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Characterization of a non-ribosomal peptide from agrobacterium tumefaciens

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Characterization of a non-ribosomal peptide from *Agrobacterium tumefaciens*

By Graham Strub

Honors Thesis

In

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The current efforts underway to sequence the genome of *Agrobacterium tumefaciens* have led to the discovery of a large region of DNA (52kb) with a strong homology to several polyketide synthetase genes. This was a matter of great interest to the *Agrobacterium* project; no current literature documents the production of a polyketide or non-ribosomal peptide from *Agrobacterium*. Furthermore, the multi-domain enzymes coded by these polyketide synthetase genes in other bacterial species are responsible for synthesizing several toxins, many which act as antibiotic agents (8, 9, 12, 16). This was also of great interest, since *Agrobacterium* is not known to produce any toxins, and also because several of these toxins produced in other species are useful antibiotic agents in humans. The hypothesis that *Agrobacterium* produces such a compound is not surprising; *Agrobacterium* is a well known plant pathogen and opportunistic human pathogen and lives in the soil where it must compete with various other microbes. This project was designed to confirm that *Agrobacterium* produces a polyketide or non-ribosomal peptide, and to determine, if possible, its structure and function.

The original sequence of genomic DNA resembling polyketide synthetase genes was found, with the help of the sequencing efforts of Cereon Genomics, to be a small part of a large cluster of genes. The overall structure of this large gene cluster was found to be 3 large operons, each encoding several genes with multiple domains with different activities (Fig 1), and the overall sequence was found to be homologous to several other polyketide synthetase genes via BLAST searches (Fig 2).

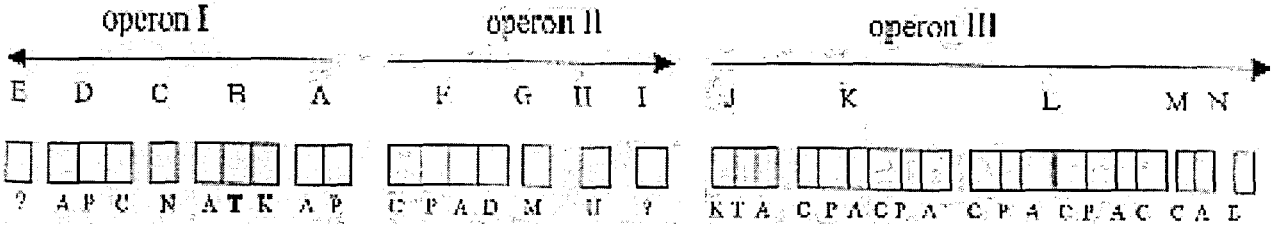


Figure 1: Each gene is shown as a series of connected boxes representing the regions encoding domains with different activities. Domain abbreviations: A, acyl carrier protein; C, amino acid condensation; D, dehydrogenase; E, thioesterase; K, B-ketoacyl synthase; M, methyltransferase; N, monooxygenase; P, amino acid activation; T, acyl transferase; ?, unknown function.

A:

WG4B1F

-3

L	A	G	V	F	S	L	K	D	A	M	F	L	V	L
A	R	G	R	L	M	Q	G	C	A	P	G	G	M	L
A	V	M	L	P	E	A	D	L	R	A	K	L	G	D
G	L	E	I	A	A	V	N	G	P	G	S	C	V	V
A	G	D	T	E	V	L	H	R	F	A	E	R	L	E
K	D	G	V	G	C	R	L	L	R	T	S	H	A	F
H	S	A	A	M	E	P	I	L	E	E	F	A	G	L
F	Y	G	I	A	L	S	A	P	A	I	D	V	M	S
N	L	S	G	N	W	L	R	A	D	E	A	C	D	P
Q	Y	W	T	Q	H	L	R	R	A	V	N	F	S	D
G	I	R	H	S	L	E								

Figure 2: A: Amino acid sequence entered into BLAST database.

