Transposon Mutagenesis of a Violacein Producing *Janthinobacterium sp.*

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Introduction:

The *Janthinobacterium* genus consists of gram negative, aerobic β-proteobacteria, commonly isolated from soil and freshwater sources such as springs, lakes and rivers (Pantanella et al., 2006). Although commonly found in the environment, *J. lividum* can cause fatal infections including septicemia. The purple pigment, violacein, associated with *Janthinobacterium* and *Chromobacterium* a close relative, is controlled by the violacein biosynthesis operon containing genes *vioA-D* (August et al. 2000). Much is known about the spectral and chemical characteristics of violacein, but little is known about the role that it plays in microbial physiology and metabolism. Developing a genetics system in this organism would facilitate the study of violacein knock outs allowing the further characterization of violacein producing organism and specifically the role that violacein plays in metabolism. This report will describe the initial steps in developing a transposon mutagenesis system in a violacein producing *Janthinobacterium.*

Methods:

Janthinobacterium Isolation, Culturing and Characterization:

Violacein producing bacteria were enriched for from a mixture of garden soil and soil from the side of the road in Woods Hole, Ma (Figure 1). As previously described in the Microbial Diversity Lab Manual 2014, organic rich soil was loosely packed into a petri dish and moistened with a solution 50 ug/ml cycloheximide in water. 10-15 rice grains were placed on top of the soil and the dish was allowed to incubate at room temperature for about 7-10 days until purple patches arose on the rice grains (Figure 2). Purple patches were picked from rice grains with a toothpick and streaked on to 5YE (5 grams yeast extract per liter of water, 15 gof agar) plates. Plates were examined under the dissecting microscope and documented (Figure 3). An antibiotic resistance test was preformed with kanamycin at 0, 10, 20, 30, 40, 100 ug/ml to determine wild type natural resistance to kanamycin. 5 ml of 5YE media

was added to autoclaved culture tubes and inoculated with 50 ul of an overnight culture and incubated at 27°C with shaking at 200 rmp. OD600 readings were taken on a 0.5 ml aliquot after 18 hr of incubation (Table 1).
The pigment associated with our isolate was determined using a simple ethanol extraction method and spectrophotometric measurements by Marton Szoboszlay (Figure 4). A nucleotide BLAST search was preformed on the whole genome sequence with the 16S ribosomal RNA sequences database.

Conjugation and Selection for Transposition Events:

Individual cultures of *E. coli* containing the pRL27 or pSC189 plasmid were grown in LB liquid media, supplemented with 300 uM diaminopimelic acid (DAP) and kanamycin 25 ug/ml, at 37°C with 200 rpm shaking overnight (Figure 5, Jiao et al., 2005, Larsen et al., 2002). Plasmids were provided by the Newman lab at California Institute of Technology. An culture of *Janthiniobacterium* in 5YE was incubated at 27°C with 200 rpm shaking overnight. 1 ml of the donor culture was washed with 1 ml of 5YE twice to remove kanamycin. 300 uM filter sterilized DAP was added to 5YE plates by sterile spreading and allowed to soak into the agar for 10 min at 30°C before plating. Different ratios of donor : recipient were used to determine which ratio would be most effective. Ratios of 1:1 (200 ul : 200 ul) and 1:4 (100 ul : 400 ul) were plated and efficacy was qualitatively determined. 200 ul of donor : recipient mixture were spotted onto a 5YE plate containing DAP and allowed to incubate for 16 hours at 30°C. After incubation cells were removed from the plate using two methods: 1) 1 ml of 5YE was added to the plate and plate was lightly scraped to resuspend cells in media. The media was then pipetted off the plate and into an eppendorf tube. The entire 1 ml could not be retrieved. 2) Cells were scraped of the plate with the side of a pipette tip and resuspended in 1 ml of 5YE in an eppendorf tube. 200 ul of cells were plated onto 5YE containing 1% glycerol and 25 ug/ml kanamycin. A dilution series (10^{-1}-10^{-5}) of the cell slurry was preformed to achieve single colonies, which facilitated picking mutant colonies. Plates were incubated at 30°C overnight.

