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Genetic analysis of the gene VARPA_4519 encoding a nonribosomal peptide synthetase crucial for surfactant production in Variovorax paradoxus EPS

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GENETIC ANALYSIS OF THE GENE VARPA_4519 ENCODING A NONRIBOSOMAL PEPTIDE SYNTHETASE CRUCIAL FOR SURFACTANT PRODUCTION IN *Variovorax paradoxus* EPS

A Thesis

Presented to the

Faculty of

California State University,

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In Partial Fulfillment

of the Requirements for the Degree

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in

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ABSTRACT

For a bacteria colony, being able to swarm is an important ability for colony growth and expansion. One process that enables bacteria to swarm more efficiently is the production of a lipopolysaccharide known as surfactant. Biosurfactants have multiple functions for bacteria including surface tension reduction and antimicrobial activity. These versatile and useful compounds are also important to human society as well. Chemical surfactants are mostly made by chemical resulting in pollution and waste. It would be more cost effective and greener for our society to use naturally occurring surfactants, and microbial surfactants are prime candidates for this sort of bioengineering. I identified a gene, Varpa_4519, from *Variovorax paradoxus* EPS that plays a role in the production of a wetting agent produced by *V. paradoxus* EPS. When I removed a large portion of the Varpa_4519 coding sequence I eliminated surfactant production. I evaluated surfactant production directly and also observed the effects of the deletion on swarming and biofilm production. The swarming ability of the mutant strain is significantly reduced compared to wild type, while the biofilm production is increased. When the knockout was complemented in *trans* by expression on a plasmid surfactant production was restored. The rate of swarming across the plate was also restored by this complementation method, but the colony phenotype was not the same as wild type swarms. Biofilm production was partially restored to wild type with complementation as well.

These results support the hypothesis that Varpa_4519 plays an integral role in the production of the wetting agent from *V. paradoxus* EPS.

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CHAPTER ONE

INTRODUCTION

Swarming

Swarming motility is defined as movement on a surface requiring surfactant and flagella (Jamieson et al, 2009). In laboratory culture swarming is an important mechanism used by bacteria to increase colony coverage of high nutrient environments and quickly increase biomass (Fraser & Hughes, 1999). Swarming is a differentiation process in which bacteria enter into an excited state of constant movement (Fraser & Hughes, 1999). The mechanism for swarming is driven by nutrient factors (Willems et al, 1991), and is not based on specific development stages of the bacterial life cycle (Fraser & Hughes, 1999). Swarming motility is often triggered by a type of chemical signaling known as quorum sensing (Fraser & Hughes, 1999) or density dependent gene regulation. This chemical signaling is what tells the colony of bacteria to continue swarming or to stop swarming, which is also modulated by various end factors (Whitehead et al, 2001; Xavier et al, 2010).

Swarming by *Variovoras paradoxus* EPS has been investigated to understand the genes involved and the mechanisms for swarming motility. The results from Jamieson et al. showed that *V. paradoxus* EPS produced a variety of swarming physiologies when grown on various surfaces, producing a variety of phenotypes depending on the different nutrient conditions. This is indicative of a

complex response to growth conditions (Jamieson et al, 2009). It has also been shown that *V. paradoxus* EPS produces a wetting agent that reduces the surface tension of media (Jamieson et al, 2009).

Surfactant Production

Biosurfactants are amphipathic molecules that lower surface tension. These molecules are structurally diverse and can interact with both aqueous solutions and hydrocarbon mixtures (Desai & Banat 1997). One method for categorizing biosurfactants is to look at the origin and chemical composition, which includes non-microbial biosurfactants like pulmonary surfactant. Pulmonary surfactant is a lipoprotein complex generated by type II alveolar cells and functions to increase pulmonary compliance and prevent atelectasis. The structures of these surfactants include amino acids, peptides, anions, cations, and saccharides. The major classes of biosurfactants are lipopetides, glycolipids, phospholipids, fatty acids, polymeric surfactants, and particulate surfactants (Peypoux et al, 1999).

Bacteria secrete many of compounds necessary for various functions. Such functions include communication, virulence, and colonization of environments. During swarming motile bacteria produce surfactant, and enabling them to move more easily across a surface. This is important because it allows a colony to reach more nutrients and promote cell growth. Colonies of bacteria that are able to swarm on media produced more cells than colonies that are

unable to swarm (Willems et al, 1991), providing an ability to outcompete other bacteria that cannot swarm. Surfactants can also work as an environment stabilizer which would allow bacteria to survive in conditions otherwise uninhabitable. *Bacillus licheniformis*produces surfactants that contribute to temperature, pH, and salt stability within the bacteria and its surroundings (7), allowing for survival in harsh environments.

The typical lipopeptide biosurfactant is a cyclic peptide with a fatty acid chain attached. Some of these possess known antibiotic activity like bacitracin and surfactin (Konz et al, 1997; Gautam & Tyagi, 2006). Other uses include detergents like household cleaning products and emulsifiers like those used to clean up oil spills. An example of a biosurfactant used for emulsifying is emulsan, from *Acinobactercalcoaceticus*, which is the strongest emulsifier known (Gautam & Tyagi, 2006). It has also been shown that *Saccharomyces cerevisiae* produces a protein that is an excellent emulsifier (Gautam & Tyagi, 2006).

Biological Importance of Surfactant

Surfactants are used as detergents, emulsifiers, food processing, and soap, and in many different processes, creating a great deal of commercial demand (Reyrat et at, 1998). The problem with the production of massive amount of chemically derived surfactant is the amount of toxic waste that accompanies it. For this reason the identification of new naturally occurring surfactants or the development of greener production is desirable. Bacteria have

been frequently identified as sources of naturally processed surfactant and as a host for genetic engineering of surfactant producer (George & Jayachandran, 2009; Ron & Rosenberg, 2001).

Bacteria are good sources for naturally produced surfactant, or biosurfactant. Recent problems like the BP oil spill have driven the search for new surfactant producers able to help with bioremediation. This means looking for microbes that naturally thrive in contaminated environments. Batista et al were able to isolate bacteria from petroleum contaminated soil (Batista et al, 2006). In their study they used quick screening techniques to find a number of isolates that could be producers with potential industrial applications. The industrial scale production of biosurfactant is done by collecting the released surfactants from culture media by purification protocols (Batista et al, 2006).

Lipopeptide Surfactant

There are many well studied lipopeptide surfactants. The antibiotic surfactin is one, which is produced by *Bacillissubtilis* (Peypoux et al, 1999). Surfactin is produced by a Non-Ribosomal Protein Synthetase (NRPS) and its synthesis is coded by the *srf* operon. This operon is estimated to be around 25kb and codes for the peptide synthetase (Ehling-Schulz et al, 2005; George & Jayachandran, 2009). Genes such as *srfA* encode proteins composed of repeating domains that facilitate the production of a lipopeptide. Other such operons include T*yc*ABC of *Bacillisbrevis* (Ledbetter & Greenberg, 2000), and

SrfA of *Bacillus subtilis* (Ledbetter & Greenberg, 2000). This class of operon is often regulated by quorum sensing, which is the cell to cell communication in a bacterial population based on cell density (Daniels et al, 2004). Cell density plays a crucial role in determining when specific genes get turned on and (Whitehead et al, 2001). This signaling system is used in many bacteria to control expression of a number of virulence factors, biofilm formation, and swarming motility (Caiazza et al, 2007; Kievit et al, 2001).

Nonribosomal Protein Synthetase

Many soil bacteria produce peptides that are biologically active and important in medicine, industry, and scientific research (Neu, 1996; Desai & Banat, 1997). Some of these products include toxins (plant and animal), surfactants, antibiotics, and immunosupresents which all play large roles in the world today (Neu, 1996). Many biologically active peptides are synthesized non-ribosomally. The proteins responsible for the building of these peptides are called non-ribosomal peptide synthatases (NRPS). These proteins are capable of producing, cyclic, and branched linear structures. This is done by sequential addition of amino acids with thioester intermediates; the process is finalized by the various ways such as cyclization, the action of thioesterase, or by transferring the peptide chain to a functional group such as a fatty acid (Stachelhaus & Marahiel, 1995). The process by which these peptides are formed is the thiotemplate mechanism, which is non-ribosomal and non-RNA dependent. In

this process the amino acid residues are transferred from amino acid adenylates to thiol groups of a peptide synthetase. The peptide synthetase gathers the residues in sequence on a phosphopantetheine arm.

The NRPS structure determines the size and arrangement of the peptide product (Marahiel, 2009). NRPS proteins have a large modular organization with various modules containing catalytic activities needed to assemble the peptide product, as demonstrated in figure 1 (Marahiel, 2009).

The main module is the adenylation domain, which causes the target amino acid to become an adenylate. The activated substrate is bound to the thiolation domain covalently or to the carrier protein. The carrier proteins contain sites for attachment of the respective cofactor. The condensation domain catalyzes the elongation of the peptide by forming a peptide bond between the amino acids (Stachelhaus et al, 1998). The condensation domain is located at the N-terminal ends of modules accepting the acyl groups from the preceding module (Stachelhaus et al, 1998). Finally a carboxyl-terminal thioesterase domain catalyzes the release of the finished peptide. This release of the peptide can also be triggered by the cyclization or addition of monomer units such as lipids (Marahiel, 2009).

Genetics of Nonribosomal Peptide Synthetase

Multiple genes are required to encode the various enzymes needed for a bacterium to produce peptides with biological activities (Stachelhaus & Marahiel,

1995). While these peptides need various enzymes to put them together, some were shown to be regulated by a single gene. Lindum et al. showed that a signal was needed to produce the surfactant serrawettin W2. The signal was based on quorum sensing, but the activation of *swrA* gene produced the putative peptide synthetase for serrawettin W2.

Even though there are many structurally diverse NRPS products there is a conserved mode of synthesis (Marahiel, 2009). The enzymes are made up of modular sections where the module carries out a synthetase function (Marahiel, 2009). An NRPS operon produces functions and modules that add various amino acids or perform various actions (such as phosphorylation) on the protein being constructed. The end product works its way down the assembly line of modules to the termination module (Marahiel, 2009).

Non-ribosomal synthesis of a peptide is a highly conserved process in bacteria (Marx & Lidstrom, 2002), enabling homology based approaches to identify in a step wise manner the specific genes responsible for making the sythetases. For example, Konz et al. utilized a knock out approach to identify the genes (*bacA-bacC*) involved in the synthesis of bacitracin (Konz et al, 1997).

Variovorax paradoxus EPS

Variovorax paradoxus EPS is a gram negative aerobic microorganism and is found in diverse soil samples including mine spoil and clay (Piotrowska-Seget et al, 2005; Willems et al, 1991). It has also been found in the oral cavity of

humans (Anesti et al, 2005). As the name implies, *Variovorax paradoxus* EPS has also been found to have the capability for many biochemical transformations (Jamieson et al, 2009; Phel et al, 2012). *Variovorax paradoxus* EPS has been shown to degrade hazardous chemicals such as oil, fuel, herbicide and pesticides (Kamagata et al, 1997; Phel et al, 2012; Smith et al, 2005). There is also evidence to show that *V. paradoxus* EPS is associated with plant growth and promotion (Belimov et al, 2009; Maimaiti et al, 2007). The ubiquity and biotransformative potential of this organism, as well as its potential importance in the soil makes *Variovorax paradoxus* EPS an interesting organism for genetic studies.

Variovorax paradoxus EPS possesses a number of characteristics that are also seen in pathogenic bacteria such as *Pseudomonas aeruginosa,* including swarming motility, biofilm formation, and biosurfactant production (Phel et al, 2012). These traits are involved in diverse activities in nature, including plant associated behaviors. The focus of this work is the production of biosurfactant that it was discovered to produce (figure 3) and the role of this wetting agent in surface associated behavior.

The production of biosurfactant is a prerequisite for several types of motility, including sliding and swarming motility (Fraser & Hughes, 1999, Xavier et al, 2010). Both of these modes of surface movement have been identified as crucial in many environments.

Allelic Exchange in *Variovorax paradoxus EPS*

Manipulating DNA has become a common practice in microbiology. Various molecular techniques enable us to determine species, examine gene expression patterns, and figure out what the functions of individual genes are. Creating a knock out mutant is an important step in understanding the link between gene and function (Lindum et al, 1998; Stachelhaus et al, 1998). The creation of the mutant is one part, but being able to identify the mutant is also a factor for being able to study it. Creating a mutant using a selectable marker will enable the study of the knock out mutant while insuring that you are only working with the mutant.