B:

Organism	Gene w/ similarity to our sequenc	Probability of random chance
Amycolatopsis mediterranei	rifamycin polyketide synthase	3e-34
Nostoc sp.	NosB polyketide synthase	3e-33
Streptomyces natalensis	polyketide synthase	9e-30
Mycobacterium tuberculosis	probable polyketide synthase	3e-29
Sorangium cellulosum	EpoA polyketide synthase	8e-29
Sorangium cellulosum	epoD polyketide synthase	3e-28
Mycobacterium leprae	polyketide synthase pksF	4e-28
Sorangium cellulosum	polyketide synthase	5e-28
Sorangium cellulosum	EpoF polyketide synthase	7e-28
Sorangium cellulosum	polyketide synthase	7e-28

Figure 2: B: Matches to sequence entered in A.

Based on the arrangement of the different activity domains, it was predicted that that the three operons encoded a biosynthetic pathway for the synthesis of a molecule with 7 amino acids and 2-3 fatty acid residues. This is not surprising based on the models of other similar biosynthetic pathways, such as the pathway for the synthesis of erythromycin (Fig 3). The sequence and specificities of each activation domain were compared using a predictive model (21) to propose an overall amino acid structure for the polyketide/non-ribosomal peptide product (Fig 4). This structure was used in conjunction with various publications (1, 2, 3, 4, 6, 7, 10, 14, 20) to design a protocol for the isolation and purification of the product of the encoded enzymes.

Obtaining a pure sample of enzyme product from a cell culture is no easy task; the extracellular fluid surrounding growing cells contains various nutrients, waste products, and any other secreted molecules. Typical toxins produced by other microorganisms are also extremely small and exhibit a wide range of solubility properties, often making simple extractions of organic molecules extremely difficult. Another important consideration in the purification of any gene product is the expression of the genes in question. Because *Agrobacterium* has never been shown to produce any polyketides or non-ribosomal peptides, it was our hypothesis that the genes responsible for the production of any such compounds are repressed under normal conditions. Therefore, in order to begin the investigation of our genes in the life of *Agrobacterium*, it was first necessary to determine when our discovered genes were turned on.

Because we have the sequence of our genes, it was possible to construct a mutant in which we could monitor the expression of our genes. Using a small segment of the *pks*B gene on a PRL119 plasmid containing a promoterless *lac* Z operon, we constructed a mutant which would express the *lac* Z gene whenever the *pks* B gene was turned on (fig 5).

Before we could monitor for gene expression, we first had to hypothesize some conditions where we would expect the cells to express the genes. If the enzyme products indeed produce a toxin, then we want to replicate conditions under which toxin production would be necessary. We hypothesized that the cells would make a toxin if they sensed competition in the environment, so we decided to grow the cells in a minimal culture to simulate other organisms competing for food. Cultures were grown in M9 minimal media under antibiotic control and containing XGAL, and very low *lac* Z expression was noted between days 7-14. This pattern of gene expression agrees with our hypothesis; namely that the expression of a toxin would be appropriate as food supplies diminish.

