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Partial Expression of the VbsS gene in Rhizobium Leguminosarum ATCC 14479 and In-Silico Analysis of the vbs Gene Cluster in Various Microorganisms

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Partial Expression of the *VbsS* gene in *Rhizobium Leguminosarum ATCC 14479* and *In-Silico* Analysis of the vbs Gene Cluster in Various Microorganisms

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by

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ABSTRACT

Partial Expression of the *VbsS* gene in *Rhizobium Leguminosarum ATCC 14479* and *In-Silico* analysis of the vbs gene cluster in various microorganisms

by

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Iron is extremely important for many organisms. Despite its abundancy, it exists in insoluble forms that makes its usability difficult. Some organisms secrete siderophores, low molecular weight compounds, that can chelate iron and convert it into usable forms for cells. One such organism, *Rhizobium leguminosarum,* is a nitrogen fixing symbiont proteobacteria that infects leguminous plants. The genome of *Rhizobium leguminosarum ATCC 14479,* which infects the red clover, *Trifoli pratense*, has previously been completely sequenced in our lab. Our lab has identified several genes in this strain involved in the biosynthesis of a siderophore, vicibactin. The protein product of one of those genes, VbsS, is hypothesized to be a non-ribosomal peptide synthase. It has been attempted to knockout the VbsS gene utilizing the 'splicing by overlap extension' method. Additionally, an *in-silico* analysis of the genome revealed the Vbs genes in *R. leguminosarum ATCC 14479* strain were similar to genes in found in the proteobacterium *Phyllobacterium sp. 628* and the fungi *Aspergillus fumigatus Af293*.

Introduction

Iron

Iron is one of the most essential trace elements required for the growth and survival of virtually all living organisms. It is utilized during biofilm production, electron transfer, oxygen metabolism, gene expression, and enzyme catalysis (Ahmed, E., & Holmström, S. J. 2014). More than one hundred enzymes participating in primary and secondary metabolism require iron containing cofactors such as heme groups or iron-sulfur clusters. Excessive iron intake in humans can damage the gastrointestinal system and lead to symptoms of iron toxicity, such as nausea, diarrhea, and stomach pain (Abhilash, K. P., Arul, J. J., & Bala, D. 2013). On the other hand, iron deficiencies can lead to the development of blood anemia. This is because in the human body, the largest amount of iron is used for hemoglobin synthesis, which produces red blood cells (Clara Camaschella 2019). In bacteria, lack of iron can cause significant damage and stress to cells. In iron-depleted conditions, *Mycobacterium smegmatis,* exhibits decreased DNA and RNA levels. In another bacteria, *Bacillus subtilis*, iron starved conditions halt DNA synthesis. In some plants, iron deficiency causes chlorosis, which produces the yellowing of leaves due to the lack of the chlorophyll pigment (Messenger, A., & Barclay, R. 2002). Despite its importance to so many different microorganisms, iron sequestration is very difficult.

Iron is the fourth most abundant element on earth. By mass, it is the most abundant element, comprising of almost 80% of the entire outer and inner Earth's crusts, and yet, organisms find it very difficult to incorporate this for their survival. In the environment, iron exists in two states, the reduced ferrous ion (Fe^{2+}) or the oxidized ferric iron (Fe^{3+}). Fe^{2+} is very soluble at neutral pH and in anoxic environments, but in the presence of molecular oxygen and other oxidizing agents, Fe^{2+} rapidly oxidizes into Fe^{3+}. Fe^{3+} is a highly insoluble element at

neutral pH, making it very difficult for organisms to incorporate it for their growth and survival. In the presence of water, Fe^{3+} forms ferric oxide, a red precipitate that naturally causes rust. During the early years of life on Earth, there was almost no oxygen present in the Earth's atmosphere. Iron existed in its Fe^{2+} form and was abundant in large bodies of water. With the evolution of photosynthesis, oxygen was introduced into the Earth's atmosphere, and this allowed the conversion of Fe^{2+} to Fe^{3+}. Fe^{3+} is very toxic, as it can react with oxygen to produce reactive oxygen species(ROS), which can damage membranes, lipids, proteins, and DNA (Emery, T. 1982; Caza, M., & Kronstad, J. 2013). In moist environments, Fe^{3+} can also spontaneously react to produce precipitates of oxides, phosphates, hydroxides, and sulfates, all of which have very low solubilities (Caza, M., & Kronstad, J. 2013). Today, Fe^{3+} is the dominant form of iron that exists naturally in Earth's oxygenated atmosphere, and as a result, organisms are forced to discover ways to incorporate the insoluble Fe^{3+} into their systems.

Iron Acquisition

In the environment, the free usable iron concentration is estimated to be 10^{-18} M (Emery, T. 1982). For many bacterial pathogens, however, this concentration in lowered to 10^{-24} M, because mammalian hosts limit iron availability. The hosts can employ several iron binding proteins, such as transferrin and lactoferrin, to reduce the amount of free ferrous iron in the environment. In order to survive, bacterial pathogens must retain an internal iron concentration of at least 10^{-6} M. In order to maintain this threshold, pathogens have developed three different methods to sequester iron from their environment. First, they can produce reductase enzymes that reduce the insoluble ferrous iron to soluble ferric iron. The *Archaeoglobus fulgidus* bacterium uses this method via assimilatory ferric reductase enzymes to help chelate free external ferric iron and reduce it to ferrous iron, which can then be incorporated into intracellular

proteins (Schroeder, Imke & Johnson, Eric & Vries, Simon. 2003). The second method is to lower the pH of the environment, creating an acidic environment that can easily facilitate the conversion of ferric iron to ferrous iron. The pathogenic fungus *Neurospora crassa* uses this mechanism by excreting hydroxy acids into the environment, accumulating free iron onto the cell walls that can then be mobilized into the cell (Howard DH. 1999; J. Kaplan, D.M. Ward, 2013; Raymond KN, Dertz EA, Kim SS. 2003). The last, most effective, and most common method is through a shuttle mechanism that utilizes small organic molecules known as siderophores that act as high affinity iron chelators (Miethke M, Marahiel MA. 2007).

Siderophores

During times of iron deficient growth conditions, many microorganisms and plants secrete siderophores, low molecular weight ligands (<1kDa) that can aid in acquiring and transporting iron into cells. Siderophores are commonly characterized by their stability constants, which depict their binding affinities with iron. These constants range from 10^{30}-$10^{50}M^{-1}$, allowing siderophores to have a very high affinity in order to easily bind to free ferric iron in the environment (Messenger, A., & Barclay, R. 2002). Siderophores secreted by bacteria are divided into three different families based on their chemical structures: hydroxamates, catecholates and carboxylates. Most bacterial microorganisms produce catecholates, and these are known to be the most efficient and effective iron chelators when compared any other type (Raymond KN, Dertz EA, Kim SS. 2003). Enterobactin, one of the best studied catecholate, is secreted by *Escherichia coli* and *Salmonella typhimurium*. In times of iron depletion, the bacteria release this siderophore, which has a very high binding affinity constant of $10^{52}M^{-1}$. After the siderophore is released from the cell, it scavenges the environment and binds to ferric iron, creating a soluble Fe-Ent complex (Moynié, L., Milenkovic, S., Mislin, G.L.A. *et al.* 2019). This complex is then

recognized by cell receptors and is actively transported into the periplasm and eventually to cytoplasm, where the ferric iron is released from the complex and is metabolized for use. This active transport has been further studied and is found to have been presumably supported by a TonB-ExbB-ExbD energy dependent transducing system.

