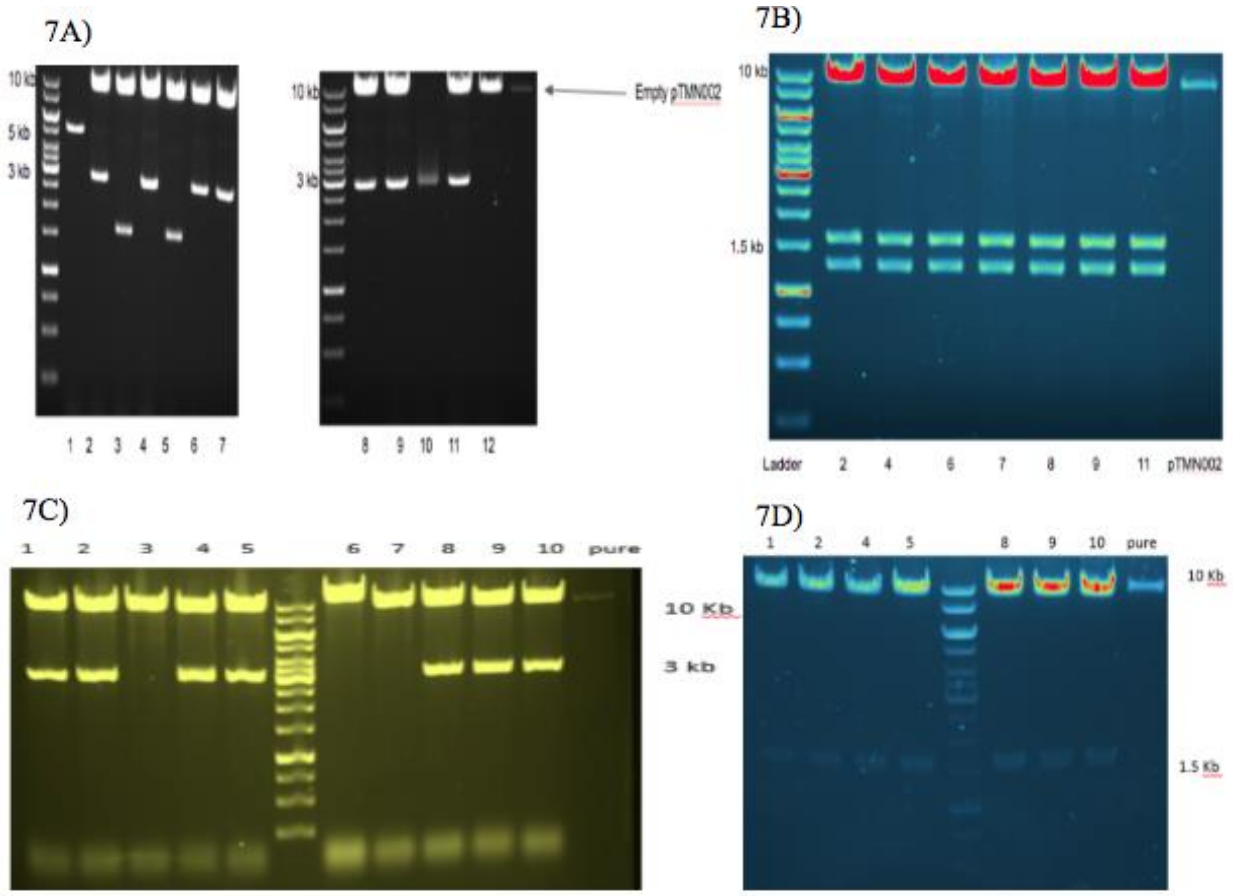

Figure 7. Gel electrophoresis of pTMAD003 and pTMAD004 digestions. (**A**) pTMAD003 double digested with XbaI and HindIII. (**B**) pTMAD003 triple digested with XbaI, HindIII and EcoRI. (**C**) pTMAD004 double digested with XbaI and HindIII. (**D**) pTMAD004 triple digested with XbaI, HindIII and EcoRI.

Attempts to transform *S. dimorphogenes* with pTMAD003 and pTMAD004

After confirmation, pTMAD003 and pTMAD004 were individually transferred into *S. dimorphogenes* by conjugation using *E. coli* ET12567/pUZ8002 as donor strain. Unfortunately, despite several attempts, this conjugation procedure was unsuccessful. Therefore, further experimentation and troubleshooting are necessary to determine as to why the conjugation was unsuccessful as well as how to increase success rates.

Discussion

Sugars are constituents of most important molecules in living organisms.[8] Many bioactive molecules contain sugars as part of their structures.[10] Although they are very important to life, the overconsumption of sugars have led to new crises, such as increased cases of heart disease, diabetes and obesity. Thus, investigation of α-amylase inhibitors, e.g., trestatins, has significant importance.

This report describes our preliminary efforts to gain insights into the biosynthesis of trestatins in *S. dimorphogenes*. As the first step, we focused on genes that encode putative glycosyltransferases, one of which is expected to have unique pseudoglycosyltranseferase activity. To investigate the function of these genes, a gene inactivation approach was used. As a prerequisite, we confirmed that the *S. dimorphogenes* strain used in this study produces trestatins. In addition, we have successfully constructed the plasmids necessary for gene inactivation in *S. dimorphogenes*. However, in spite of multiple attempts, the desired mutants were not obtained. Future work will focus on alternative transformation systems, including implementing a new type of mating system. This conjugative system involves a helper *E. coli* strain carrying a conjugative plasmid, a donor strain carrying a constructed plasmid (pTMAD003 or pTMAD004), and recipient *S. dimorphogenes* spores.

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