Allelic exchange is a common occurrence among diploid organisms. During the generation of gametes when homologous chromosomes line up crossing over events occur which generate new variation in the genetic material of future offspring. Microbes on the other hand have haploid genomes, and asexual reproduction. Thus, for variation by homologous recombination to take place in bacteria they need an outside source. For example, if a bacteriophage infects a microbe and proceeds to synthesize new phages there is a chance while packaging genetic material into new viruses it packs chromosomal DNA from the host by mistake. This process, generalized transduction, results in the introduction of that chromosomal DNA into a new host which can then be inserted or exchanged (via crossing over) with the new host upon infection.

The idea to create an allelic exchange in bacteria using plasmids and cloned genes stems from the idea of creating variation within bacteria using an outside source. The allelic exchange created by placing a cloned gene on a suicide plasmid and allowing a crossover event to take place will allow the development of directed variation among studied bacteria. The gene of interest will be replaced with an allele that contains a selectable marker in place of the active portion of the gene product, flanked by host sequence. The plasmid containing the construct is a suicide plasmid, which means that it cannot replicate in the targeted host. Selection for resistance to the marker followed by testing to eliminate single crossover creation of merodiploids leads to the selection of a strain where the wild-type gene of interest has been replaced with a selectable null mutant. Using the *cre-lox* system (Stachelhaus & Maraheil, 1995) further provides us with the flexibility to remove the selectable marker by Cre recombinase action, resulting in an unmarked deletion. This can be used to verify that the phenotype is related solely to the loss of the functional gene in question. And can also allow for serial deletion of multiple genes in the host without requiring use of multiple markers.

Being able to target genes like this has become easier with the prudence of genome sequencing. While the high throughput sequencing of whole genomes enables huge amounts of data to put explored; most of it is hypothetical analysis based on homology of already known genes (Lambert et al, 2007; Marx & Lidstrom, 2002). While knowing the potential relationship to previously known

homologous genes can generate a starting point, more extensive analysis is needed to understand a new genes function. This is done by creating a knockout and looking at the phenotypic change, or looking at the expression levels of the gene under various conditions.

RT-qPCR

Cells regulate gene expression in decisions related to survival and growth (Caiazza et al, 2007). To understand the regulation of genes within a cell a method is needed to quantitatively measure expression. qPCR is used to determine the level of gene expression accurately and quantitatively by measuring mRNA levels from bacterial cells. The process involves prepping RNA from cells and reverse transcribing it into cDNA that can then be amplifyied via PCR. The major difference in qPCR from PCR is that the amount of amplification of the DNA is measured after every cycle. The levels of DNA can then be related to levels of mRNA which in turn express the level of gene expression. This widely used process is the preferred method for quantifying mRNAs from microbial samples (Caiazza et al, 2007).

The process works by integrating a fluorescent dye into the PCR reaction which is linked to the DNA. This can vary depending on the company that produces the qPCR machine and can either be a molecule integrated into the DNA product or a fluorescence molecule (dye) that binds to completed dsDNA.

In either case the fluorescence level of the reporter dye/probe is measured quantitatively.

One of the drawbacks to qPCR is that the process is complicated by the possible loss of mRNA during sample preparation and by the inefficiencies in reverse transcription. In order to counter the loss of mRNA the quantities are normalized to a reference gene, which is constitutively expressed. Since bacteria don't have any good constitutively expressed genes for a reference an exogenous mRNA strand (Firefly Luciferase) is added to the sample before the reverse transcription takes place. The reverse transcription is a process by which the mRNA obtained from samples is transcribed back in to DNA, otherwise known as complimentary DNA (cDNA). This will give us a known about of luciferase cDNA to compare to the mRNA levels of our organism. This step enables the accurate comparison of gene expression levels across multiple growth conditions (Caiazza et al, 2007).

RT-qPCR gives proportional measurements of the amount of cDNA in the sample, which is related to the amount of mRNA acquired from the beginning. The level of mRNA under different conditions will tell whether the gene is being up regulated, down regulated, or not changed in the different environments compared to the standard expression levels of the exogenous mRNA. Taking the Ct values, this is the cycle number when the generated curve crosses a set threshold. The Ct value is then converted to fold expression and compared to a standard. When looking at this under different growth conditions the expression

of a particular genes regulation can be analyzed. The lower the Ct value the higher the concentration of cDNA template in the reaction. This is exemplified in figure 2 as a sample of expression levels of DNA. By comparing Ct values, which is done using the Pfaffl method for comparative transcript analysis (Ron & Rosenberg, 2001), you can determine relative expression levels.

CHAPTER TWO

MATERIALS AND METHODS

DNA Manipulation in *E. coli*

The development of a mutant strain of *V. paradoxus* EPS required the use of *E. coli* to accomplish DNA manipulation. *E. coli* was grown in LB media in broth cultures and Petri dishes at 37°C. *V. paradoxus* EPS was grown in YE media at 30 °C for both for both broth and Petri dishes.

Visualization of Biosurfactant in *V. paradoxus* EPS

A wetting agent was produced and visualized by *V. paradoxus* EPS on 0.5% and 0.3% agarose, YE media (figure 3). The visualization of the wetting agent came under variable growth conditions of *V. paradoxus* EPS from previous experiments.

Swarming Assay

For the swarming media 1.25g of agarose was added to 235ml sterile water, then autoclaved. After it was taken out of the autoclave and cooled to about 60°C the following were added to it: 2.5ml 100X freshwater base, 2.5ml 20% succinate, 5ml 1M MOPS, 2.5ml 10% casamino acids, 2.5ml 20% glucose, 250ul Na_2SO_4, 250ul Trace metals, 250ul Trace vitamins (ATCC). When using for complementation assay 125ul Gentamicin was added to the media. The

antibiotic will be excluded if doing a normal swarming assay. All measurements for swarming were done on 3 plates with 3 colonies on each, giving a total of 9 samples per strain of bacteria.

Biofilm Assay

For the biofilm assay a colony was selected from a freshly streaked YE plate and inoculated into an overnight culture in 50% YE broth. This was done for all strains of *V. paradoxus* EPS. The overnight culture was grown for 24 hours. At the 24 hour mark the culture was diluted 1:20 with 50% YE broth. From there 100ul of the diluted culture was added to a 96 well plate. The 96 well plate's lid was treated with Triton X (Jamieson et al, 2009) and dried. Outer well were filled with sterile water, and the inner wells filled with the diluted culture. 96 well plates containing the diluted cultures were grown for 24 and 48 hour time points, at which point they were treated and analyzed as per protocol (Jamieson et al, 2009; Phel et al, 2012)

Construction of Allelic Exchange Vector

A homolog to the *swrA* gene associated with serrawettinW2 in *Serratia liquefaciens* was found. This homolog responsible for biosurfactant production was used to BLAST search (Basic Local Alignment Searc Tool) to find similar NRPS genes in *V. paradoxus* EPS. The gene sequence identified as the most likely target was Varpa_4519. Using the sequence of the identified potential

surfactant gene primers were developed using a sequence analytical tool called Geneious (table 2). To create the construct for directed deletion primers were development for the amplification of the flanking regions as well as the whole gene (figure 8). Each primer was designed to incorporate a specific restriction site. The N terminus flanking DNA had EcoRI and NdeI restriction sites inserted on opposite ends, while the C terminus had ApaI and AgeI. This was so the N and C terminus could be inserted in series and not be digested out later.

For the purification of genome DNA Promega Wizard® genome purification kit was used as per manufacturer's directions. The flanking regions of Varpa_4519 including the N and C terms of the putative enzyme were amplified from purified genome. Based on primer sequence and location the N term was 1.5kb and the C term as 1kb (figure 9). This was done by using 1ul of purified *V. paradoxus* EPS genome, 12.5ul GoTaq green, 1ul of each primer diluted to 0.25uM (reverse and forward), and 10.5ul of DNA grade water. PCR reaction samples were examined by gel electrophoresis and purified after verification using the Promega Wizard® SV Gel and PCR Clean-up System.

The purified flanking segments were cloned into pCR 2.1 using the Invitrogen TOPO TA cloning kit. The new vector containing one of two flanking DNA segments was then transformed into *E. coli* using Lucigen® chemically competent cells and protocol. From the products stock cultures were made from colonies that showed correct insertion of the gene, verified by sequencing

(Laragen). The plasmid was purified using Promega Wizard® *Plus* SV Minipreps DNA purification system and kept in the -20 °C freezer.

The construction of the pCM184 vector containing the N and C term flanking DNA was prepared by subcloning the fragments from PCR 2.1 and ligating them into pCM184. This was done in a series of restriction enzyme treatments. After each digestion sample was verified by electrophoresis, followed by extraction and subsequent ligation. The completed pCM184 including the N and C terms was verified by electrophoresis (figure 10). The completed plasmid would be inserted into wild type *V. paradoxus* EPS and screened for potential double cross over event, as depicted in figure 11. The plasmid constructs used for the generation of the mutant strain and all complementation strains are shown in table 5.

Electroporation of *E. coli*

Electroporation cuvettes were placed on ice for 15 minutes while the ligation reaction was deactivated. All components used in electroporation were put on ice to keep everything cold during the process. Electrocompetent cells were thawed on ice, and divided into cooled microcentrifuge tubes in 25ul aliquots. 1ul of plasmid was added to thawed cells and mixed gently. Mixture of cells and plasmid were then added to chilled electroporation cuvettes with 0.1mm gap. Cuvettes containing competent cells and plasmid were then pulsed by the Micropulser Electroporator, which is set to 1.8 kV for bacteria and pulsed. After

10 seconds 975ul of recovery medium was added to electrocompetent cells the solution was transferred to culture tube, and shaken at 37°C for 1 hour. After the 1 hour recovery process cells were then plated out on LB Ampicillin or LB kanamycin culture plates and then stored in 37°C incubator for 20 hours.

Creation of pSMV8 Vector and Verification of Genomic Insertion

To improve the efficiency of allelic exchange with pCM184 the insertion segment containing the C and N term flanks of Varpa_4519 and the kanamycin gene were amplified directly from completed PpCM184 plasmid and ligated into pSMV8. The ligation was performed with a SmaI digested pSMV8 and the amplified PCR product from PCM184. pSMV8 was then transformed into electrocompetent *E. coli* and grown on LB kanamycin media for blue/white screening. White colonies were chosen for verification of the purified plasmid by redigesting with SmaI. This new tactic would only require a single crossover event and the subsequent excision of the excess plasmid via the sacB gene (figure 12).

Once the allelic replacement construct was verified it was transformed into electrocompetent *V. paradoxus* EPS. Electrocompetent *V. paradoxus* EPS was prepared as described in previous work (Piotrowska-Seget et al, 2005). The complete pSMV8 plasmid containing the insert from PCM184 was then transformed into wild type *V. paradoxus* EPS and grown on YE Kanamycin plates. Once a successful insertion of the plasmid was found the knockout was

completed by growing the strain of *V. paradoxus* EPS on 5% sucrose media to induce the toxicity of the sacB gene (Reyrat et al, 1998), which is also on pSMV8. The sucrose counterselection forced the excision of the sacB gene from the genome of *V. paradoxus* EPS creating a clean knock out of Varpa_4519 (figure 12). This prospective knockout was tested for kanamycin resistance (figure 13) and gentamicin sensitivity (figure 14) to verify complete removal of the unwanted section of pSMV8 from the genome, and also verified by PCR (figure 15). The primers generated to amplify the sacB gene are on table 3.

Creation of Delta Surf Complementation Strains

The mutant strain of *V. paradoxus* EPS created by knocking out Varpa_4519 was labeled delta surf. Further constructs were created for the complementation of delta surf by cloning the complete gene (Varpa_4519) onto pBBR-MCS5. The vector pBBR-MCS5 is a vector with an origin of replication for *V. paradoxus* EPS and a gentamicin resistance gene. Strains were created of both with type and delta surf strains with pBBRMCS5 alone and pBBRMCS5 plus Varpa_4519 (table 5). The complementation strains were labeled as follows: wild type + pRJF006, wild type + pBBRMCS5, delta surf + pRJF006, delta surf + pBBRMCS5. The electro-transformation was conducted as previously described (Piotrowska-Seget et al, 2005), and each strain was verified by SmaI digestion of purified plasmid and gel electrophoresis.

Generation of Growth Curves

Generation of growth curves was performed and strain specific growth rates were calculated. This was done as described in previous work from the lab (Jamieson et al, 2009). The assays done to evaluate the effects from the loss of production of a wetting agent were a biofilm assay and swarming assay. These assays were also done the same as described in previous work in the lab (Jamieson et al, 2009; Phel et al, 2012).