TonB-ExbB-ExbD

Siderophores (500-1500 Da) cannot easily diffuse through porins located on the outer membrane, as these channels have an exclusion limit of about 600Da (Nikaido H. 1994). A majority of the siderophores, when bound to extracellular iron, form the siderophore-Fe complex, too large to easily passively diffuse through porins channels. Instead, these complexes are recognized by outer membrane transporters that utilize an active transport system that derives its energy from the proton motive force located in the inner membrane. Structurally, gram-negative bacteria are composed of an outer membrane, periplasmic space, and an inner membrane with integral proteins (Nikaido H. 2003). The outer membrane does not possess a source of energy that is vital in order to transport extracellular iron actively into the cell. This mechanism is only present in the inner membrane, which is the location of the proton motive force (pmf) that forms an electrochemical gradient for the formation of ATP. This energy must somehow be transferred to the outer membrane receptors, allowing them to actively transport the siderophore-Fe complex into the cell. Gram-negative bacteria solve this dilemma through the use of the TonB-ExbB-ExbD system, studied extensively in *E.Coli* (Higgs, P.I., Larsen, R.A. and Postle, K. 2002). This system, comprising of 3 main proteins, couples the pmf from the inner membrane with active transport of siderophore-Fe complexes across the unenergized outer membrane. TonB is the main energy transducing complex. ExbB and ExbD are two accessory proteins located in the inner membrane that are thought to harness the proton motive force

energy to TonB (Ollis et al 2012). The TonB-ExbB-ExbD proteins exist in the ratio of 1:7:2 (Carter et al. 2002).

TonB is a periplasmic protein and appears as a dimer anchored to the inner membrane by its hydrophobic N-terminal domain (Miethke M, Marahiel MA. 2007). It contains a proline rich region in its C terminal, allowing it to span the periplasmic space and contact the outer membrane receptors. ExbB, consisting of three transmembrane domains, is a 26.1 kDa protein and appears to be used as a scaffolding on which TonB and ExbD assemble (Karlsson et al 1993). It is also the only protein of the three that is stable when expressed by itself and is also required for stabilization of both TonB and ExbD (Skare JT, Postle K. 1991). A study has shown that the ExbB protein prevented degradation of the TonB protein (Fischer et al 1989). The third protein, ExbD, similar to TonB, is composed of a carboxy-terminal periplasmic domain (more than 60% of the protein), a single TMD, and a short cytoplasmic amino terminus (Ollis et al 2012). It assists in the energy transduction by causing creating a link with and structural changes in TonB. A previous study has shown that a mutation in TonB leads to a failure of the crosslinking of ExbD with TonB (Larsen et al 1994).

After the siderophore binds to free iron, it has to then bind to the cell's outer membrane receptors, known as TonB-dependent transporters (TBDTs). Structurally, the TBTDs are composed of a 22 β-stranded transmembrane β-barrel and an N-terminal known as the plug, which blocks molecules from freely entering and exiting the cell. In the N-terminus of the plug domain resides a structure known as the TonB box (Noinaj et al 2010). The binding of the siderophore-Fe complex with the TBDTs is signaled to TonB, which transduces energy from the pmf in the inner membrane. Previous studies have shown that this coupling of TonB conformation to the proton gradient is mediated by interactions between ExbB and the TonB

amino terminal (Larsen et al 1999;Noinaj et al 2010). In the next step, the "charged" TonB interacts with components of the outer membrane (Larsen et al 1999). Ton B has to interact with the TonB box in order to relay the energy to the TBTFs. As a result, the TonB box undergoes a substrate dependent conformational change in order to interact with TonB. This interaction is vital in in order to transduce energy from TonB. In the absence of substrate, the Ton box adopts a folded conformation (Kim et al 2007). In the last step, TonB releases its stored potential energy and assumes the 'discharged' conformation, driving a conformational change in the outer membrane receptor that results in the release of the siderophore-Fe complex into the periplasmic space. The exact mechanism of how it is transported through the periplasm is unknown (24), but afterwards, it is transported through the inner membrane via an ATP binding cassette transporter. Powered by the hydrolysis of ATP, the siderophore-Fe complex is transported across the inner membrane to the cytoplasm, where the complex is broken down, Fe3+ is reduced to Fe2+, and iron is eventually metabolized by the cell (Kim et al 2007).

Rhizobium leguminosarum

R. Leguminosarum is an aerobic, gram negative bacillus. It is a nitrogen-fixing symbiont found in the root nodules of legume plants. Nitrogen in the atmosphere is present in form of nitrogen gas(N_2), but most plants can utilize only the reduced form of this element (Zou et al 2019). Nitrogen fixing bacteria, like *R. leguminosarum*, can use the nitrogenase enzyme in order to convert inert atmospheric nitrogen into ammonia(NH_4), which plants can readily assimilate into their cells. In return, the host plant allows *R. leguminosarum* to create nodules within its roots. Residing in the roots, the bacteria is now able to fix nitrogen for the host plant (Zou et al 2019; Datta, B., & Chakrabartty, P. K. 2014). This symbiotic relationship is iron dependent because iron is essential for nodule formation and synthesis of leghemoglobin, nitrogenase

complex, ferredoxin and other electron transport proteins required by the nitrogenase system during symbiosis (Guerinot, ML 1991). During iron-deficient conditions, *R. leguminosarum* can secrete siderophores that can scavenge the environment for iron.