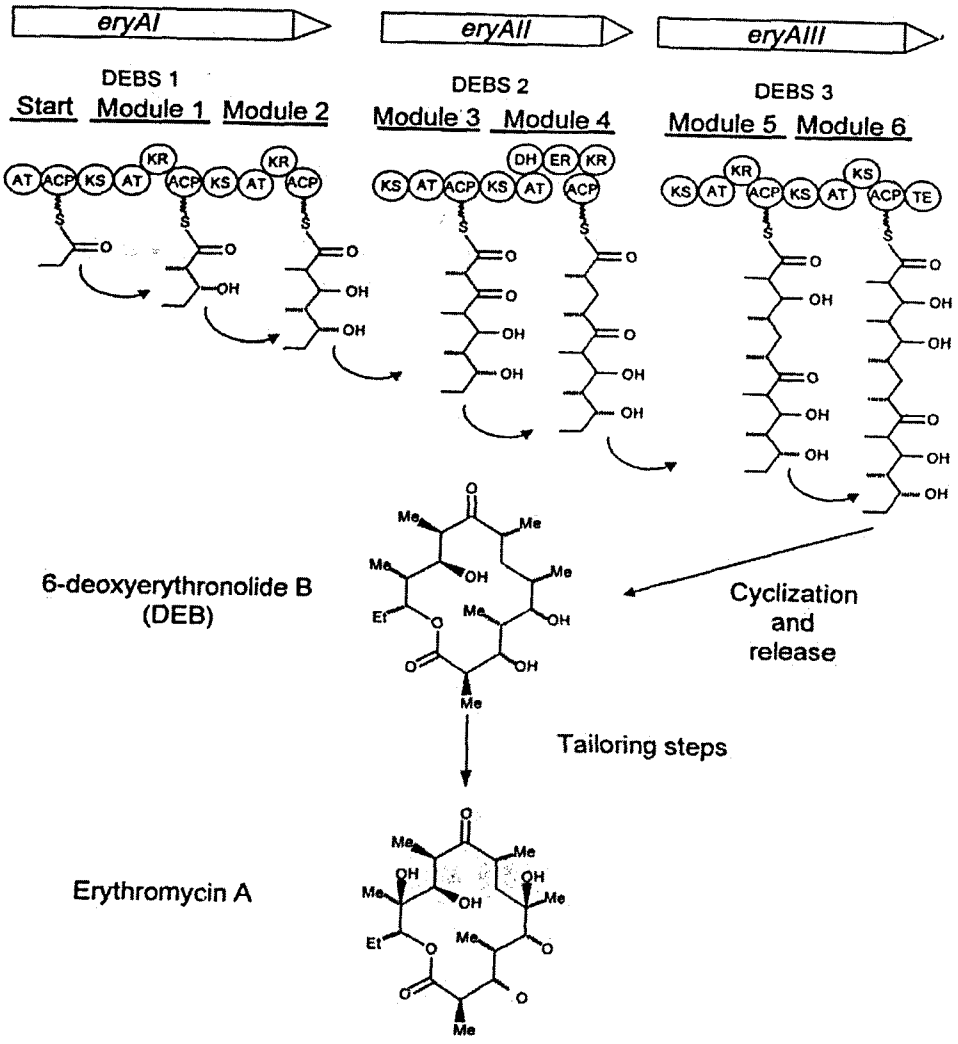


Figure 3: The biosynthetic pathway of the formation of Erythromycin A, an antibiotic non-ribosomal peptide. Note the similarities in gene structure.

Before proceeding with attempting to purify any products of the cells, we wanted to conduct an assay to monitor for any antimicrobial activity in cells that had grown in minimal media for 7-14 days. In order to do this, it was necessary to construct a disruption mutant in the *pks* gene cluster. This would allow us to monitor antimicrobial activity due to only our genes in question. A *pks* B disruption mutant was constructed via homologous recombination (Fig 6) and was used in various assays outlined in this paper.

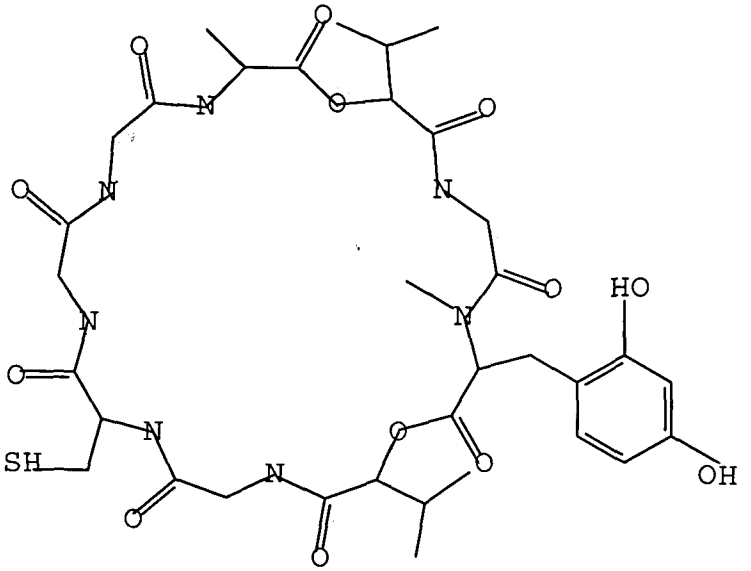


Figure 4: A putative structure for the product of the polyketide synthetase genes in *Agrobacterium* based on a predictive model (21). The model cannot account for fatty acid residue structure, so only the amino acid backbone has been included.