Visualization of Biosurfactant and Drop Collapse Assay

Visualization of surfactant production was performed by placing 10ul of overnight culture on 0.5% agarose YE media a grown for 24hr. Further visualization of the surfactant was performed by the drop collapse assay (figure 17). Taking swarming media after the 48 hour time point and adding a drop of 1:100 diluted methylene blue solution. This was added to the center of each plate containing wild type + pBBRMCS5, wild type + pRJF006, delta surf + pBBEMCS5, and delta surf + pRJF006 strains. The presence of surfactant would produce a collapsed drop by showing a spreading and expansion of said drop. A negative result is indicated by the lake of spreading indicating no reduced surface.

RT-qPCR

RT-qPCR was performed on RNA collected from wild type *V. paradoxus* EPS using Fisher and Qiagen RNA purification kits. Swarm assay plates consisted of three types of growth media for RT-qPCR: Ye (2.5 g/L yeast extract); Sp (FW media with glucose and casamino acids); and Suc (FW with sodium succinate and NH4Cl). Each media type was done with agar at 0.5% and 1.5% (w/v) agarose, except YE which was also solidified with 0.3% (w/v) agarose. RT-qPCR analysis of RNA was done on samples collected at 24 hour (figure 4) and 48 hour (figure 5) time points. There were three samples collected for each growth condition. Each sample was run in triplicate for RT-qPCR. Due to inconsistent technical replicates 0.3%ye2 and 0.5%Suc2 were left out of the 48h set.

The purified RNA was purified with Qiagen RNA purification kit then reverse transcribed using a Promega GoScript reverse transcription kit. The resulting cDNA was then stored in the -80°C. Prior to the cDNA use, the RT-qPCR prep was done. The SYBR Green was spiked with ROX dye aliquoted out and stored in the -20 °C freezer. For the set up and preparation of the RT-qPCR reaction pipettes, tips, ice container (with ice), microfuge tubes, RT-qPCR strips and RT-qPCR strip holders were placed in the UV hood and sterilized for 30 minutes.

The reaction was prepared by adding 12.5ul SYBR Green, 1ul forward primer, 1ul reverse primer, 1ul cDNA, 9.5ul sterile water. No template and no

primer controls were included in all reactions to insure the experimental reaction was between primers and sample only. The primers used for RT-qPCR on Varpa_4519 are listed in Table 1. Once reaction mixtures were finished they were loaded into the MX3005P qPCR machine and the program was run as directed in the MxPro manual. The machine ran for 40 cycles. And samples were analyzed using the Pfaffl (Peypoux et al, 1999) method.

CHAPTER THREE

RESULTS

RT-qPCR

RT-qPCR were performed to quantify expression of Varpa_4519 under various growth conditions. While a great deal of variation in mRNA levels was observed under various growth conditions, no overall pattern in the expression of Varpa_4519 was evident. In early (24 hour) growth Varpa_4519 is expressed on 0.5%Ye and decreases in later (48 hour) growth. The opposite is true on low percentage agar plates using succinate as carbon source. On plates using the swarming assay plate recipe we observed minimal expression which suggests nutrients might affect gene expression. Expression of Varpa_4519 on 0.5% and 1.5% YE plates is increased at 24 hours and slightly decreased at 48 hours. The expression on 0.3% was increased in both the 24 hour and 48 hour time points. On 0.5% and 1.5% Succinate expression was decreased in 24 hours and similar to log phase growth at 48 hours. On sp media there was no difference between 0.5% 24 and 48 hours with log phase growth. 1.5% sp had a decreased expression at 48 hours compared to log phase. This supports earlier studies on nutrient impacts on *V. paradoxus* EPS physiology (Jamieson et al, 2009).

Surfactant Purificaion

The surfactant released by *Variovorax paradoxus* EPS has been isolated and analyzed in collaboration with Richard Belcher and David Crowley of UCR, to determine the major molecules that are associated with it as well as its mass. As shown in figure 6, the mass/charge ratio of the purified wetting agent was determined to be 679.3 m/z (Belcher et al, 2012). The wetting agent reduced the surface tension on water from (x) N to (y) N as seen in figure 7. Based on this experimental observation the critical micelle concentration of wetting agent was calculated to be 30 $\pm$ 5 mN/m.

Growth Curve Analysis

Growth curve was show to have no significant difference between each strain of bacteria as shown in figure 29. The doubling time (table 4) was also calculated from the curves and showed no significant difference between strains. The various strains were made using plasmid constructs as seen in table 5. The strains used for the growth curve were wild type, wild type + pBBRMCS5, wild type + RJF006, delta surf, delta surf+ pBBRMCS5, and delta surf + RJF006.

Comparison of Delta Surf to Wild Type

Comparison of delta surf to wild type vividly demonstrated that surfactant was no longer produced on 0.5% YE agarose/agar plates.

The knockout of Varpa_4519 significantly reduced swarming capabilities in delta surf compared to wild type. Figure 18 and 19 shows the distance traveled for each strain in the particular experiments. In both cases delta surf had significantly reduced swarming capabilities compared to wild type.

Biofilm levels were significantly higher ($P<0.05$) in delta surf than in wild type. This was shown for both trials of the experiment (figure 27 and 28).

Wetting Agent Verification in Complementation Experiments

Figure 16 shows that complementation strains grown on 0.5% agarose YE media recover surfactant production. The delta surf + pBBR1MCS5 strain is unable to produce surfactant after 24 hour (figure 16, 17) while the introduction of pRJF006 results in the restoration of surfactant clearly visible on the plate (figure 16). The presence of either the vector of the recombination plasmid did not visibly alter surfactant production on this media in the wilt type background.

Drop Collapse Assay

The drop collapse assay was used to directly qualitatively examine surfactant production on swarming assay plates. The assay was carried out on a wild type + pBBRMCS5, wild type + pRJF006, delta surf + pBBRMCS5, and delta surf + pRJF006 in triplicate plates, each plate containing 3 colonies. As shown in figure 17 the drop collapses and spreads across the media for the wild type + pBBRMCS5, wild type + pRJF006, and delta surf + pRJF006 indicating the

presence of surfactant on the media. Delta surf + pBBRMCS5 (C on figure 17) shows a drop that retains it shape, Indicative of a high surface tension on the agar and the absence of surfactant.

Swarming Complementation

When swarming complementation of Varpa_4519 was done variable results were observed. There was a partial restoration of swarming with the complementation of delta surf + pRJF006 compared to wild type; this is shown by the swarming distance in figure 21, 22, 23, and 24. The difference lies in the morphology of the colony (figure 20). The complementation of delta surf did not fill out as expected compared to the wild type. This same issue is seen in the wild type + pRJF006 strain. This suggests that having multiple copies of the gene (either on plasmids or plasmids and genome) generates a different phenotype than just having a single genomic copy. The humidity showed an inconsistency with the plates as well, this is related in the data as seen in figure 20 as well as previous data from the lab.

The lack of swarming is noticeable by delta surf + pBBRMCS5 (D of figure 25). While there is only partial complementation of delta surf + pRJF006, you can see the ability to swarm but the morphology is not the same as wild type (C of figure 25). The same trend is seen in the 48 hour photos as well, in figure 26.

There were a couple experiments that were taken out because the wild type did not swarm as expected, indicating abnormal conditions. This has been

seen before as humidity fluctuates in the incubator and might play a role in the variation seen among different plates in the same experiment.

Biofilm Complementation

Delta surf + pRJF006 showed lower biofilm levels compared to delta surf + pBBRMCS5, indicating that the replacement of Varpa_4519 lowered the biofilm levels. Figure 27 also shows that the levels were similar to wild type + pRJF006 and could be due to the issue of having multiple copies of Varpa_4519 present. This shows a partial complementation for biofilm, because we do not get the wildtype phenotype back but we show a reduction of biofilm in delta surf + pRJF006 compared to delta surf + pBBRMCS5.

These results show that Varpa_4519 plays a role in biofilm formation. For the complementation of Varpa_4519 there were some inconsistent results. These inconsistencies showed delta surf + pBBRMCS5 being similar to wild type + pBBRMCS5 and that there were also differences between wild type + pRJF006 and wild type + pBBRMCS5, both of which are indicative of the variability of this complex phenotype.

CHAPTER FOUR

DISCUSSION

Biosurfactants are widely used by modern society in a range of applications, making these biological products a desirable commodity. Being able to make such bio-molecules cheaply and efficiently would be a great help to both the planet and society by reducing the production and waste of chemically made surfactants. Understanding how bacteria create and utilize surfactants is a necessary step towards finding greener ways to produce surfactants and finding ways to harness the production capability of microbial made surfactants.

Bacteria use biosurfactants to help colony expansion, which is a necessary step for successful growth. Previous research has shown the importance of surfactant production in swarming and biofilm production (Mireles et al, 2001; Xavier er al, 2010). I have knocked out the surfactant gene Varpa_4519 generating a mutant strain that does not produce surfactant and reestablish the production of surfactant by adding Varpa_4519 back in *trans*. As shown in figures 18 and 19, the mutant strain has significantly lower swarming distances. This is further validated by figures 20, which show a dramatic colony morphology difference between the wild type and delta surf. This is similar to previous research showing lack of swarming due to lack of surfactant production (Lindum et al, 1998; Xavier et al, 2010). A bacterium that has significantly lower

potential to swarm has a severe disadvantage towards nutrient acquisition and its overall fitness.

Bacteria that lack surfactant production will have a harder time trying to access nutrients by being able to overcome the surface tension. It is also possible for bacteria to lose in competition to other microbes by lacking defense mechanisms like antibiotics, some of which are surfactants.

There is also some evidence that having multiple copies of Varpa_4519 play a role is colony morphology on swarming media. This is shown with wild type + pRJF006, as seen in figure 21, 22, 23 and 24. A potential reason for this difference might be due to having multiple copies of the gene supplied in *trans* on a plasmid, rather than the single copy in the wild type genome. This could explain why the wild type + pRJF006 produced inconsistent colony morphology on swarming media. The distance of delta surf + pRJF006 is not different than that of the wild type, but not morphologically. This is shown in figure 25 and 26, where the pattern for delta surf + pRJF006 is a tendril like spread compared to the wild types uniform circular spread. These differences may be due to subtle regulatory interactions that alter surfactant production and colony morphology. One idea in the future is to test the merodiploid as a complementation construct to look at single copy complementation.

The lack of surfactant being produced by knocking out Varpa_4519, as shown in figure 16 indicates that Varpa_4519 is crucial in the production of

surfactant. Varpa_4519 provided on a plasmid (pRJF006 if table5) reestablishes surfactant production in the mutant strain (figure 16).

Figure 27 and 28 shows that increase in biofilm production from delta surf compared to wild type. It is clear that the surfactant produced by *V. paradoxus* EPS also plays a role in biofilm formation this is consistent with previous work showing that *Salmonella enterica* that surfactant production inhibits biofilm formation (Mireles et al, 2001). This makes sense because the surfactant can act as a dispersal agent, allowing a bacterial colony to get dense enough and then spread out. Taking away the production of surfactant would make it much more difficult for the bacteria to spread across a surface. Upon complementation of Varpa_4519 the biofilm production does not reduce to the same levels as the wild type. It is evident that the addition of Varpa_4519 back into the mutant strain does reduce the amount of biofilm produced compared to delta surf and delta surf + pBBRMCS5. This partial complementation shows that Varpa_4519 plays a role in the biofilm production for *V. paradoxus* EPS. The lack of complete complementation could be due to the number of copies of Varpa_4519 present in the bacteria, similar to swarming complementation.

It is noteworthy that the consistency of these experiments depends greatly on the relative humidity in the lab. The humidity for these experiments was consistent around 19-21% (for the experiments presented in this study), but there is some evidence that swarming is affected (evidence not shown). Biofilm assays are highly variable and this phenotype warrants further examination.

There is no indication that the presence of Varpa_4519 (in genome, knocked out, or present on a plasmid) has any effect on the growth rate of *V. paradoxus* EPS. As shown in figure 29, the complementation growth rates were consistent with that of the wild type. Table 4 shows the growth rates and doubling time of *V. paradoxus* EPS, including the delta surf, wild type, and their respective complementation constructs. From this it was concluded that there is no difference between the growth rates between each strain. The fact that there is no effect on growth rates shows that Varpa_4519 is not essential for growth. This means that its primary roll would be for surfactant production and that the production of surfactant is also not necessary for growth in liquid broth.

The RT-qPCR data suggests that the expression of Varpa_4519 is dependent on growth conditions. Understanding how the conditions promote expression is a key component to understanding the function and regulation of Varpa_4519 and needs further study. Swarming and biofilm are regulated by nutrient conditions (Jamieson et al, 2009), and surfactant might be part of this regulation.