Vicibactin

R. leguminosarum has previously been shown to produce vicibactin, a cyclic trihydroxamate siderophore (Carson et al 2003). It appears to be secreted by most siderophore-secreting strains of *R. leguminosarum.* Therefore, the production of the siderophore vicibactin is not thought to be strain specific (Carson et al 2003; Ollis, A. A., & Postle, K. 2012). In the *R. leguminosarum bv. viciae* strain, the transcription of vicibactin was found to be controlled by four operons, *vbsGSO, vbsADL, vbsC, vbsP* (Hill 2014). Six of their gene products, vbsS, vbsO, vbsA, vbsL vbsC, and vbsP, play a direct role in synthesizing the cyclic trihydroxamate vicibactin. VbsS is a non-ribosomal peptide synthetase that comprises of four domains: an N-terminal condensation domain, an adenylation domain, a PCP domain, and a C-terminal thioesterase domain. Each of these domains catalyze different steps during the production of vicibactin (Carter et al. 2002). VbsO resembles a flavin-dependent monooxygenase, which are crucial in synthesizing hydroxamate- containing siderophores (Carter et al. 2002). VbsA contains a C-terminal that resembles an acyltransferase, which catalyzes condensation of D-3-hydroxybutyrate with L-ornithine (Carter et al. 2002). VbsL acts as an epimerase, responsible for the conversion of L- to D-ornithine (Carter et al. 2002).VbsC is an acetylase, needed to N-acetylation of ornithine (Carter et al. 2002). VbsP is similar to many phosphopantetheinyl transferases that attach phosphopantetheine to proteins involved in the synthesis of polyketides or non-ribosomal peptides (Carter et al. 2002).

Present Work

R. leguminosarum ATCC 14479 was isolated at Arlington Farms, VA and purchased from the American Type Culture Collection (Hill 2014). After sequencing the entire strain, our lab has isolated and structurally identified the siderophore vicibactin produced by *Rhizobium leguminosarum ATCC 14479* (Wright et al 2013). Vicibactin, produced by many strains of *R. leguminosarum*, has been shown to be the product of genes vbsS, vbsO, vbsA, vbsL vbsC, vbsL, and vbsP. It was in our best interest to knockout the vbsS gene, hypothesized to be a non-ribosomal peptide synthase, and observe the effects it has on siderophore production and iron transport. Also, *in-silico* analysis was performed on *Rhizobium leguminosarum ATCC 14479* to determine if genes similar to the vbs gene cluster exist in other microorganisms.

Methods

Genomic DNA Extraction

R. leguminosarum ATCC 14479 was first inoculated onto a Congo Red plate from a -80°C freezer stock solution that was cultured stationary at 30°C for 48 hours. The Congo Red media is commonly used for isolating *R. leguminosarum* bacteria, which absorbs the condo red dye weakly, as opposed to other bacteria, that absorb the dye strongly(Keen et al 1983). The media contained 1% mannitol, 0.05% K_2HPO_4, 0.02% $MgSO_4*7H_20$, 0.01% NaCl, and 0.1% yeast extract. It was adjusted to a pH of 6.8 using 6M NaOH and was autoclaved before use. A colony was then inoculated into a 5mL Yeast Mannitol Broth tube that was cultured on a shaking incubator at 250rpm, 30°C, for 48 hours. The genomic DNA was isolated from 1.8mL of this culture utilizing the QIAGEN DNeasy UltraClean Microbial Kit. This kit uses the mechanical

force of beating beads to help lyse the cells. The DNA released is bound to a silica spin filter, filter washed, then recovered in a Tris buffer.

Deletion of *vbsS* via Splicing by Overlap Extension

Splicing by overlap extension is a PCR-based method of recombining DNA that can be used to create *in vitro* gene deletions. A first PCR round generates two fragments, one that contains 1000bp upstream of the target gene, and another that contains 1000bp downstream of the target gene, both sharing a region of homology on their 3' ends. A second PCR round anneals and extends both fragments, thereby creating an *in vitro* deletion of the target gene. Two sets of primers were designed to perform the splicing by overlap extension method. The primers are shown in Table 1. The first set of primers, SOE1F and SOEVbsSR, were used to amplify the 1000bp upstream flanking the region of the *vbsS* gene. The second set of primers, SOE1R and SOEVbsSF, were used to amplify the 1000bp downstream flanking region. The two sets of primers underwent separate PCR reactions that each contained 1µl of genomic DNA, 5µl of GC Buffer, 5µl GC Enriched Media, .5µl dNTP, .25µl Q5 Polymerase, and 11.25µl Nuclease-free H_20 in their reaction tubes. The upstream flanking region reaction tube also contained 1µl SOE1F and SOEVbsSR, while the one for the downstream flanking region contained 1µl SOE1R and SOEVbsSF. After the first PCR, round, the flanking region fragments were viewed on a 1% agarose gel for gel extraction. A subsequent PCR round was used to create a 2000bp crossover product that contained an *in-frame* deletion of the *vbsS* gene (33). In this reaction, equal amounts of each product were used as template and SOE1F and SOE1R were used as primers (33). The results of this PCR reaction were later viewed on a 1% agarose gel for gel extraction.

Primer Name	**Primer Sequence**
SOE1F	5’ CGATGGTACCGAATCGGCCACGGTGAAGGC 3’
SOEVbsSR	5’CGTGACGTTAGCGTACGAGGACTAAGAATACGCTGGACGGTCGTC TTGACGTCGAACGC 3’
SOE1R	5’ GCTATCTAGATGCTGATCCATGTCAGCTCCG 3’
SOEVbsSF	5’CCGTCCAGCGTATTCTTAGTCCTCGTACGCTAACGTCACGGATACC TACGACCAGTTCC 3’

Table 1. Primers used for SOE PCR reactions

Agarose Gel Extraction

The products of both PCR rounds were analyzed on the 1% agarose gel with Tris base, acetic acid and EDTA (TAE) buffer to confirm expected size. The gel was viewed through the use of UV light and ethidium bromide. The DNA bands of the crossover product were cut, weighed, and placed in labeled microcentrifuge tubes. The DNA was purified using the Monarch® DNA Gel Extraction Kit Protocol. The purity of the DNA attained from the gel extraction kit was assessed utilizing the NanoDrop™ 2000/2000c Spectrophotometer. The PCR products of the second round were further purified via ethanol precipitation.

Isolation of pEX18GM

After the desired DNA crossover product was successfully amplified and purified, it was inserted into the plasmid pEX18GM. *E.coli* containing pEX18GM was first streaked on an LB plate containing gentamycin overnight at 37°C. An individual colony was then inoculated in a

5ml LB broth tube containing 50µl of 2mg/ml gentamycin, which was placed on a shaker overnight at 37°C. This solution was then resuspended in 4 microcentrifuge tubes and each centrifuged at 13,000 rpm for 3 minutes. The supernatant was discarded by gently inverting the open microcentrifuge tubes. The plasmids were then isolated following the QIAprep Spin Miniprep Kit protocol. The broth culture was resuspended with 250µl of Buffer P1. 250µl of Buffer P2 was later added and mixed by gently inverting tubes 5 times. Next, 350µl of Buffer N3 was added, mixed immediately by inverting the tubes 5 times. The microcentrifuge tube was then centrifuged at 13,000 rpm for 10 minutes, ensuring the formation of a compact white pellet. Next, 800µl of supernatant formed after centrifugation was applied to a QIAprep 2.0 Spin Column. This column was centrifuged for 30-60 seconds and the flow through discarded. The column was then washed by adding .5ml Buffer PB and centrifuged at 13,000 rpm for 30-60 seconds, and the flow through discarded. The spin column was centrifuged again at 13,000 rpm for 30-60 seconds to ensure the removal of all nuclease activity traces. Then, the column was washed with .75ml Buffer PE and centrifuged for at 13,000 rpm for 30-60 seconds. The spin column was centrifuged again at full speed for 1 minute to remove traces of the wash buffer. The spin column was then placed in a clean microcentrifuge tube and 50µl Buffer EB was added. This solution then remained undisturbed for 1 minute at room temperature, then centrifuged at 13,000 rpm for 1 minute. It was then further purified via ethanol precipitation. The purity of the plasmid DNA attained by the QIAprep Spin Miniprep Kit and ethanol precipitation was assessed utilizing the NanoDrop™ 2000/2000c Spectrophotometer.