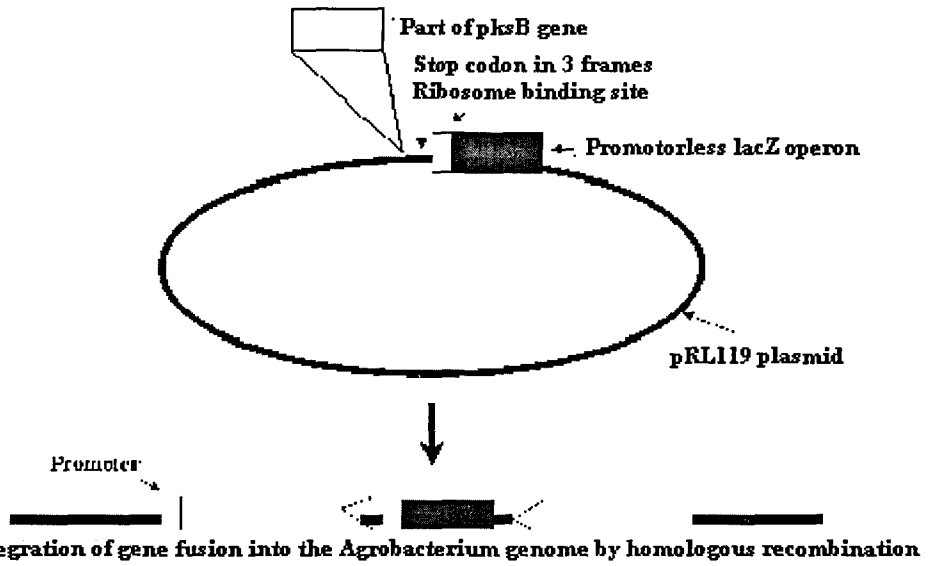


Figure 5: Homologous recombination was used to construct a mutant with a *lac* Z insertion into the *pks*B gene. Cells incorporating the insertion were selected by ampicillin resistance..

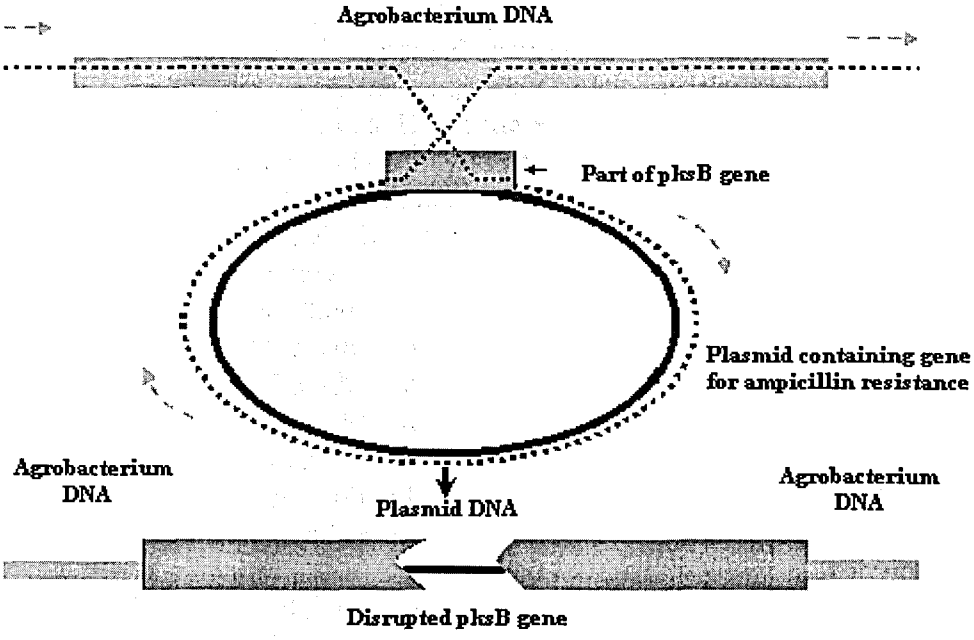
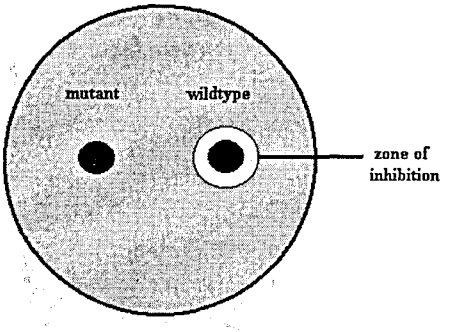


Figure 6: Construction of a *pks* B disruption mutant for use in various functional assays. A plasmid with ampicillin resistance was inserted into the *pks* B gene via homologous recombination to disrupt the product of the genes.