In conclusion, Varpa_4519 is a crucial component in the production of surfactant in *V. paradoxus* EPS. This is evident with the phenotypic change producing a lack of visible surfactant with deletion of Varpa_4519, and the reemergence of surfactant with complementation (figure 16). It is also shown to play a role in the increase in biofilm formation (figures 27 and 28) and swarming motility (figure 18, 19, 21, 22, 23, and 24). To better understand Varpa_4519's

expression it will be further studied. This will include the study of surfactant production, and composition and function in the environment. It is currently being investigated to what extent the surfactant plays a role in plant growth promotion and the expression levels of Varpa_4519 under various growth conditions.

APPENDIX A

FIGURES

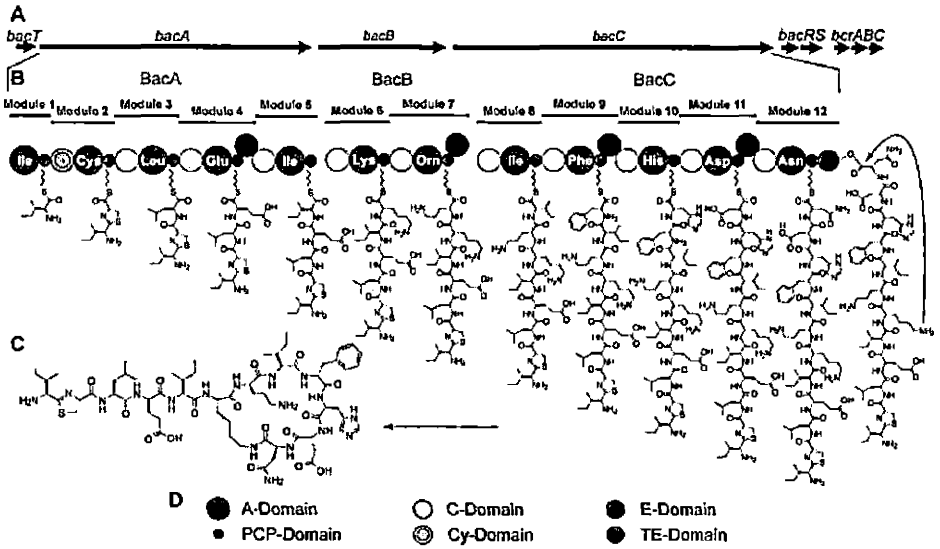

Figure 1. A prototype NRPS assembly line (Maraheil, 2009). (A) Cluster of genes involved. (B) The breakdown of the various modules in each gene and respective assembly of the cyclic branched dodecapeptide . (C) Final dodecapeptide after release from terminal module via macrocyclization. (D) Labeling for each NRPS domain.

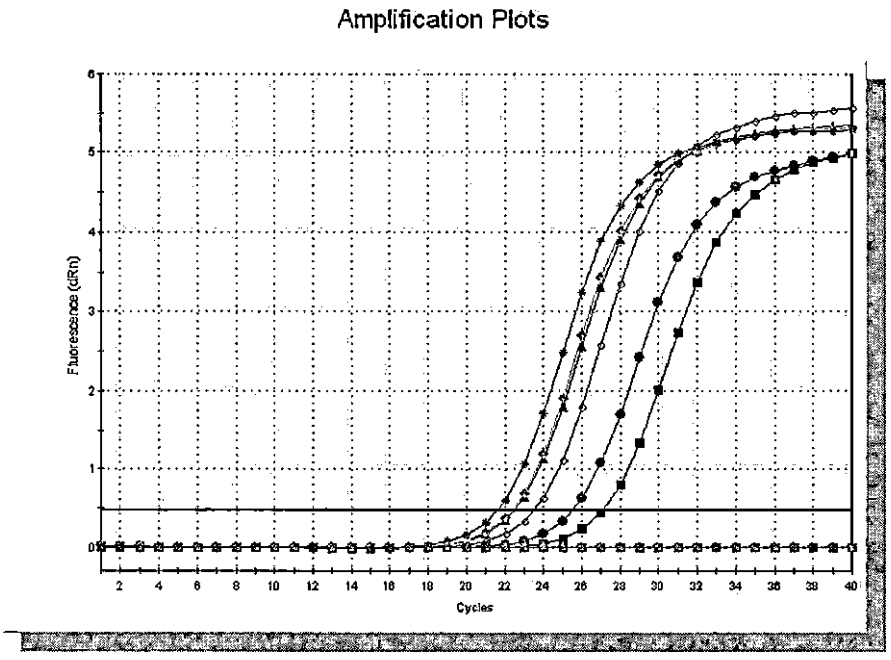

Figure 2. An example of an RT-qPCR amplification plot, done on Varpa_4519. The earlier the amplification curve starts to generate is related to the amount of cDNA present in the reaction.

Forward surf (qPCR)	5'-GATTCCGTACCTCGACCTCA-3'
Reverse surf (qPCR)	5'-GTCGATGTTCCGGGTGTAGT-3'

Table 1. Primer sequence generated for RT-qPCR reactions.

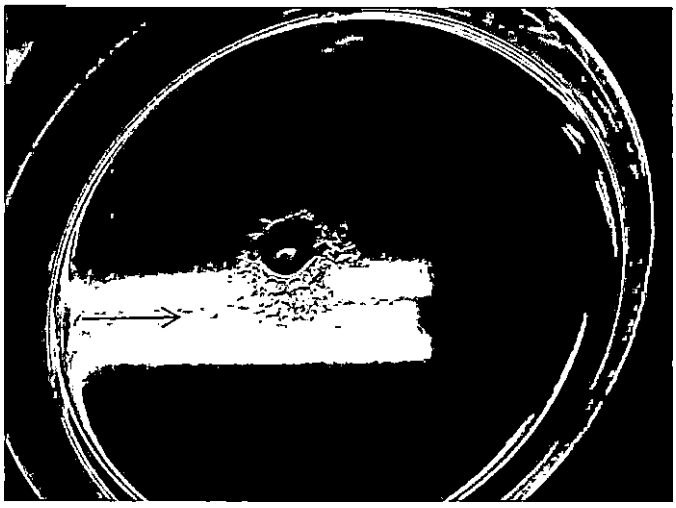

Figure 3. Visual image of the production of surfactant around a swarming colony on 0.3% agar. Colony shown is a wild type *Variovorax paradoxus* EPS. (Refraction from overhead lights is the only way to visualize the surfactant)

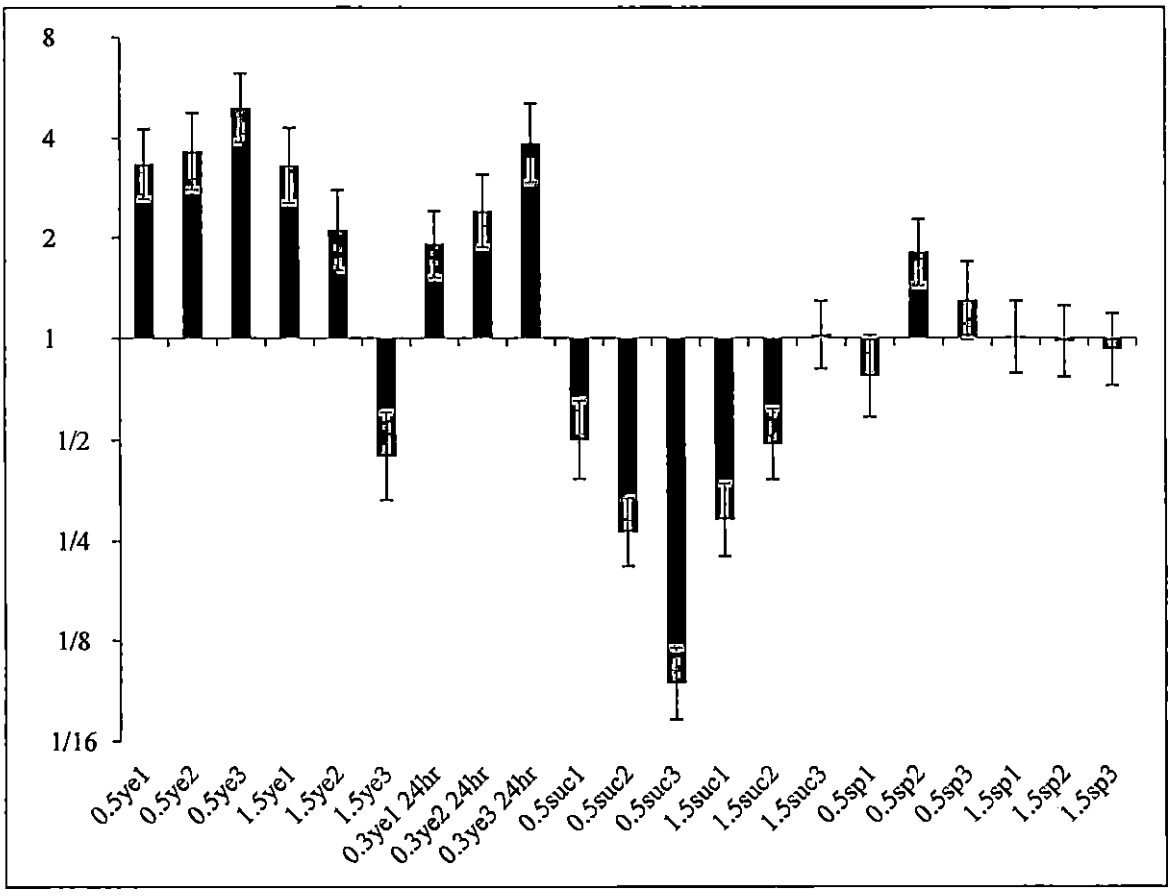

Figure 4. RT-qPCR analysis of RNA collected at 24h. The number in each sample denotes the agarose concentration for each plate (0.5%, 1.5%, and 0.3%). The growth media used is abbreviated as ye = yeast extract, suc = succinate, and sp = fresh water media with glucose and casamino acids. Each sample was done in triplicate and the fold expression (y axis) is compared to wild type in log phase broth culture.

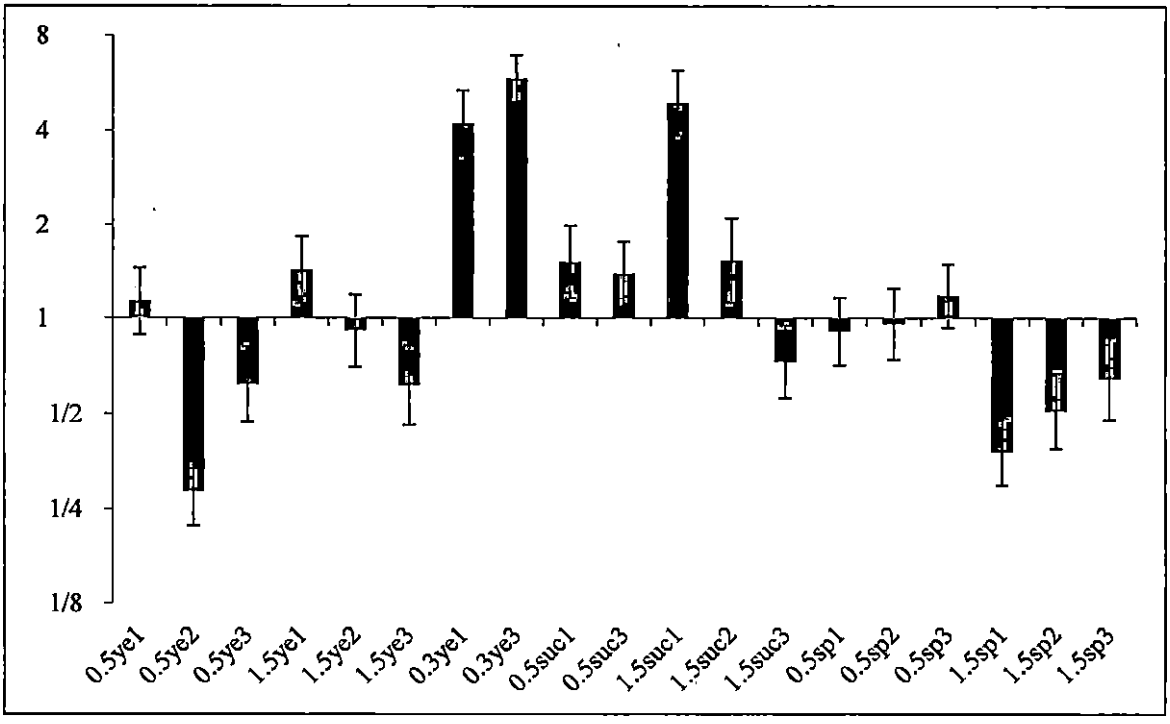

Figure 5. RT-qPCR analysis of RNA collected from samples at 48h. The number in each sample denotes the agarose concentration for each plate (0.5%, 1.5%, and 0.3%). The growth media used is abbreviated as ye = yeast extract, suc = succinate, and sp = fresh water media with glucose and casamino acids. Each sample was done in triplicate and the fold expression (y axis) is compared to wild type in log phase broth culture.