Restriction Enzyme Digestion

After the SOE crossover product and isolated pEX18GM plasmid DNA were attained, restriction digestion was performed. This procedure utilizes naturally occurring enzymes that

recognize and cleave DNA sequences at specific nucleotides. It results in the production of single stranded overhangs known as sticky ends. These sticky ends can later form base pairs and bind to complimentary strands having similar sticky ends during ligation. The NEB Restriction Enzyme Double Digestion Protocol was followed. The two time saver enzymes utilized were KpnI and Xbal. Two separate restriction digestion procedures were performed, one for the crossover product, and another for the plasmid DNA. The amount of DNA was calculated by converting ng/µL from the NanoDrop readings to µLs. To each reaction, 1µL of KpnI, 1µL of Xbal, 6.5µL of CutSmart Buffer, and nuclease free H_20 were added to create a reaction volume of 50µL. These reactions then remained undisturbed at room temperature for 40 minutes. The DNA from both reactions were subjected to column purification using the Monarch® DNA Gel Extraction Kit and the DNA concentration was measured with the NanoDrop™ 2000/2000c Spectrophotometer.

Ligation

After the SOE crossover product and the pEX18GM plasmid were restriction digested, ligation was performed. DNA ligation catalyzes the formation of covalent phosphodiester linkages on complimentary sticky ends that can join two strands together. The NEB Ligation Protocol with T4 DNA Ligase was used. The reaction was set up in a microcentrifuge tube on ice. A molar ratio of 1:3 vector to insert was utilized. The amount of crossover product and plasmid DNA was calculated by converting ng/µL from the NanoDrop readings to µLs. The microcentrifuge tube was pipetted with .7µL of the crossover product DNA, 1µL of the plasmid DNA, 2µL of the T4 DNA Ligase Buffer, 1µL of T4 DNA Ligase, and nuclease free H_20, for a final volume of 20µL. The reaction was gently mixed by pipetting contents up and down, then remained undisturbed at room temperature for 20 minutes. Then, it was heat inactivated at 65°C

for 10 minutes. The resulting ligated product was stored in a 4°C freezer until transformation into competent cells was performed.

Rubidium Chloride Competent Cells

Competent cells were prepared following the McManusLab rubidium chloride competent cell protocol (McManusLab). First, two separate solutions, TFB1 and TFB2 were prepared. The TFB1 solution was adjusted to a pH of 5.8 with dilute 1M acetic acid and the TFB2 solution to a pH of 6.5 with KOH. Both solutions were filter sterilized and were placed in 4°C freezer to cool before further use. NEB 5-alpha *E. Coli* (lacZ-) cells were streaked on an LB agar plate and incubate at 37°C for 12-24 hours. A single colony was inoculated into 20mL super optimal broth with catabolite repression (SOC) media in an Erlenmeyer flask, then placed in a 37°C shaking incubator overnight. This culture was inoculated with 2XYT media in a 1:100 ratio and was grown on a 37°C shaking incubator until its OD_{600} reached .4 to .6. The culture was immediately transferred to an iced water bath for 10 minutes. The culture was transferred to culture flasks and was centrifugated at 5000Xg in 4°C for 10 minutes. The supernatant was poured out and 100mL TFB1 per 250mL culture was added, the culture was resuspended, and was incubated in an ice water bucket for 5 minutes. The culture was centrifugated at 5000Xg in 4°C for 5 minutes. The supernatant was poured out and 10mL TFB1 per 250mL culture was added, the culture was resuspended, and then incubated in an ice water bucket for 15-60 minutes. The cell suspension was dispensed as 100µL aliquots into chilled 1.5mL microcentrifuge tubes, snap-freezed in liquid nitrogen, then stored in -80°C freezer until needed for transformation.

Transformation

The pEX18GM plasmid containing the crossover product was transformed into the prepared rubidium chloride competent cells. They were plated on X-gal containing media for identification of recombinant bacteria. First, the competent cells were thawed in an iced water bath for 10 minutes, or until all crystals were completely dissolved. 50µL of competent cells were transferred into a transformation tube and .5µL of the pEX18GM ligation product containing the crossover product was added. The cells were mixed by gently flicking the tube four to five times, then placed on ice for 30 minutes. The cells were heat shocked at 42°C for exactly 30 seconds, then immediately placed on ice for five minutes. Next, 950µL of freshly prepared LB broth was added to the cells, then placed on a shaking incubator at 37°C for one hour. Meanwhile, LB plates containing gentamycin and X-gal were warmed to 37°C. After one hour, the cells were mixed by inverting the tube four to five times. 50µL of the cells were added to each of the pre-warmed LB plates with a sterilized hockey stick. The plates were placed in a 37°C incubator for 12-24 hours. A positive control was performed to measure the efficiency of transformation and the viability of the competent cells. Competent cells were transformed with undigested pEX18GM plasmid and plated on LB plates with gentamycin, which was then placed in a 37°C incubator overnight.

Colony PCR

The white colonies that grew on the X-gal containing media were patched plated on an LB plate with gentamycin, then grown in a 37°C incubator overnight. The next day, the colonies were screened for presence or absence of crossover product DNA in the pEX18GM plasmid constructs via colony PCR. Eight 25µL PCR reactions were set up for eight of the colonies that had grown overnight. In each PCR tube, .65 µL of the SOE1R primer, 11.5µL of nuclease free

water, a portion of the colony using a sterilized toothpick, and 12.5µL of the Taq 2X master mix was added. The tubes were placed in the thermocycler with the following conditions: initial denaturation 94°C for 2 min, 30 cycles [95ºC for 30 sec, 55ºC for 30 sec, 68ºC for 1 kb/min], final extension at 68ºC for 10 min, hold at 4ºC. The colony PCR results for all eight colonies were then run on a 1% agarose gel. Due to COVID-19 limitations, the transformed plasmids have not yet been extracted in order to perform restriction digestion.