Screening for Violacein Production:

Several methods of screening for violacein production were used to determine which would most readily facilitate pigment formation and screening. After about 24 hr single colonies were visible, but there was minimal pigment formation. Colonies were picked with sterile toothpicks and inoculated into 96 well plates containing 100 ul of 5YE with 1% glycerol and 25 ug/ml kanamycin. Plates were incubated at 27°C and monitored daily. After 24 hours of incubation in the 96 well plate, the samples were inoculated onto 5YE solid media plates using the 48 pronged frogger (Figure 8 and 9). After 2-3 days of incubation on plates, the original dilution series revealed very distinct phenotypes in certain colonies, from which genomic DNA was extracted (Figure 5 and 6).

Genomic DNA Extraction, Arbitrary PCR and Identification of Mutants:

DNA was extracted from the colonies by picking a colony and resuspending it in 20 ul of PCR grade water and boiling in the thermocycler for 10 min. Promega GoTaq Hot Start Polymerase was used for the arbitrary PCRs according to manufactures instructions. As previously described, the following sets of primers were used in different combinations; pSC189: Mar3 (5-CTTCTTGACGAGTTCTTCTGA GC-3) and Mar4 (5-TAGGGTTGAGTGTTGTTCCAGTT-3) (Jiao et al., 2005). pRL27: pRL27Extdx (5-CCAGAAAGTGAGGGAGCCA-3) or pRL27Extsx (5-GACAACAAGCCAGG GATG-3) with one of the following degenerate primers SS9arb1 (5-GACCAC GAGACGCCACACTNNNNNNNNNNCATGC-3), SS9arb2 (5-GACCACGAGA CGCCACACTNNNNNNNNNNACTAG-3), or SS9arb8 (5-GACCACGAGACGC CACACTNNNNNNNNNNGATAT-3). The following conditions were used for the first round of PCR amplification: 95°C for 2 min, then five cycles of 94°C for 30 s, 30°C for 30 s, and 72°C for 60 s, followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 60 s. For the nested PCR, five microliters of the initial PCR product was used as template for a second round of amplification with the following cycles: 95°C for 2 min, 30 cycles of 95°C for 30 s, 55°C for pSC189/50°C for pRL27 both for 30 s, and 72°C for 45 s using a nested primer unique to the mini-Tn*5* end pRL27Intdx1

(5-GAGTCGACCTGCAGGCATGC-3) or pRL27Intsx (5-CGCACT GAGAAGCCCTTAGAGC-3) and a primer with sequence identity to the 5' GC clamp of the arbitrary primer arb3 (5-GACCACGAGACGCCACACT-3) (Lauro et al., 2008). Then nested PCR was rerun with both 1 or 2 ul of initial PCR product. The samples that produced promising products were sent off for sequencing to determine the gene that was disrupted by the transposon. Sequencing was preformed with pRL27Intdx1 and arb3 primer set in both directions.

Results:

Janthinobacterium Isolation, Culturing and Characterization:

The antibiotic resistance growth test OD600 measurements are reported in Figure 4. All of the samples in the kanamycin resistance test were visually evaluated, while only several of the OD600 readings of the cultures were measured. Since 20 and 30 ug/ml had the same OD reading and very minimal growth, 25 ug/ml was used in the selective media. This antibiotic growth experiment should be repeated with the same range of kanamycin, but with replicates. The OD600 of all the kanamycin concentrations should be measured. A growth curve of all the concentrations could also be helpful to further characterize this strain.