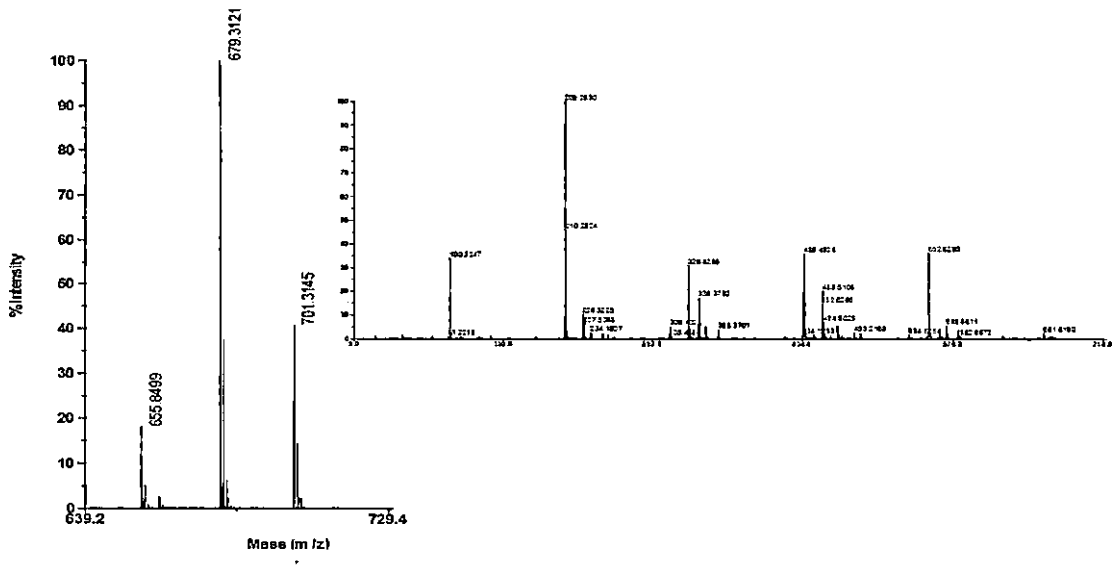

Figure 6. Mass spectra of the wetting agent from *V. paradoxus* EPS. Inset shows the MS/MS of the compound found at 679.3 m/z. the peak at 679.3121 m/z is the purified wetting agent. The peak at 701.3145 is a salt control. The upper right portion shows the lack of a peak at 679.3121. The run was done without the purified wetting agent showing that the wetting agent is the cause of the peak at 679.3121 m/z.

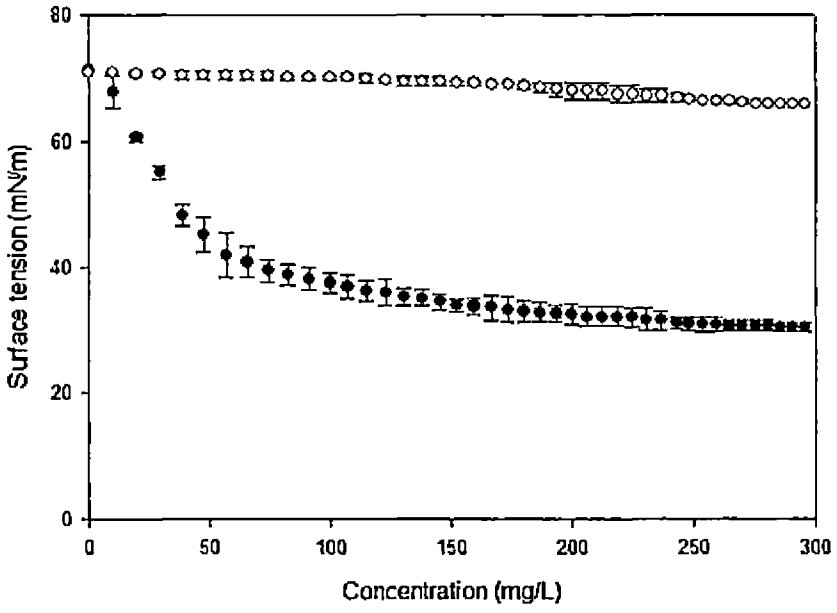

Figure 7. Reduction of water's surface tension with variowettin compared to control. Solid dots represent the wetting agent variowettin. Empty dots represent the water control. As higher concentrations of the wetting agent were added you can see the surface tension decrease.

N term forward primer	5'-GAATTCACTGCTGAACCTGGAAGACC-3'
N term reverse primer	5'-CCCCATATGGGTGCGAATCAGTATCT-3'
C term forward primer	5'-GGGCCCATGCCGGTCGCAATCTAC-3'
C term reverse primer	5'-ACCGGTCGCGGCCTCGAAATCTT-3'

Table 2. Sequences of primers used in PCR reaction for Varpa_4519

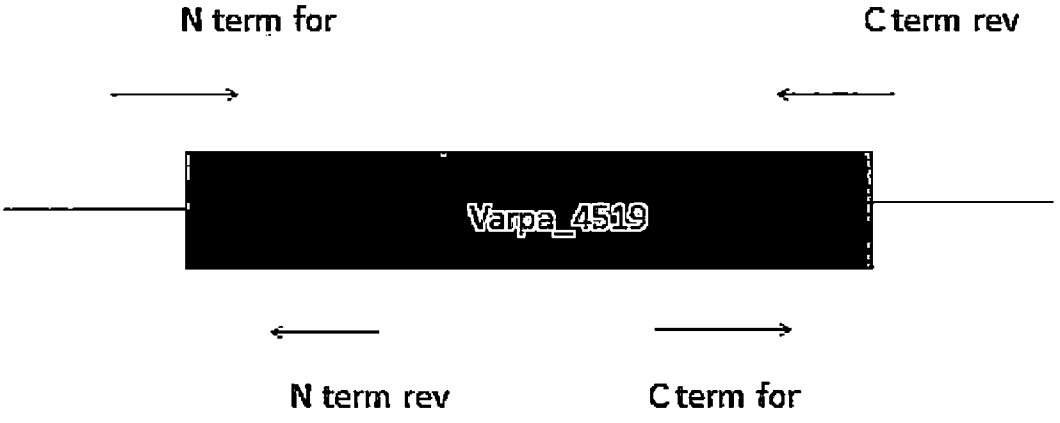

Figure 8. diagram showing the primer setup for amplifying flanking DNA for Varpa_4519. Rev = reverse primer and for = forward primer.

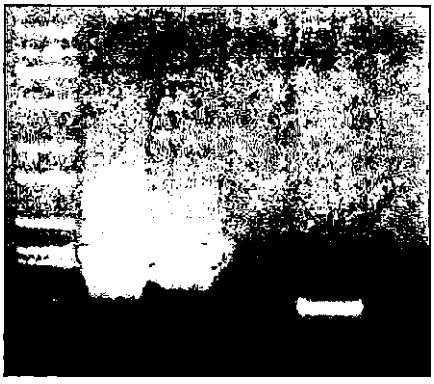

Figure 9. A picture of an electrophoretic gel showing correct sized PCR products for N and C term DNA segments of Varpa_4519. N term is 1.5Kb (shown as bands next to ladder) and C term is 1Kb (shown as single band on far right).

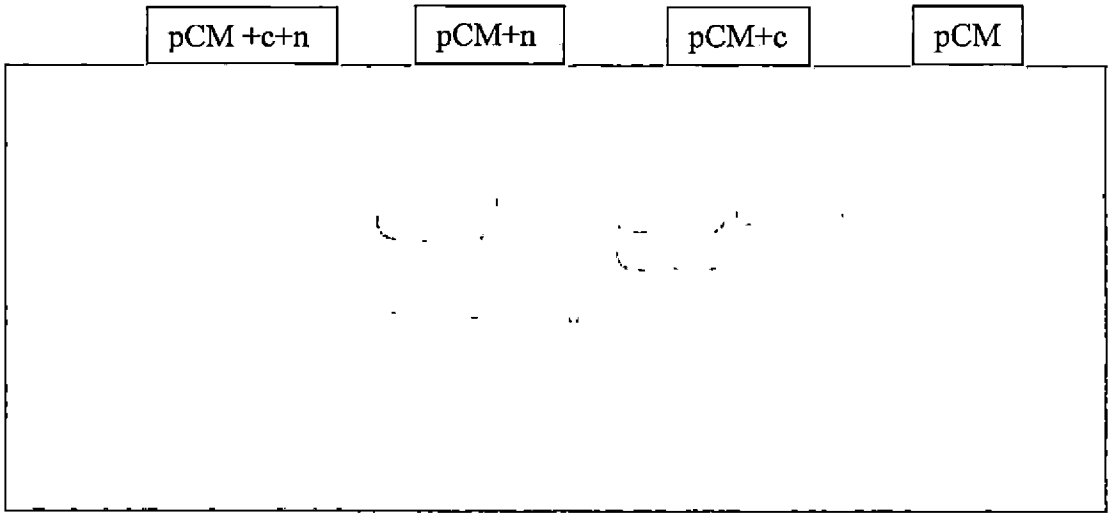

Figure 10. Verification of correct and complete pCM184 with both flanking DNA segments for C and N term. Total cut plasmid size should be 9Kb. Due to added restriction sites allowing for a *Hin*DIII digest to check entire plasmid size in multiple segments we can see that the first and second column contain the complete plasmid.

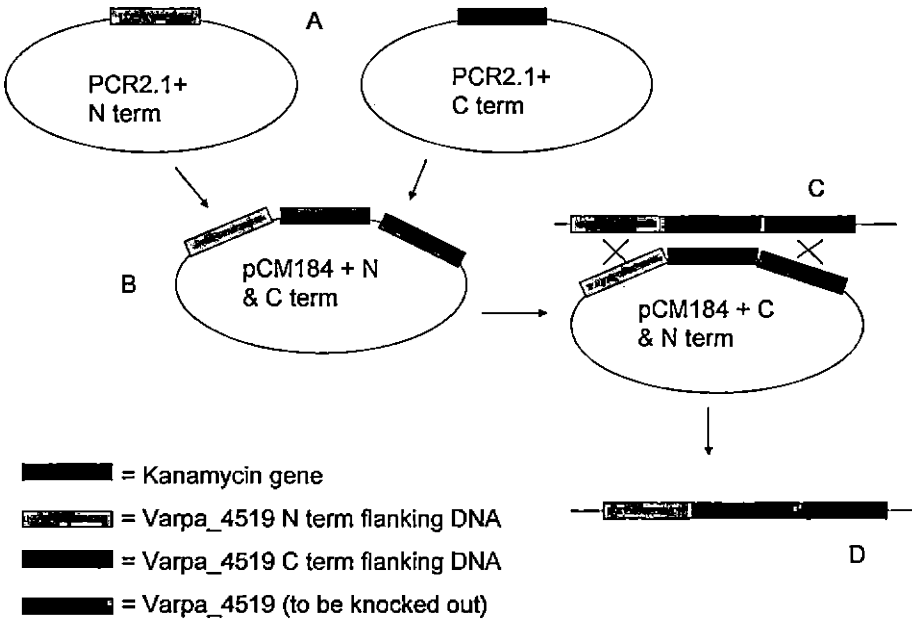

Figure 11. Shows the process for creating a mutant in *Variovorax paradoxus* EPS. A) is the amplified PCR products of both the Varpa_4519 flanking segments denoted as C and N terms. B) the completed knockout vector Varpa_4519 flanking DNA segments are ligated to. C) the allelic exchange between knockout vector and wild type Varpa_4519. D) the resulting mutant gene that contains Kanamycin.