In-Silico Analysis

With the advent of technology and online databases, researchers are able to utilize *in-silico* analysis methods to work with their genes of interest. The National Center for Biotechnology Information (NCBI) is one such online database that holds numerous sequenced genomes and provides integrated access to sequence, mapping, taxonomy, and structural data. The NCBI database was utilized to obtain the full genomic DNA sequence of *Rhizobium leguminosarum ATCC 14479.* The genome was subjected to *sequence analysis,* a technique used to extract information about its biological structure, function, and properties. A subset of this technique utilizes *sequence alignment,* a feature that compares two or more genomic sequences to find regions of nucleotide sequence homology. *NCBI BLAST* was used to compare our genomic sequence of interest with that of various microorganisms. The antiSMASH database was also utilized to find regions of homology between the gene cluster of interest and other known gene clusters. The genomic sequence of the *vbs* gene cluster in *R. leguminosarum ATCC 14479* was subjected to *sequence alignment, NCBI BLAST, and antiSMASH* to find organisms that contained similar genomic sequences.

Results

Deletion of *vbsS* via Splicing by Overlap Extension

The *vbsS* gene of *R. leguminosarum ATCC 14479* was successfully deleted following the splicing by overlap extension (SOE) method. All four primers contained the following properties: melting temperature (T_m) of 60°C according to the formula 4(C+G)+2(A+T), 19-20 nucleotides, 50-60% GC content, and start and end with 1-2 G/C pairs. The first round of SOE PCR produced the upstream and downstream regions flanking *vbsS*. The second round of PCR produced the crossover product that contained an *in-frame* deletion of the *vbsS* gene. PCR products from both rounds were tested for DNA concentration using the NanoDrop™ 2000/2000c Spectrophotometer. Nucleic acids and proteins have a maximum absorbance at 260nm and 280nm, respectively. Other common contaminants absorb at 230nm. The 260/280 ratio represents DNA purity with any protein contaminants, and should ideally be ~ 1.8. The 260/230 ratio represents DNA purity with other contaminants, and should ideally be ~ 2.2. The results are given in Table 2. The products of the second PCR round were further purified via ethanol precipitation, and their concentration was again measured using the NanoDrop™ 2000/2000c Spectrophotometer. The results are shown in Table 3. Only the *VbsS* Crossover Product Sample 1 was utilized throughout the rest of the experiment due to its high DNA purity.

	Concentration (ng/µL)	260/280	260/230
Upstream(5') Fragment Sample	**35.5**	1.92	.28
Downstream(3') Fragment Sample	**48.7**	.973	1.14
VbsS Crossover Product Sample 1	**33.6**	1.95	.09
VbsS Crossover Product Sample 2	**41.2**	1.83	.85

Table 2. NanoDrop Results of Both Rounds of SOE PCR from 1µl of sample

	Concentration (ng/ µL)	260/280	260/230
***VbsS* Crossover Product Sample 1**	**54.1**	**1.85**	**2.59**
VbsS Crossover Product Sample 2	9.4	2.03	9.45

Table 3. NanoDrop Results of Ethanol Precipitation of SOE Crossover Products from 1µl of sample

In addition to the measuring DNA concentration and purity, a 1% agarose gel was run to confirm the size of the crossover DNA in relation to the *vbsS* flanking fragments. The crossover product should be about 2.2kb, as it is a combination of the flanking fragments. Lane 1 contained the 1kb standard ladder. Lane 2 contained the upstream 5' fragment and was approximately 1.2kb. Lane 2 contained the upstream 3' fragment and was approximately 1.2kb. Lane 3 contained the SOE crossover product and was about 2.5kb. The flanking regions' size was approximately 1000 base pairs, as they amplified DNA 1000 base pairs upstream and downstream of the *vbsS* gene. The SOE crossover product was deemed successful as its size

around 2.4kb, and it was the addition of the 3’ and 5’ fragments. All of the DNA fragments were stored at 4°C until further use.

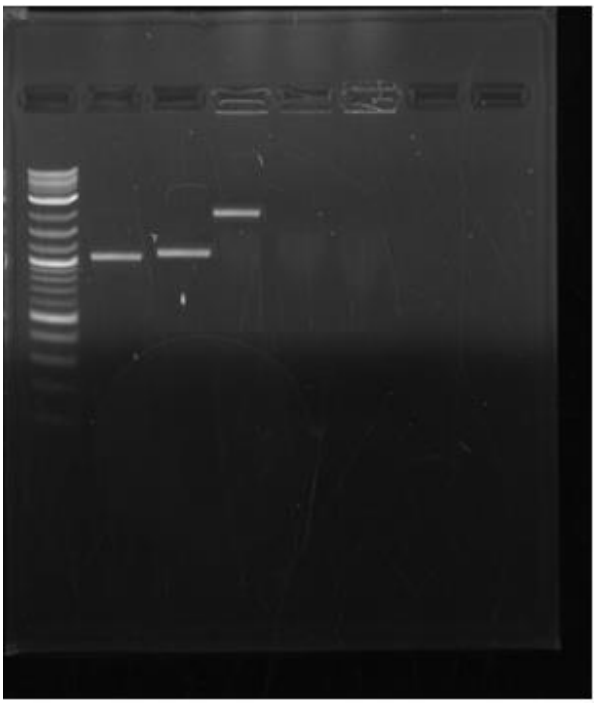

Figure 1. 1% Agarose gel of vbsS flanking regions and crossover product. Lane (1) kb ladder, lane (2) 5’ fragment, lane (3) 3’ fragment, lane (4) SOE crossover product

Isolation of pEX18GM Results

The next step was to isolate a plasmid vector that could carry the *vbsS* crossover product. pEX18GM was chosen for several reasons. It contained a Gm^R selectable marker, a counter selectable *sacB* marker, a Multiple Cloning Site (MCS) in the lacZα allele, and 10 different restriction sites (Hoang et al 1998). It is a circular plasmid containing 5,831 base pairs. The pEX18GM vector is shown below (Figure 2).

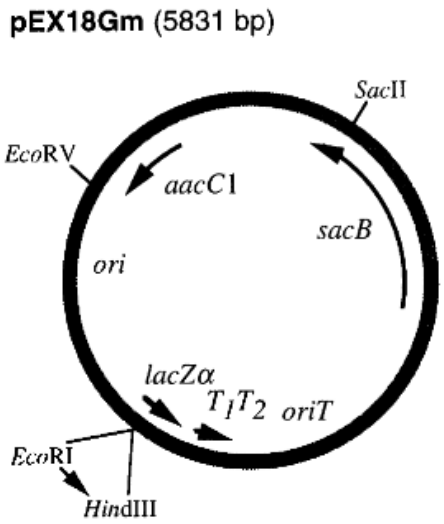

Figure 2. Vector map for pEX18GM (35)

The pEX18GM was isolated using the QIAprep Spin Miniprep Kit. The plasmid DNA purity and concentration was measured using the NanoDrop™ 2000/2000c Spectrophotometer. The results are shown below in Table 4.