Wildtype cells were grown on minimal plates, and plugs from these plates were transferred onto lawns of other microbes to monitor for any inhibition activity. A small zone of inhibition was produced by a day 14 plug transferred onto a lawn of *Staphylococcus* (Fig 7), but the size of the inhibition zone was not significant enough to conclude that our genes had anything to do with antimicrobial activity. The plugs did not inhibit the growth of any other microorganisms (data not included). This data does not refute or support the claim that *Agrobacterium* produces a toxin, so efforts were then made to attempt to purify the organic components of the supernatants of growing cells. This would make it possible to directly monitor the effects of secreted molecules on other organisms.

Figure 7: Zone of inhibition produced by plugs of wildtype cells on lawns of *Staphylococcus*. The figure at right is not to scale, in fact the zone of inhibition produced was smaller than indicated here. The transferred plugs of *Agrobacterium* had been grown for 14 days on minimal plates.



The overall purification scheme designed for the products of *Agrobacterium* was based on the purification of various non-ribosomal products from various bacterium. In a general respect, the majority of these compounds precipitate under acidic conditions when present in high concentrations. It was therefore our goal to grow large cultures of bacteria for 7-14 days (the amount of time our *lac* Z disruption took to express our genes) and then attempt to precipitate out our non-ribosomal product. Cell cultures, however, contain various other compounds that may precipitate under these conditions, and if all growing cells are not completely removed the supernatants can become contaminated with lysed cellular products. Therefore, in order to determine which compounds might represent our gene products, we designed a comparative assay that used the *pks* B- disruption in conjunction with the wildtype. 1L cultures of wildtype and *pks* B- cells were grown for 7 days, at which time 500ml of each culture was removed and centrifuged at high speed. The resulting supernatants were stored at 4°C. For the next 7 days, 1ml of each culture was removed, centrifuged, and refrigerated. At day 14, the remaining cultures were centrifuged and supernatants were stored for further experimentation.

Now that supernatants had been obtained for days 7-14 for both strains of cells, the purification process could begin (note: the large portions of obtained supernatants were from days 7 and 14. The 1ml cultures collected between these days were obtained to monitor if the concentrations of any cell products increased over these days). The solutions were made acidic (pH 2.0) by adding concentrated HCl, and the solutions were stored for 48hrs at 4°C. The solutions were then centrifuged (13,000rpm) and supernatants were discarded. The resulting pellets were washed several times with acidic water, washed 3 times in hexane to remove any free fatty acids or alcohols, and then dissolved in alkaline water. The resulting solutions were frozen in liquid nitrogen and lyophilized. The collected pellets were stored at -20°C for further experimentation.

The pellets were finally dissolved in diethyl ether (after trying various organic solvents) and run on silica TLC paper under various mobile phases to asses for the presence of any organic solvents. Results for days 7-14 TLC are included below (Fig 8).

It seemed clear that several organic molecules were present in our lyophilized pellets from the wildtype cells. Upon high magnification, we discovered an area in each sample (indicated with arrows) that appeared to increase in concentration between days 7-14. This was consistent with both our hypothesis and the results of our *lac* Z disruption assays (XGAL breakdown increased over the 7-14 day period, indicating an increase in gene expression). This became our primary area of interest. However, this spot on the TLC plate is too close to other spots to purify this compound in the current mobile phase. Therefore, efforts were made to discover a mobile