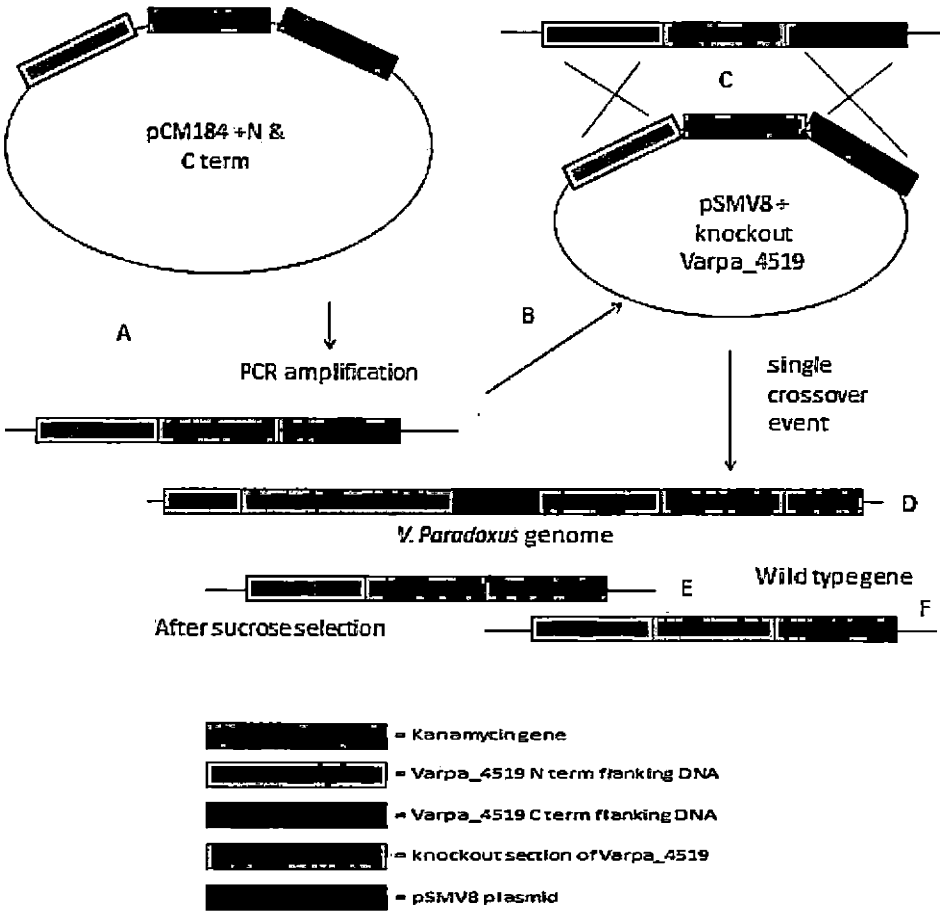

Figure 12. Shows the process for creating a mutant in *V. paradoxus*. A) the amplified PCR products of the pCM184 containing both N and C term flanking segments and the kanamycin gene in between. B) insertion of PCR product into new vector pSMV8. C) the allelic exchange between knockout vector and wild type Varpa_4519, only a single crossover was needed. D) the resulting genome after vector insertion. E) the resulting genome after the sucrose treatment producing the sacB gene excision. F) Shows the other potential outcome from sucrose treatment, which is the wild type gene sequence

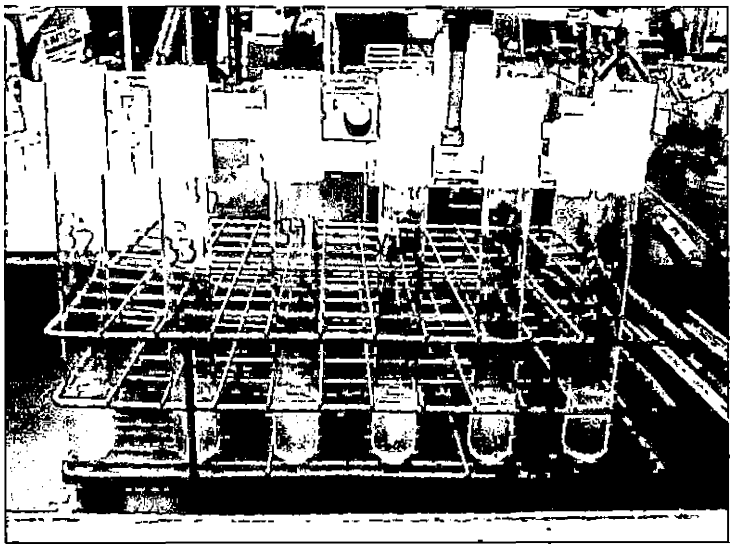

Figure 13. Potential Varpa_4519 mutants grown in the presence of kanamycin. Positive growth indicates that the pSMV8 plasmid was successfully inserted into the genome of *V. paradoxus*.

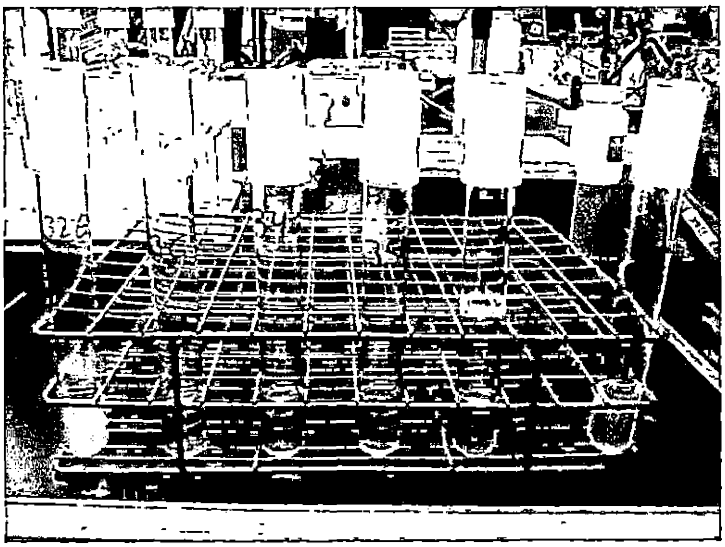

Figure 14. Potential Varpa_4519 mutants grown in the presence of gentamicin. The lack of growth indicates that the gentamicin gene has been removed with the rest of the plasmid due to sacB toxicity.

forward	CACGGCTGGACGGAAGTCG
Reverse	ATCGCGCGGGTTTGTTACTGA

Table 3. Forward and reverse PCR primers for the sacB gene in pSMV8.

Figure 15. PCR product of the sacB gene from single crossover mutant 22 (far right band). The empty lanes (left to right)are from the wild type and delta surf indicating a lack of sacB in the genome. Showing evidence of removal of pSMV8, and leaving just the kanamycin marker in the genome of *V. paradoxus.*

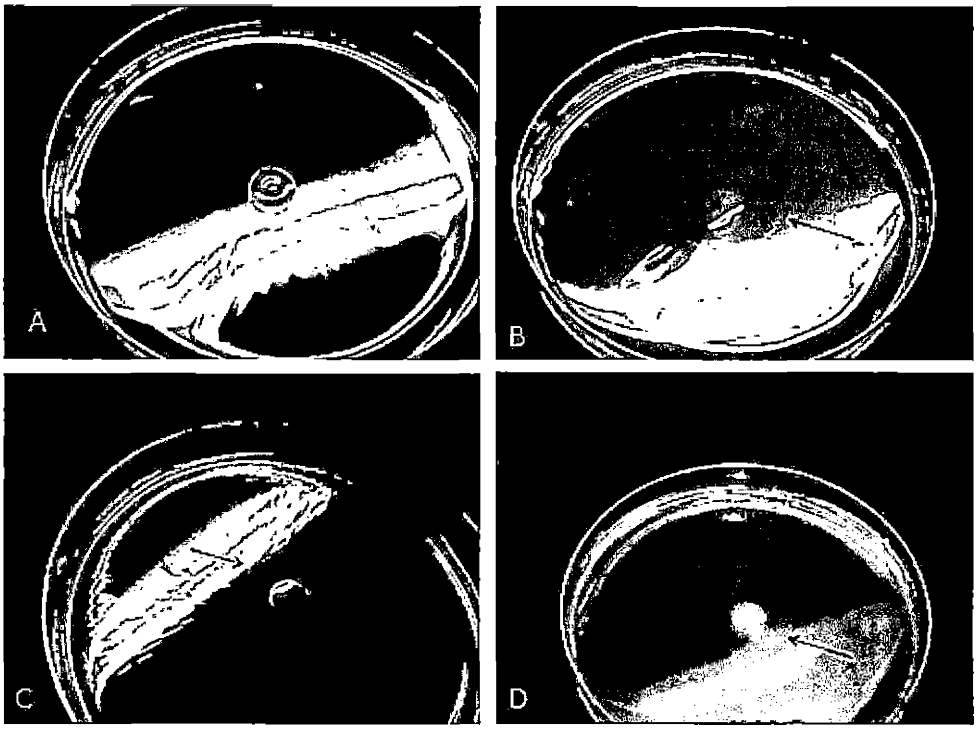

Figure 16. Visual complementation of the wetting agent. A) 35V. B) 35I. C) WTI. D) WTV. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5. A shows a lack of wetting agent present, while B-D show the wetting agent present. This shows the complementation of the production of the wetting agent in delta surf (B).

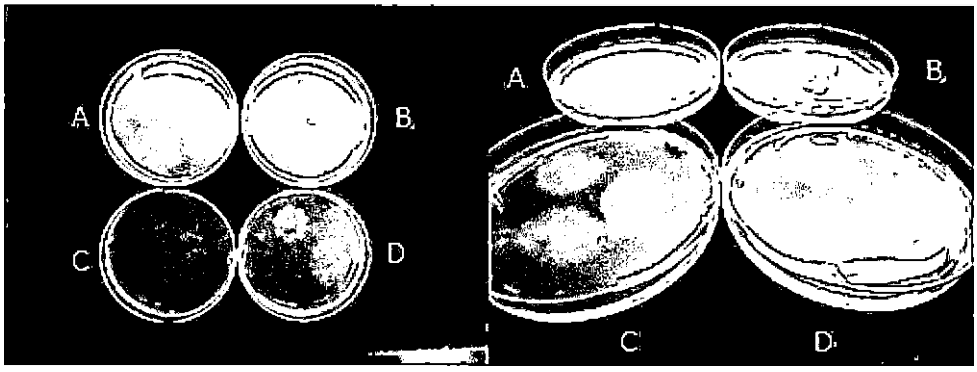

Figure 17. Drop collapse assay. Left) top view of delta surf and wild type swarming plates. Right) angled view of the same plates. A) WTV B) WTI C) 35V D) 35I for both the left and right photo. A, B and D all show collapsed drops in center of plates while C shows a firm budding of the drop. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5.

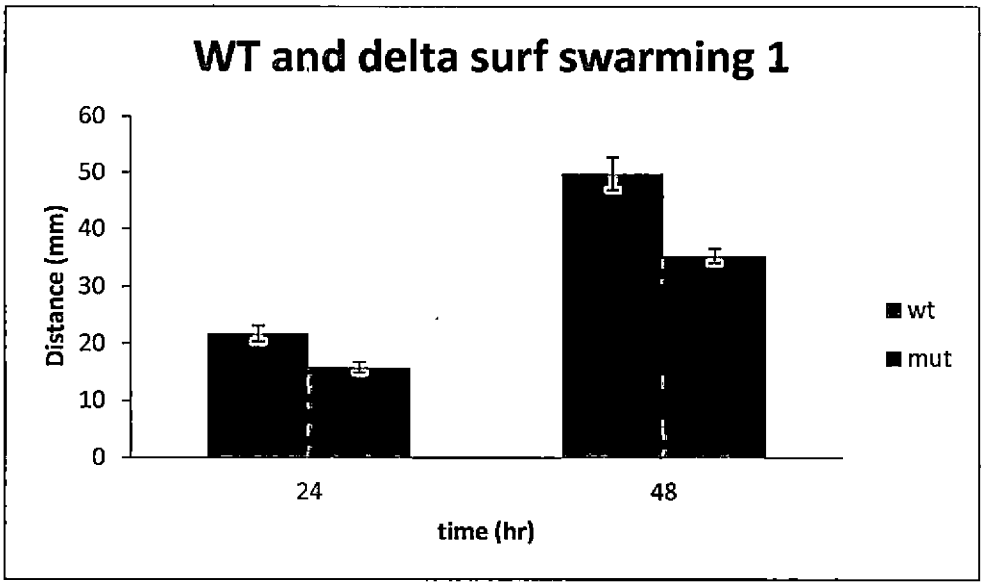

Figure 18. Swarming distance of wild type and *V. paradoxus* EPS vs delta surf (mut in graph) at 24 and 48 hour time points. All swarms were measured in sets of 9 samples, with error in all cases ± SEM. ($P<0.05$ for all samples)