The pigment produced by our isolate was compared to the UV spectrum of known violacein molecules (Figure 4). The comparison shows that there are similar peaks from 200-300 nm and a smaller but present broad peak from 450-700 nm. Although these spectra are similar further analysis of the pigment should be preformed to verify its structure and homology to violacein. A putative violacein biosynthesis operon was identified in the genome, but further investigation of conserved domains and gene expression should be preformed to verify violacein production. The nucleotide BLAST against the 16S ribosomal RNA sequences database revealed that *Janthinobacterium lividum* strain DSM 1522 is the closest relative with an E-value of 0.0.

Conjugation and Selection for Transposition Events:

The conjugation of the *E.coli* host and *Janthinobacterium sp.* was successful. On selection media there were many colonies that grew from both of the different transposon containing plasmids. The efficacy of the different ratios used was only evaluated qualitatively. Both ratios contained what seemed to be a similar of number of mutants. The colonies that were produced from the pRL27 transposition were much smaller and grew a lot slower than those from the pSC189 transposition. For both transposons, the plates containing the dilution of 10^{-4} had the most evenly distributed colonies facilitating the picking of individual colonies (Figure 6). Both of the methods for removing the conjugated cells from the plates seemed to be equally effective.

Screening for Violacein Production:

The different types of screens were more or less effective depending on the phenotypes. The initial plating of serial dilutions of the mutants was effective to look for pigment mutants and wrinkly structures in the colonies. The liquid media in the 96 well plates would have been effective for a biofilm formation screen with a dye such as safranin that is a different color than violacein (Figure 7 and 9). The volume of media used in the 96 well plates should be increased to at least 200 ul and possibly parafilmed around the edges to prevent desiccation. The frogging of the liquid media onto solid media would be an effective way to do large volume screen for different phenotypes, but took longer due to the time required to allow the 96 well plates to grow and the frogged colonies to grow. There are pros and cons to each type of screen that should be further investigated and optimized.

Genomic DNA Extraction, Arbitrary PCR and Identification of Mutants:

Arbitrary PCR yielded a potential band from a pRL27 mutant that was sent for sequencing but at the time of this report, the results had not yet been returned. The results from the PCR will allow for the further confirmation of an effective transposition event, and verify the genomic DNA extraction and arbitrary PCR

method for the following set of primers: Initial PCR reaction - pRL27Extsx and SS9arb1, and for the nested PCR reaction pRL27Intsx and arb3 (Figure 10).

Future Directions:

As the field of environmental microbiology continues to move forward, there will need to be a shift away from being restrained by the use of model organisms and a movement towards the genetic manipulation of environmental isolates. This is what has been successfully demonstrated, although not optimized[1]. There are many interesting questions that have not yet been probed regarding the physiological or metabolic role that violacein plays in *Janthinobacterium* and other violacein producers. There are other more environmental microbiology directed questions that could be elucidated with a violacein knock out including the nature of the symbiosis of *Janthinobacterium* and amphibians and the benefits that potentially ward off the chytrid fungus (Brucker et al. 2008). Establishing a system for genetic manipulation of environmental isolates will facilitate a further exploration of the physiology, metabolism, symbiosis or competition, nutrient cycling and much more of many organisms that have only been superficially probed.

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[1] For those of you that have read thus far with this much attention to detail you are probably interested in an aspect of this project forward and might want to move it forward. Please feel free to contact me, afsarno@asu.edu, as I'm happy to help or put you in touch with those who can. And if Kurt D. is around for the genetics portion of the course, he owes you a beverage ☺

comical support. To Dr. Brooke Jude for discussions on violacein producers, and their genetics, isolation, phenotypes. I would like to thank my teammate for life Aaron Thompson for all his support. I would like to acknowledge my funding sources, NSFGRFP, American Society for Cell Biology, Planetary Biology Internship Scholarship and the William Townsend Porter Scholarship.