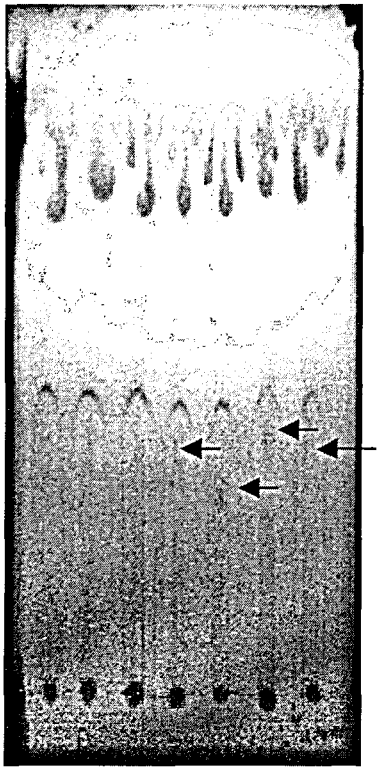


Figure 8: Thin layer chromatography of dried pellets dissolved in diethyl ether under a 1:1 v/v diethyl ether/pentane mobile phase. Each spot represents a pellet obtained from each day (7-14 left to right). Note the increase in concentration of the indicated area as the days progress.

phase that would separate the spot in question from the other dissolved molecules. After several trials, pure chloroform proved the best in isolating the spot in question. Several attempts including pure chloroform (indicated with an arrow) are included (Fig 8).

Now that we had developed a way to separate the molecule in question from the other dissolved compounds, we wanted to assay the disruption mutant to see if the expression of this compound differed in any way. We would expect that if the indicated spot was indeed the product of our disrupted genes, our mutant cell supernatants should produce different results. This is exactly what we found. When pellet samples from both widtype and mutant cells were run in parallel, the spot found on the wildtype plate was completely absent in the mutant assay (Fig 9).

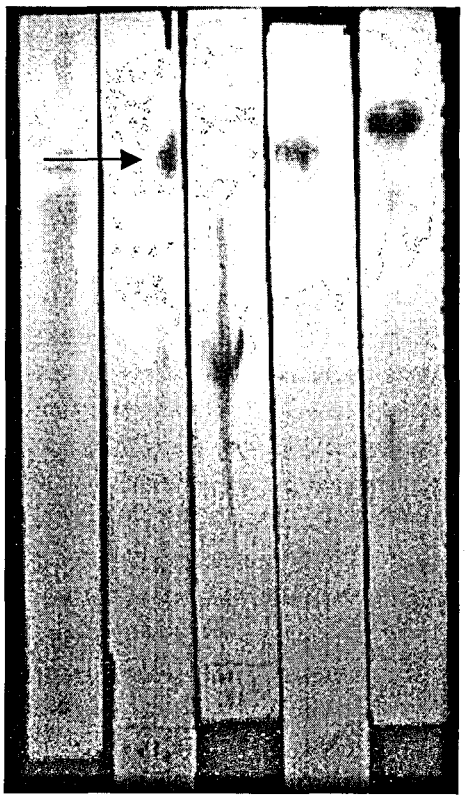


Figure 9: TLC of WT pellets in various solvents in attempts to separate organic compounds. Clearly, the second plate from the left (indicated) produced the best separation (chloroform mobile phase), so this mobile phase was selected for all further experimentation. Spots were resolved with short wave UV.

All results obtained so far supported our hypothesis that a: the pellets we obtained may contain the product of our genes, and b: we had obtained a suitable purification scheme that would allow us to separate whatever this compound was. There was no way of proving that the spot was the product of our genes; but most of the evidence pointed in this direction. It then seemed appropriate to move onto a more rigorous purification scheme that would allow us to collect some of the purified product for analysis.

In order to collect a crudely purified product, a 10cm elution column containing silica gel was constructed. The remaining pellets (both WT and mutant for days 7 and 14) were dissolved in 1ml of chloroform and poured into the elution column. The column was eluted with diethyl ether, and 50 1ml samples of elutant were collected for analysis. Each 1ml sample was spotted on TLC plates and resolved with short wave UV (data not included). Samples 3-6 in both WT and mutant assays (both days 7 and 14) resolved a spot, coordinating with the resolved spot at the top of the plate in figure 10. In addition, the WT day 7 and day 14 pellets resolved a spot in samples 14-17 (note: the spot in day 14 pellet was much darker, indicating a higher concentration as in figure 8, with no corresponding spot in the mutant). These samples were combined, resulting in a 3ml