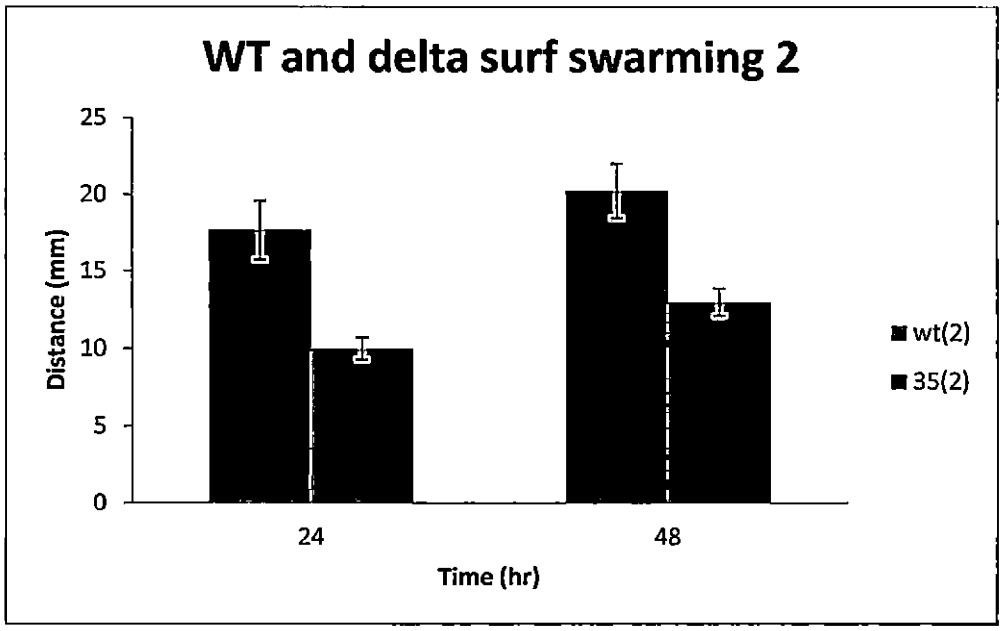

Figure 19. The second experiment of Swarming distance of wild type *V. paradoxus EPS* vs delta surf (mut in graph) at 24 and 48 hour time points. All swarms were measured in sets of 9 samples, with error in all cases ± SEM. ($p<0.05$ for all samples)

Figure 20. Swarming motility colonies of wild type and delta surf at 24 and 48 hour time points. A) 24 hour swarming phenotype of wild type. B) 24 hour swarming phenotype of delta surf. C) 48 hour swarming phenotype of wild type. D) 48 hour swarming phenotype of delta surf

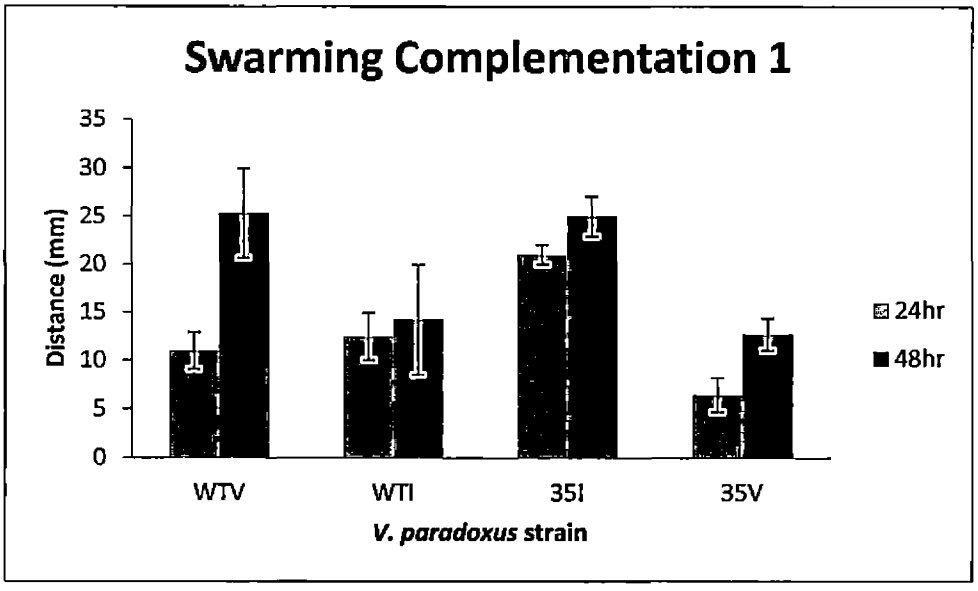

Figure 21. 24 and 48 hour measurements of swarming colonies containing the vector PBBRMCS5 with or without the full Varpa_4519 gene inserted. All swarms measured in sets of 9 samples, with error in all cases ± SEM. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5. ($P<0.05$ for all comparisons except when comparing WTV/WTI and WT/35I at 24hr. $P<0.05$ for all comparisons at 48 hours except 35V/35I, 35V/WTI, 35I/WTV, and WTV/WTI.)

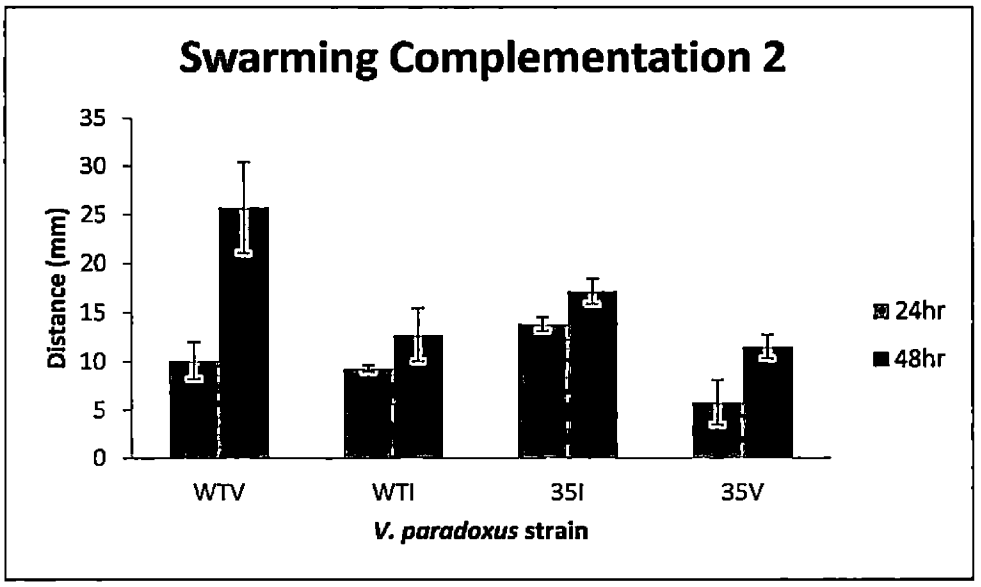

Figure 22. 24 and 48 hour measurements of swarming colonies containing the vector PBBRMCS5 with or without the full Varpa_4519 gene inserted. All swarms measured in sets of 9 samples, with error in all cases ± SEM. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5. ($P<0.05$ for all comparisons made between strains)

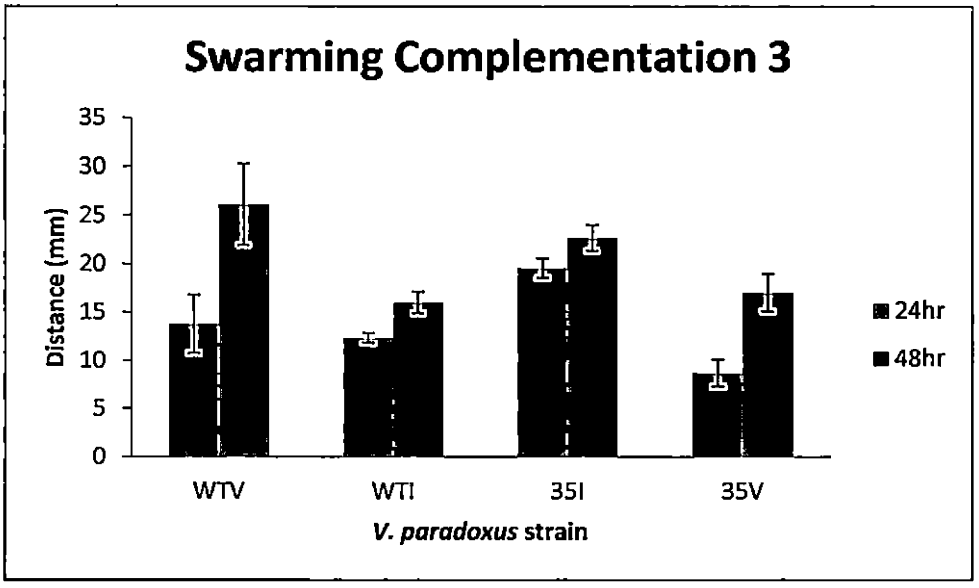

Figure 23. 24 and 48 hour measurements of swarming colonies containing the vector PBBRMCS5 with or without the full Varpa_4519 gene inserted. All swarms measured in sets of 9 samples, with error in all cases ± SEM. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5. ($P<0.05$ for all comparisons made except between 35I/WTI and 35V/WTI at 48 hours)

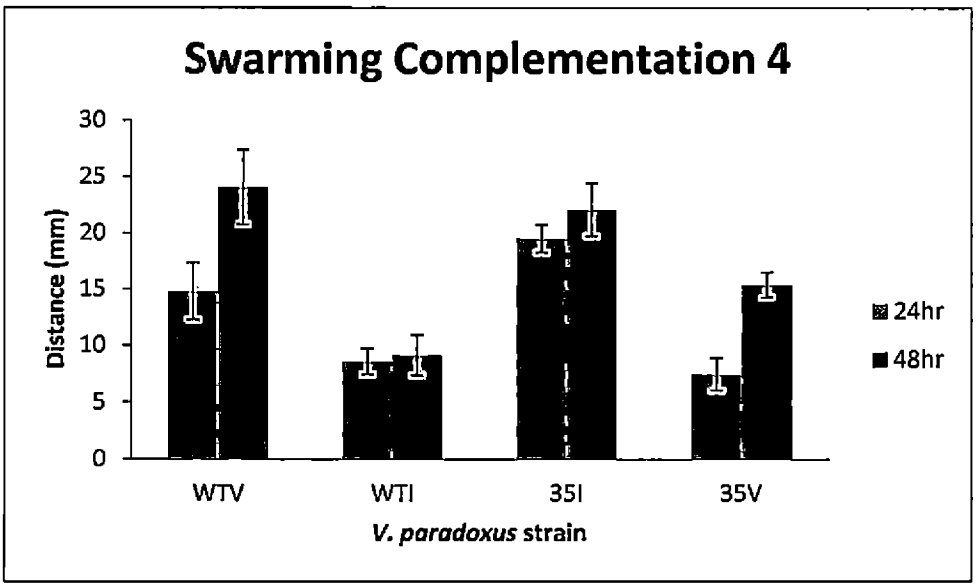

Figure 24. 24 and 48 hour measurements of swarming colonies containing the vector PBBRMCS5 with or without the full Varpa_4519 gene inserted. All swarms measured in sets of 9 samples, with error in all cases ± SEM. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5. ($P<0.05$ for all comparisons made except between 35I/WTV at 48 hours)

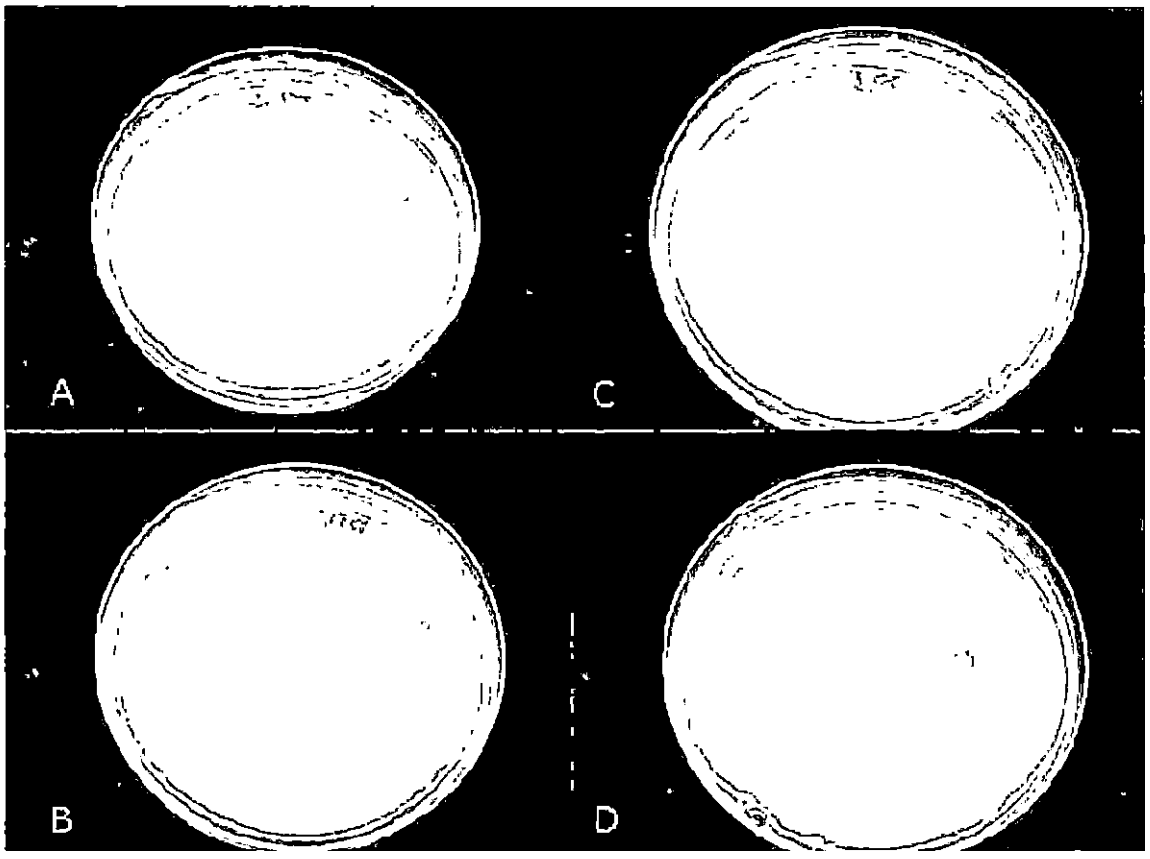

Figure 25. 24 hour complementation swarming phenotype of *V. paradoxus* EPS strains. A) WTI. B) WTV. C) 35I. d) 35V. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5.