	Concentration (ng/uL)	260/280	260/230
pEX18GM Sample 1	**86.5**	1.97	2.06
pEX18GM Sample 2	**57.6**	1.98	1.99

Table 4. NanoDrop results of pEX18GM isolation from 1µl of sample

Restriction Enzyme Digestion Results

The next step was to perform restriction digestion. Two of the same restriction enzymes can cleave both the plasmid vector and the gene of insert at specific sites in order to produce matching single stranded overhangs. The crossover product and pEX18GM were restriction digested with restriction enzymes KpnI and Xbal. These enzymes were previously incorporated into primers utilized for the SOE method. KpnI has a palindromic sequence of GGTACC and

was incorporated into the 5' froward primer (SOE1F) that amplified the region upstream of *vbsS.* XbaI has a palindromic sequence of TCTAGA and incorporated into the 3' reverse primer (SOE1R) that amplified the region upstream of *vbsS.* After restriction digestion was performed, a 1% agarose gel was used to assess the results. After confirmation of results, the DNA bands were excised and purified from the agarose gel using the Monarch® DNA Gel Extraction Kit. The DNA concentration and purity of the crossover product and pEX18GM was measured with the NanoDrop™ 2000/2000c Spectrophotometer. The results are shown in Table 5. Next, ligation was performed in order to join the single stranded overhangs and incorporate the gene of interest into the vector plasmid to create recombinant DNA.

	Concentration (ng/uL)	260/280	260/230
vbsS Crossover	14.4	2.16	.16
pEX18GM Plasmid	9.2	1.85	.39

Table 5. NanoDrop results after restriction enzyme digestion from 1µl of sample

Transformation Results

The next step was to perform transformation to insert the recombinant DNA into competent host cells. Cells were made competent so they can easily uptake foreign DNA. Naturally, cell membranes are negatively charged, so they repel negatively charged DNA. The rubidium chloride salt treatment can neutralize these negative charges and allow foreign DNA to move closer to the cell. Subsequently heat shocking these cells creates temporary pores in the membranes, allowing foreign DNA to enter. After the pEX18GM plasmid containing the crossover product was transformed into NEB 5-alpha *E. Coli* (lacZ-) cells, they were screened

for presence of recombinant DNA via the blue and white screening technique. This technique relies on the principle of α-complementation. The host *E.coli* cells contain deleted amino acids in their *lacZ* gene, creating a non-functional β-galactosidase enzyme, that normally breaks down the chromogenic substrate X-gal into galactose and an insoluble blue pigment (4-chloro-3-brom-indigo). These host cells take up foreign DNA that encodes the deleted amino acids (α-peptide) and create a fully functional β-galactosidase enzyme through complementation. Studies have shown that introducing a multiple cloning site (MCS) into the α-peptide can help screen for recombinant DNA. A gene inserted in a region other than the MCS doesn't disrupt α-complementation and a functional β-galactosidase enzyme is produced, allowing the pigment from X-gal to produce blue colonies**.** A gene properly inserted into a plasmid vector's MCS can disrupt α-complementation and a functional β-galactosidase enzyme is not produced, ensuring the white color of colonies. Several successfully recombinant white colonies were produced and cultured on separate LB plates overnight. colonies had also grown on the positive control LB plate containing competent cells transformed with undigested pEX18GM. This ensured that the competent cells produced were successful, as they were easily able to uptake foreign DNA from their environment. The figure for the positive control is shown below in Figure 2.

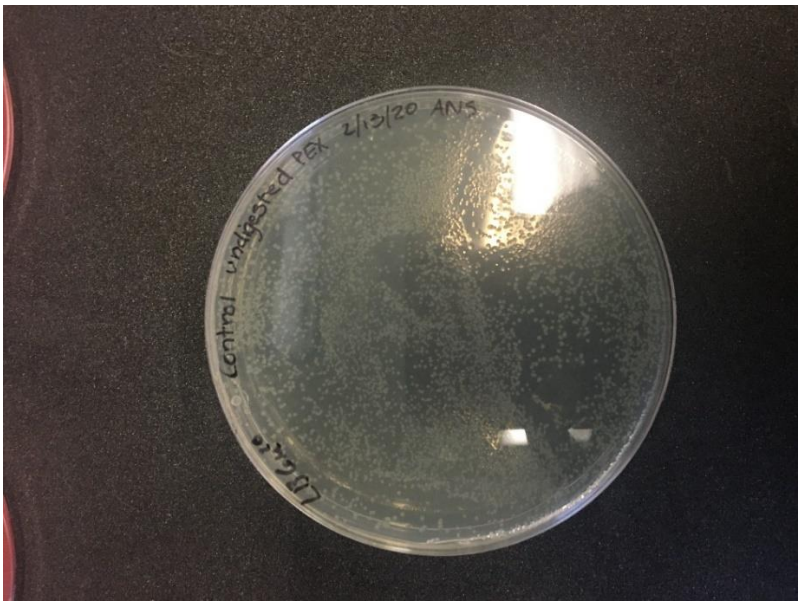

Figure 2. Positive control testing viability of Rubidium Chloride competent cells

Colony PCR Results

After the pEX18GM plasmid was transformed into the host *E.coli* cells, it were tested for the presence of the *vbsS* crossover product via colony PCR. Colony PCR is the method of detecting the presence or absence of the inserted gene in plasmids directly from bacterial colonies. It utilizes the insert flanking primers to amplify the gene of interest. The SOE1R primer was used to detect the presence of the *vbsS* crossover product within the pEX18GM plasmid. The colony PCR products that were run on a 1% agarose gel are shown below in Figure 3. Lane 1 is the kb ladder, lane 2 contains the 3' fragment, and lanes 3-10 contain the 3' fragment from each of the eight colonies. All of the eight colonies amplified at the 3' region, confirming that they contained the *vbsS* crossover product.

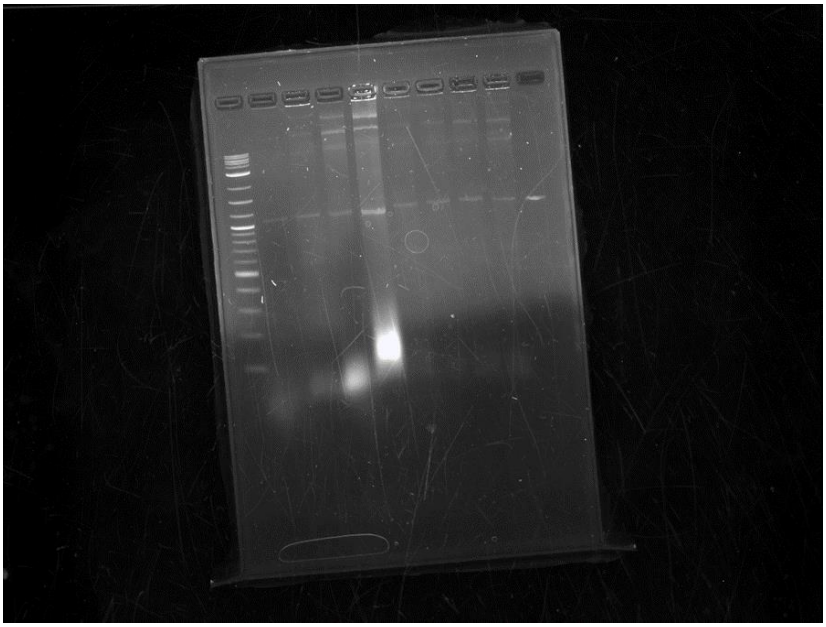

Figure 3. Colony PCR products on 1% agarose gel. Lane (1) kb ladder, lane (2) SOE 3' fragment, lanes (3-8) 3' fragment of colonies