References:

August, P.R., Grossman, T.H., Minor, C., Draper, M.P., Mac Neil, I.A., Pemberton, J.M., Call, K.M., Holt, D. et al. 2000. Sequence analysis and functional characterization of the violacein biosynthetic pathway from Chromobacterium violaceum. J Mol Microbiol Biotechnol **2:**513–519.

Brucker, R.M., Harris, R.N., Schwantes, C.R., Gallaher, T.N., Flaherty, D.C., Lam, B.A., Minbiole K.P.C. Amphibian Chemical Defense: Antifungal Metabolites of the Microsymbiont *Janthinobacterium lividum* on the Salamander *Plethodon cinereus.* 2008. J Chem Ecol **34:** 1422-1429.

Jiao, Y., Kappler, A., Croal, R.L. and Newman, D.K. 2005. Isolation and Characterization of Genetically Tractable Photoautotrophic Fe (II)-Oxidizing Bacterium, *Rhodopseudomonas palustris* Strain TIE-1. Applied and Environmental Microbiology. 71: 4487-4496.

Larsen, R.A., Wilson, M.M., Guss, A.M. and Metcalf, W.W. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that in functional in a wide variety of bacteria. Arch Microbiol. 178: 193-201.

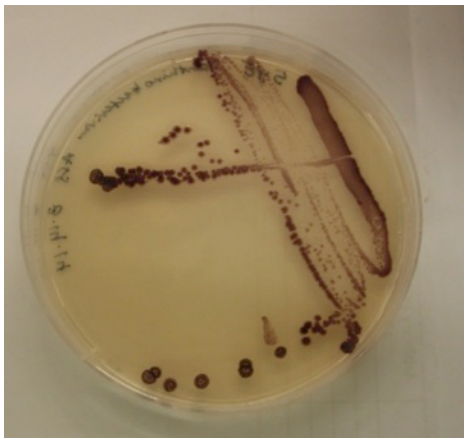
Lauro, F.M., Tran, K., Vezzi, A., Vitulo, N., Valle, G. and Bartlett D.H. 2007. Large-Scale Transposon Mutagenesis of *Photobacterium profundum* SS9 Reveals New Genetic Loci Important for Growth at Low Temperature and High Pressure. J. Bac 109:1699.

Pantanella, F., Berlutti F., Passariello, C., Sarli, S., Morea, C. and Schippa, S. 2006. Violacein and biofilm production in *Janthinobacterium lividum*. J. of Applies Micro **102:** 992-999.

Figure 1) Soil sampling locations in Woods Hole, Ma. indicated by stars.

Figure 2) Picture of violacein producing enrichment in rice grain on top of soil in petri dish.

A)



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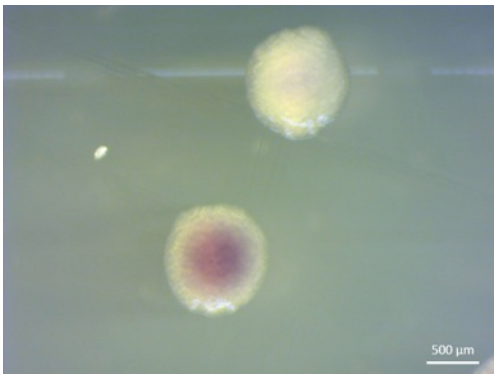