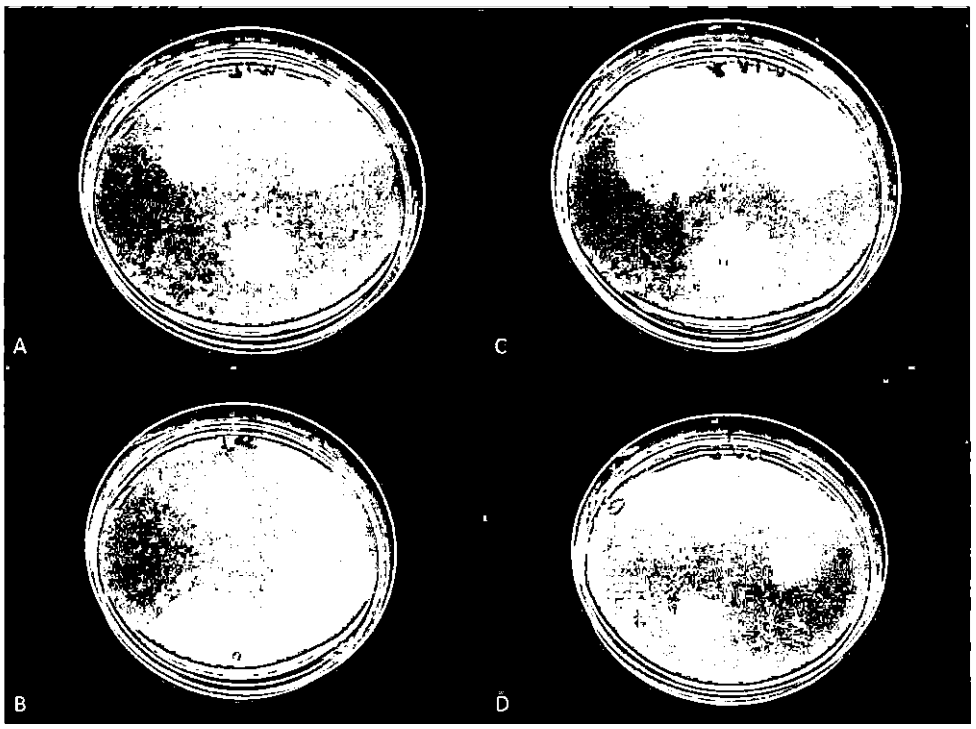

Figure 26. 48 hour complementation swarming phenotype of *V. paradoxus* EPS strains. A) WTI. B) 35V. C) WTI. D. 35V. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5.

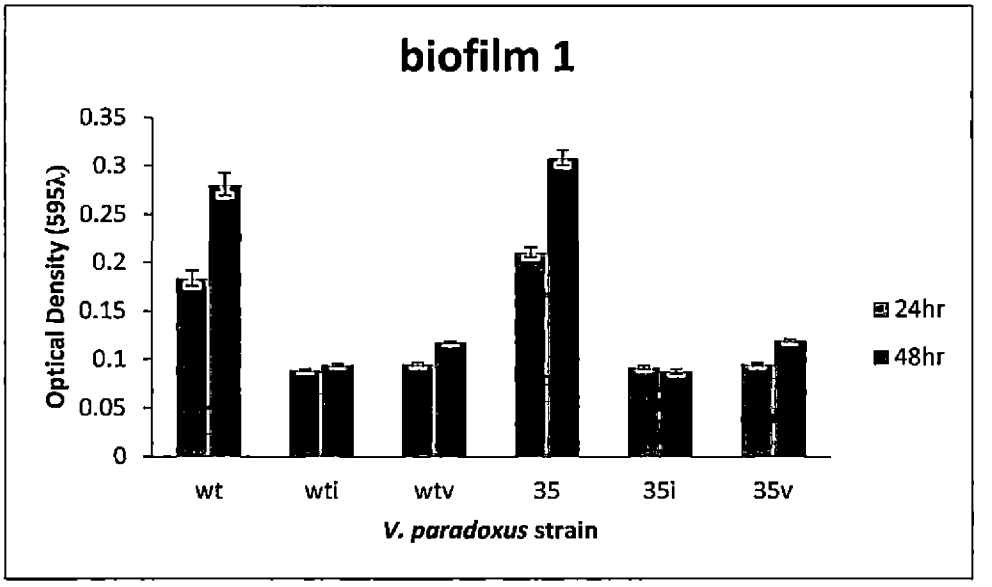

Figure 27. Biofilm levels determined by optical density levels for 24 and 48 hours or each strain of *V. paradoxus* EPS. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5. ($P<0.05$ for all comparisons except WTI/35I, 35I/35V, and WTV/35V at 24 hours. $P<0.05$ for all comparisons except WTV/35V at 48 hours)

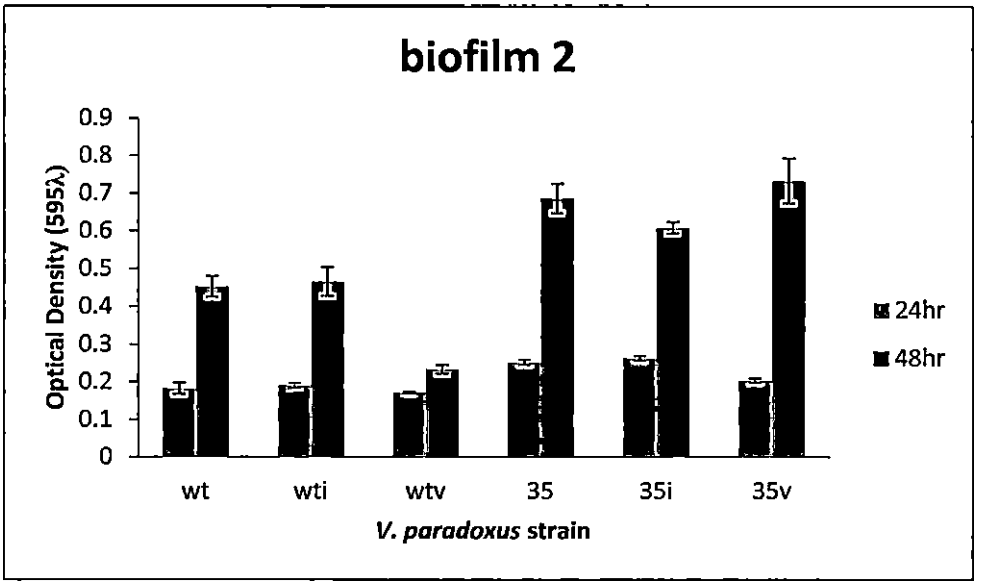

Figure 28. biofilm levels determined by optical density levels for 24 and 48 hours or each strain of *V. paradoxus* EPS. WTI = wild type + pRJF006, WTV = wild type + pBBRMCS5, 35I = delta surf + pRJF006, 35V = delta surf + pBBRMCS5. ($P<0.05$ for all comparisons at 24 and 48 hours)

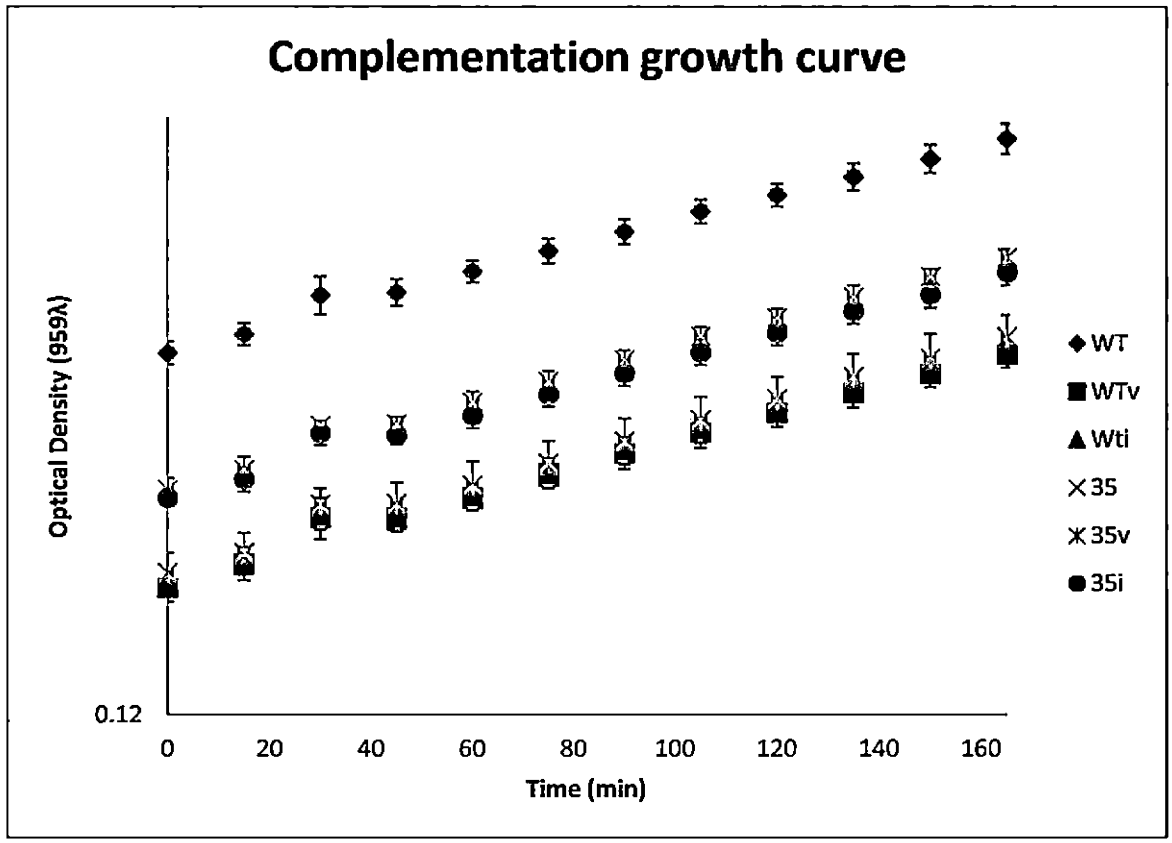

Figure 29. Growth curve of the log phase of mutant and wild type *V. paradoxus* EPS and their complementation constructs. No significant difference was shown between any of the curves.

	Growth rate	Generation time
WT	0.0015	462.1
WTv	0.0016	433.22
Wti	0.0016	433.22
35	0.0016	433.22
35v	0.0016	433.22
35i	0.0015	462.1

Table 4. Growth rates of the wild type, delta surf and their respective complementation constructs. Growth rate was calculated by taking the data points from the exponential growth phase and converting it into a log form graph, then taking the slope of the line. Generation time was calculated from the growth rate using the equation $\ln 2/k = t_D$

PCR 2.1 + N term of Varpa_4519	pRJF001
PCR 2.1 + C term of Varpa_4519	pRJF002
pCM184 + N term of Varpa_4519	pRJF003
pCM184 + N & C terms of Varpa_4519	pRJF004
pSMV8 + amplified insert from pCM184 (insert contains N & C terms of Varpa_4519 flanking a kanamicin gene)	pRJF005
pBBRMCS5 + Varpa_4519	pRJF006

Table 5. The plasmid constructs used to make the mutant strain and complementation strains of *V. paradoxus* EPS.

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