In-Silico Analysis Results

The *NCBI* online database was utilized to search through microorganisms' genomic sequences that contained genes similar to those within the vbs gene cluster. The pathogenic fungi *Aspergillus fumigatus Af293* produces Fusarinine C, a siderophore that is structurally similar to vicibactin, as shown in Figure 4 (Hissen et al 2005). Fusarinine C is controlled by genes *sidA, sidD, sidG, sidF,* and *sidC* that reside on various chromosomes (Hissen et al 2005). The genes responsible for fusarinine C and vicibactin were subjected to *NCBI Protein BLAST* to identify regions of homology within their gene sequences. The genes, their protein products, and similarities are shown below in table 6. Three of the six proteins responsible for vicibactin production were found to contain some degree of homology with the proteins for fusarinine C. The vbsA and sidF proteins had the highest homology percentage; their sequence alignment is shown in Figure 5. Although similarities for the rest of the proteins were not very high, it should

be highlighted that *R. leguminosarum ATCC 14479* is a prokaryotic bacteria and *Aspergillus fumigatus Af293* is a eukaryotic fungus. Having even some form of homology between 2 very different types of organisms is very surprising and this can aid future studies comparing the two organisms.

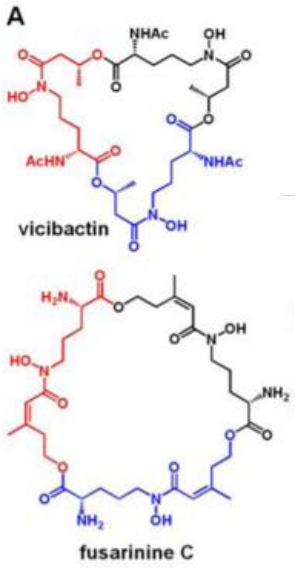

Figure 4. Structural similarity of Fusarnine C and Vicibactin (Wright et al 2013)

Fusarinine C Proteins	Vicibactin Protein Similarity with Corresponding Fusarinine C genes
sidA- Monooxygenase	*vbsO*- Monooxygenase (25%)
sidD- NRPS	*vbsS*- NRPS (28.9%)
sidF- Acetyltransferase	*vbcA*-Acetyltransferase (44%)
sidC- NRPS	*vbsS*- NRPS (26.8%)

Table 6. Protein Similarity of Fusarinine C and Vicibactin

GNAT family N-acetyltransferase [Rhizobium leguminosarum]

Sequence ID: WP_166478620.1 Length: 329 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 130 to 320 GenPept Graphics Next Match

Score	Expect	Method	Identities	Positives	Gaps
173 bits(439)	5e-49	Compositional matrix adjust.	86/195(44%)	116/195(59%)	4/195(2%)

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            HP RP KP     +Y R+IP L++  +  V D + +  L+ FH+W ND +V   W + G
Sbjct  130  HPRRPAKPK--GTIYRRFIPWLEREIAFTVADLETD--LEAFHRWMNDEQVNTIWEDAGS  185

Query  302  LEHHRNYLRQLHEDKHVLCLFGRFDDFPFSYFEVYWAKEDHYGAHYDAGDYDRGRHSLVG  361
            ++ HR  L     D HVL L G F D PF YFEVYWAKED  G  YDA DYDRG H  +G
Sbjct  186  IDKHREILEARIADPHVLPLIGSFADIPFGYFEVYWAKEDRLGPFYDADDYDRGWHVAIG  245

Query  362  ESSVRGAYRVNAWWSSLIHYIFLDEPRTMCVVGEPKATNTTVLSYENAHGLTVQKYVDLG  421
            E   RG   ++AW  SL+H+IFLD+PRT  +VGEP+A++   +   +  G    K+ D
Sbjct  246  EPGYRGKKWISAWLPSLMHFIFLDDPRTKRIVGEPRASHEQQIRNLDRSGFAKVKHFDFP  305

Query  422  HKRSVHVYCSREKWF  436
            HKR++ V   RE++F
Sbjct  306  HKRALLVMLGRERFF  320
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Figure 5. Protein sequence alignment of sidF and vbsA proteins. Subject: *R. leguminosarum ATCC 14479*, Query: *Aspergillus fumigatus Af293*

The unclassified gram-negative, aerobic *Phyllobacterium sp. 628* also produces a siderophore. Via *NCBI Blast*, several genes responsible for vicibactin were similar to ones found on this Phyllobacterium strain, specifically, *vbsS*, *vbsO*, *vbsA*, *vbsL*, and *vbsP*. Table 7 shows these results. The antiSMASH database was also used to generate a color-coded protein sequence of the similarities between Vicibactin and *Phyllobacterium sp. 628*, which generated a 66% gene cluster identity. This is shown below in figure 6. The proteins that had the highest homology percentages have their sequence alignments shown in Figures 7 and 8. Similar to the vicibactin genes, the genes responsible for *Phyllobacterium's* siderophore were within close proximity. The genes in both of the bacteria also exist in the same order, which could play a role in their protein homology.

Phyllobacterium sp. 628 siderophore proteins	Vicibactin protein similarity with corresponding *Phyllobacterium sp. 628* genes
Amino acid adenylation domain containing protein	*vbsS*-Amino acid adenylation domain containing protein (77%)
SidA family monooxygenase	*vbsO*- Lysine-6-monooxygenase (65%)
GNAT family N-acetyltransferase	*vbsA*- Acetyltransferase (58%)
MBtH family NRPS accessory protein	*vbsL*- Polyketide synthase (80%)
Phosphopantetheinyl transferase family protein	*vbsP*- Phosphopantetheinyl transferase (37%)

Table 7. Protein Similarity of *Phyllobacterium sp. 628's* siderophore and Vicibactin

Figure 6. Gene cluster similarity of *Phyllobacterium sp. 628's* siderophore and Vicibactin