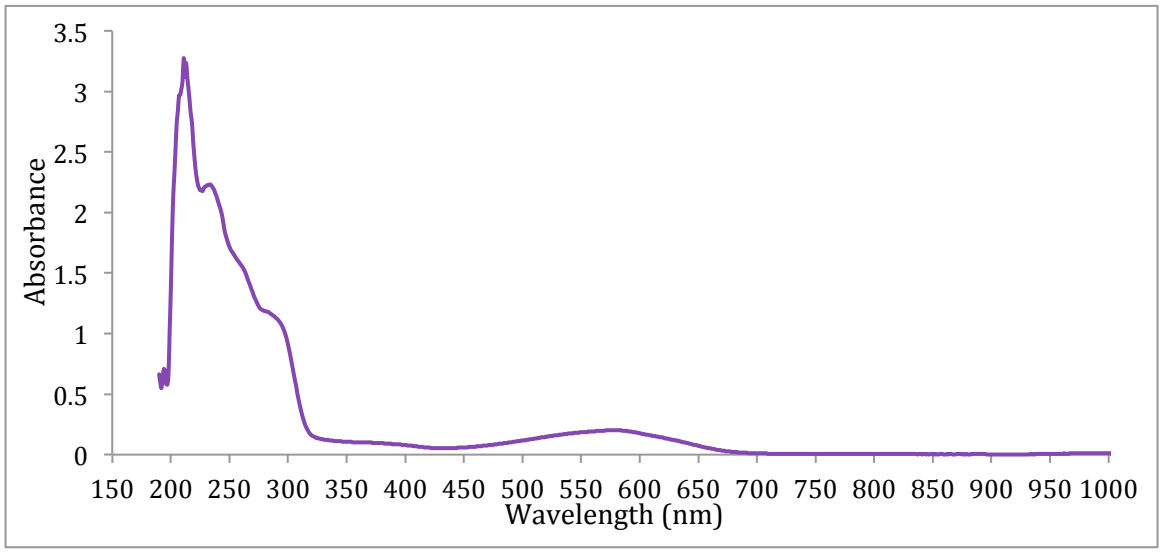
C)

Figure 3) A) Wild type plate of streaked *Janthinobacterium sp.* on 5YE plate. B) Younger colonies of wild type *Janthinobacterium sp..* Photos taken by Ben Roller with dissecting microscope. C) Older colonies of wild type *Janthinobacterium sp..* Photos taken by Ben Roller with dissecting microscope.

Kanamycin concentration (ug/mL)	OD600
0	2.50
10	0.128
20	0.026
30	0.020

Table 1) OD600 readings taken on the spectrophotometer in a plastic disposable cuvette.

A)



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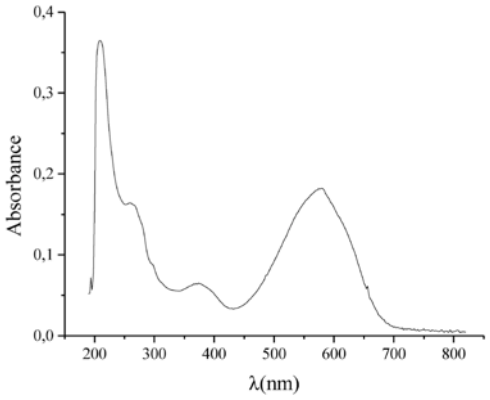


Figure 4) A) Putative violacein pigment from ethanol extraction absorbance spectrum, preformed by Marton Szoboszlay. B) Figure from Dias Jr. et al., 2002.

A) B)

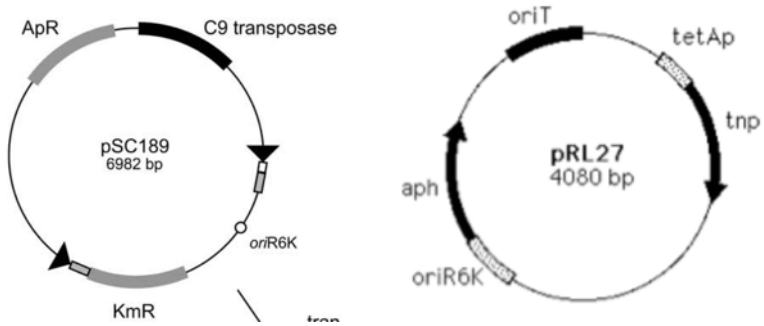
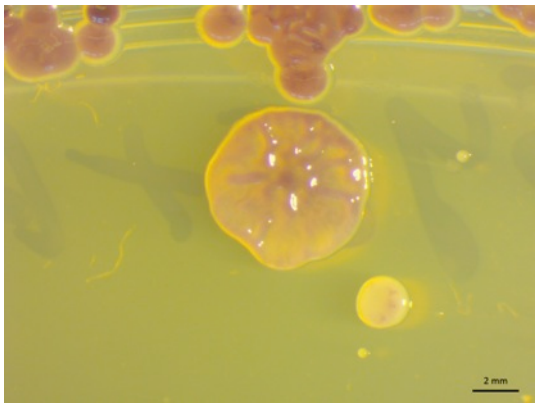