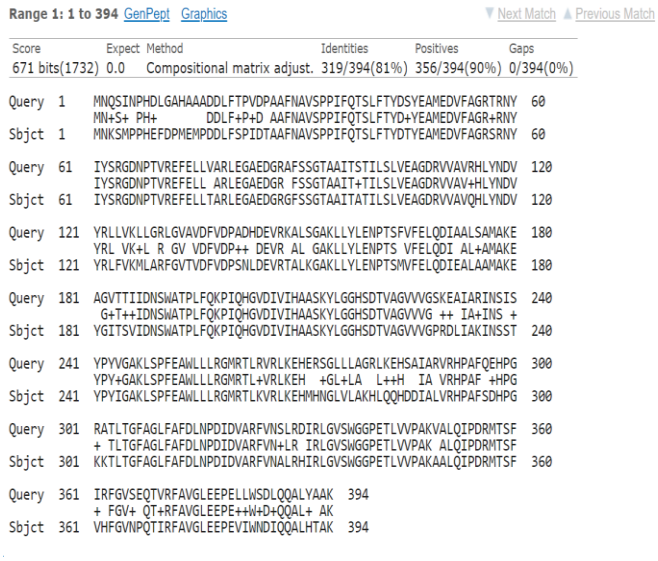

Figure 7. Protein sequence alignment of MtbH family NRPS accessory protein and vbsL.

Subject: *Aspergillus fumigatus Af293*, Query: *R. leguminosarum ATCC 14479*

SidA/lucD/PvdA family monooxygenase [Phyllobacterium sp. 628]

Sequence ID: WP_188342263.1 **Length:** 448 **Number of Matches:** 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 448 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
616 bits(1589)	0.0	Compositional matrix adjust.	293/448(65%)	352/448(78%)	5/448(1%)

```
Query  1    MTVAFARRDA-----EPLDIAGIGIGPSNLSLACLFESVPEIRSRFFERRDSFDWHPGMM  55
            MT  FA  D      E LD+AGIG+GPSNLSLA L + V ++++ F+ERR +FDWHPGMM
Sbjct  1    MTAMFANNDHARQPNETLDLAGIGVGPSNLSLAALLDGVGDMKAHFYERRTNFDWHPGMM  60

Query  56   MPGVELQSSFLKDLVTPVLPTSRWSFVSYLVAHKRLYAFLNANYEAVPRQEFARYLAWVA  115
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Sbjct  61   LPDVELQSSYLKDLVTPVMPTSPWSFVSYLVAHKRLYAFLNAHYDAVPRREFAQYLAWVA  120

Query  116  NGVDGLRFGTEIRDVEHRDDRFFLRFDNGQEEARNLVIGTGSSPFVPDWAKPFLGADCFH  175
              ++ + F T +R+V H  DRF +RFDNG   A+N+V+GTG++PF+P WA PFLG  CFH
Sbjct  121  GQLNNVSFDTPVREVNHDKDRFVVRFDNGSVAAKNIVLGTGTTPFMPSWATPFLGDRCFH  180

Query  176  NSEAKSRLGDLGAARIVVVGGGQSGGEVVEALLGDPGSMTELTWISRRHNFEPINDTPFS  235
            NSEAK RL  L A+RI V+GGGQSGGEVVE++L     + EL W SRR NFEPINDT FS
Sbjct  181  NSEAKFRLPGLNASRIAVIGGGQSGGEVVESILNSKSELKELNWFSRRTNFEPINDTAFS  240

Query  236  NQVFSPEYVQAYLKLGGEQKQAALKNSILTSDGLSISTIHSIYRRLYALRYLEPSTLNAS  295
            NQVFSPEYV AYL L  +QK  ALK SILTSDGLS+STI+SIYRRLY+LRYLE   + A
Sbjct  241  NQVFSPEYVYAYLNLNNDQKLEALKASILTSDGLSLSTINSIYRRLYSLRYLEGRDIEAK  300

Query  296  LAPNRDVIQMERNGNAYRLIVRNQFDGGIEVLHADAVVLATGYRFRLPDALGSLGERITL  355
            L+PNRDVIQ+E + + YRLIVRN+FDGG+EV HADA+VLATGY+F+LP+AL  L +RI
Sbjct  301  LSPNRDVIQVEADKDGYRLIVRNRFDGGVEVAHADAIVLATGYQFKLPEALSGLQDRIQF  360

Query  356  DRNGYPLLNDDYTMQWTGPRNNRLFAQNAGRYSHGIADSQLSLMAWRSATIVNTLLGRQH  415
            DRN  P LND+Y + W GP+ NR+FAQNAGRYSHGIADSQLSLMAWRSA I+NT+ GR H
Sbjct  361  DRNSRPALNDEYCLNWNGPKQNRIFAQNAGRYSHGIADSQLSLMAWRSAHIINTMTGRDH  420

Query  416  FDAEPDNGQLAWATETASAMPHQVRAGY  443
            FD EP + Q+ W +    A+   V A Y
Sbjct  421  FDLEPHDSQINWLSSGHEALGQSVAAEY  448
```

Figure 8. Protein sequence alignment of MtbH family NRPS accessory protein and vbsO. Subject: *Aspergillus fumigatus Af293*, Query: *R. leguminosarum ATCC 14479*

Discussion

The objective of this research was to construct a *vbsS* gene knockout in *Rhizobium leguminosarum* ATCC 14479, in order to determine its gene product's effect on vicibactin production and iron transport. The *vbsS* gene was successfully deleted from the *Rhizobium leguminosarum ATCC 14479* genome through the 'splicing by overlap extension' method. The crossover product was inserted into pEX18GM, through the KpnI and BamI restriction enzymes. The digested products were then ligated together using DNA ligase, with which joins the two DNA fragments together. The recombinant plasmid was transformed into competent *E.coli* (lac

Z-) cells, which were then plated on X-gal containing media, screening for cells that contained plasmids with the crossover product. Cells were also subjected to colony PCR as a second screening method for recombinant plasmids. Analyzing the results revealed that the plasmids taken up by the host *E.coli* cells contained the correct *vbsS* crossover product.

For future studies, the transformed cells should once again be screened for successful insertion of the crossover product into the pEX18GM plasmid. The plasmids should then be isolated and restriction digested to confirm the insert DNA size. The cells can later undergo tri-parental mating in order to incorporate the crossover product into the genome of *Rhizobium leguminosarum ATCC 14479*. They should be screened for potential merodiploids and true mutants should be selected. These *vbsS* mutant *R. leguminosarum* cells and the wild type cells can later be tested for differences in siderophore production and iron transport.

In-silico analysis of the fusarinine C and vicibactin genomic sequences resulted in a low protein homology. Future studies can research other factors that can lead to structural similarity, such as biosynthetic mechanisms and iron transport methods. Comparison between the genes responsible for vicibactin and *Phyllobacterium sp. 628's* siderophore also resulted in a low protein homology but its location alignment was very similar. Future studies can narrow down *Phyllobacterium sp. 628's* classification to reveal its species and type of siderophore produced. This can aid in narrowing down the similarities between the two bacteria.

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