Figure 5) Transposon containing plasmid maps. A) Chiang and Rubin, 2002. B) Larsen et al., 2002.

A)



B)

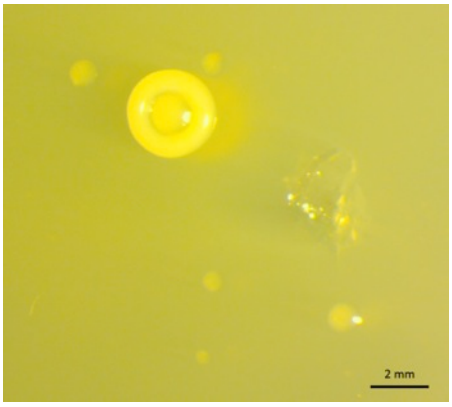
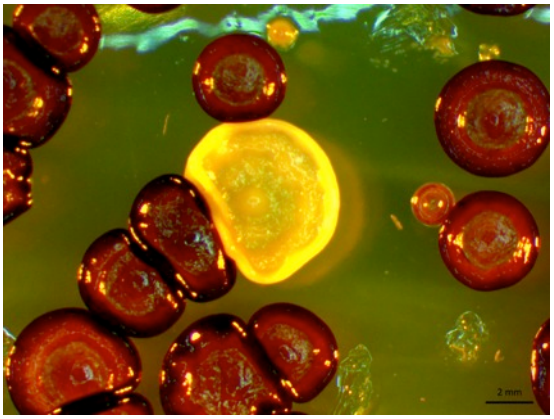


Figure 6) A) pRL27 mutant colony on 5YE 1% glycerol with hyper wrinkled phenotype. B) pRL27 mutant colony on 5YE with slow growth and little pigment production.

A)



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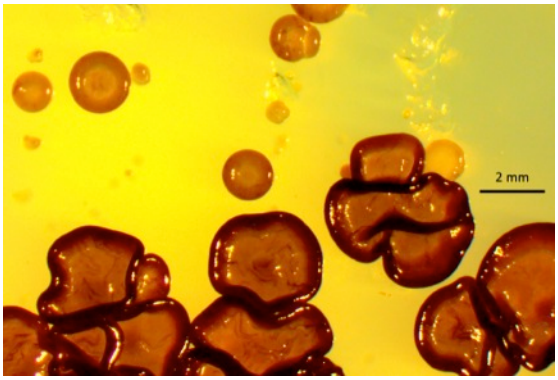


C)

Figure 7) A) pSC189 mutant colonies on 5YE 1% glycerol plates, central white colony could be a violacein producing deficient. B) pSC189 mutant colonies on 5YE 1% glycerol plates, upper left colonies have a slow growing phenotype. C) pSC189 mutant colonies on 5YE 1% glycerol plates, with hyper wrinkled phenotype.

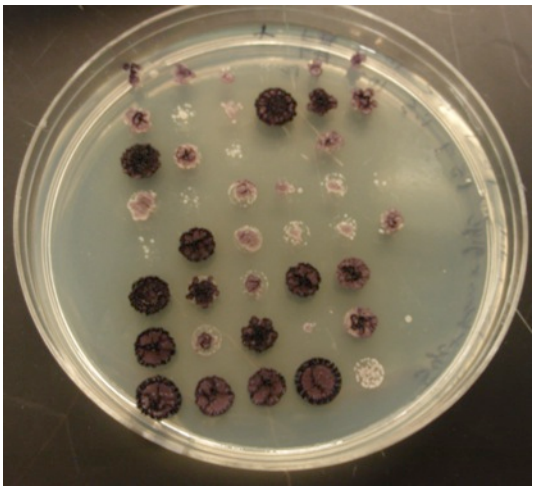


Figure 8) pSC189 mutants that were inoculated from a 96 well plate containing single colony picks, using a 48-pronged frogger.

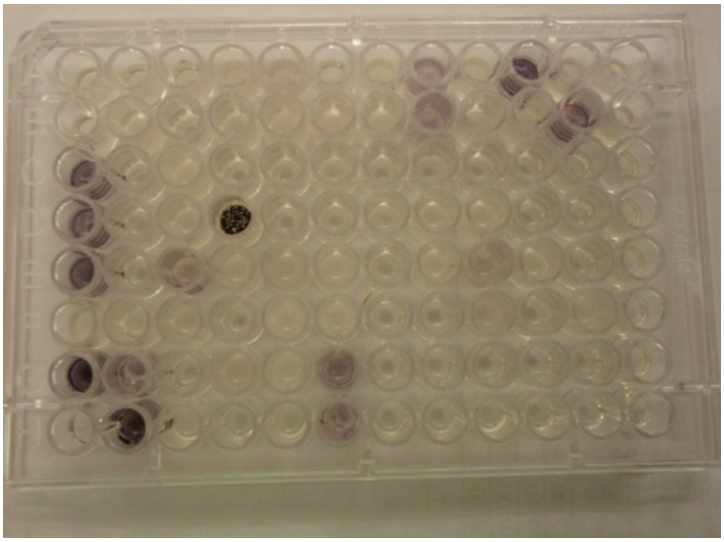
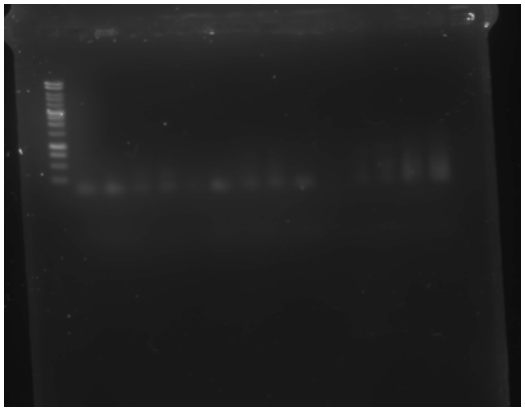


Figure 9) 96 well plate containing incubating single colony picks in 100 ul 5YE 1% glycerol.

A)



B)

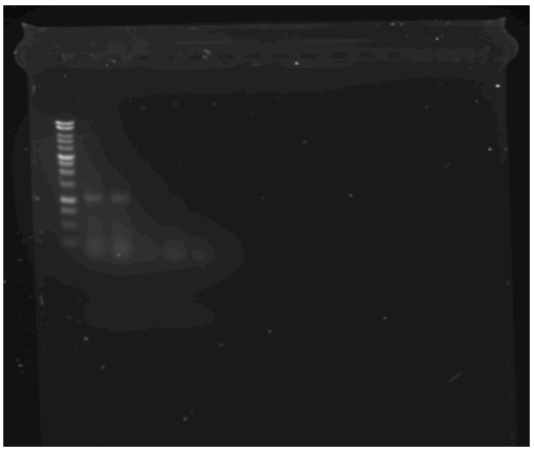


Figure 10) 1% agarose gel containing nested PCR products. Lane: A, 1) 1 kb ladder, 2-13) pRL27 mutant phenotypes 14,15) pSC189 mutant phenotypes. B, 1) 1 kb ladder, 2-7) pSC189 mutant phenotypes.