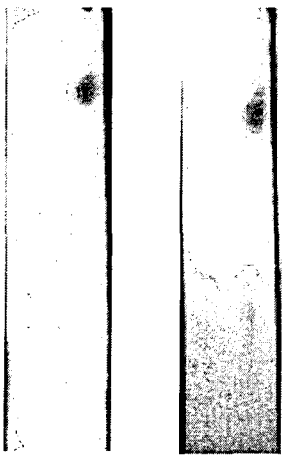


Figure 10: TLC of wildtype (right) and mutant (left) pellets dissolved in ether and run in chloroform mobile phase. Note the indicated spot on the wildtype plate that is absent in the disruption plate. Spots were resolved with short wave UV.

sample for both days 7 and 14 for the wildtype. For demonstration purposes, samples 14-17 in the mutant were also combined, and all four resulting solutions were spotted on TLC plates and run in a chloroform mobile phase (Fig 11).

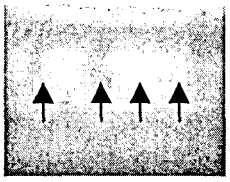
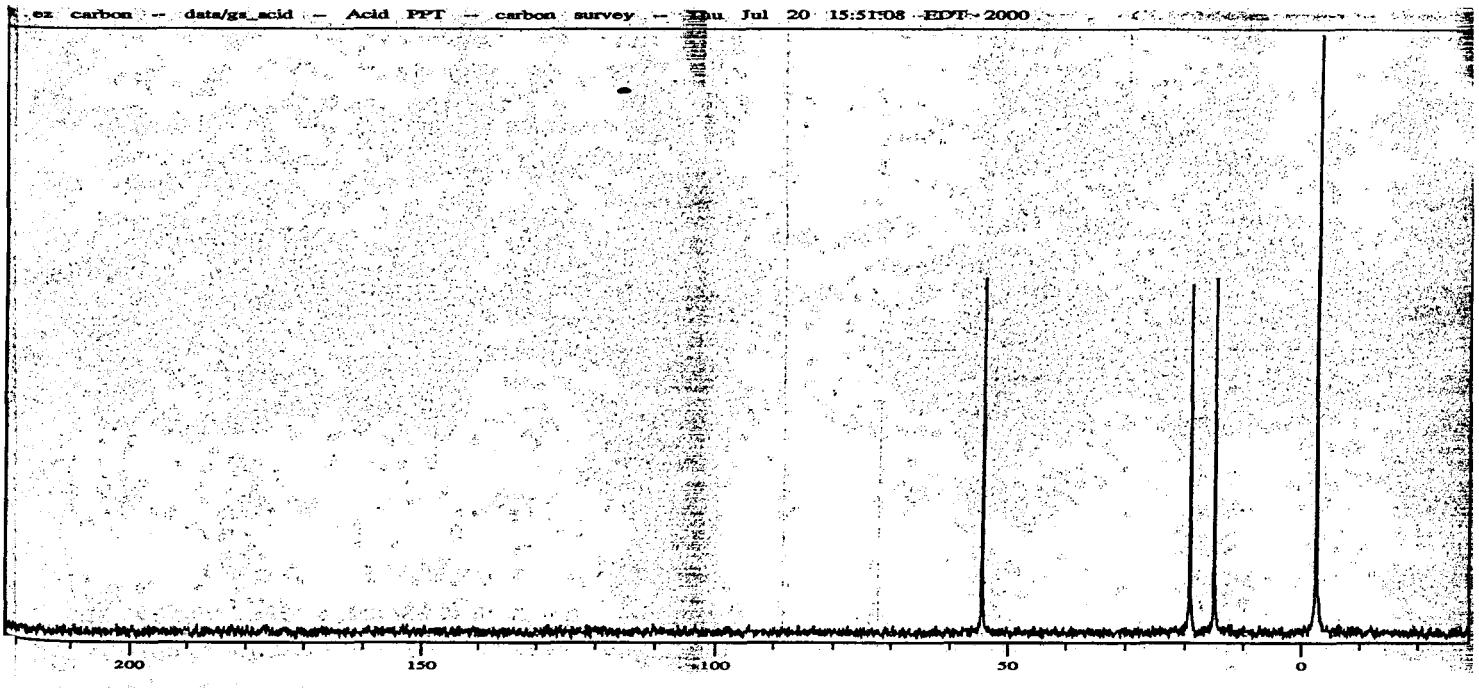


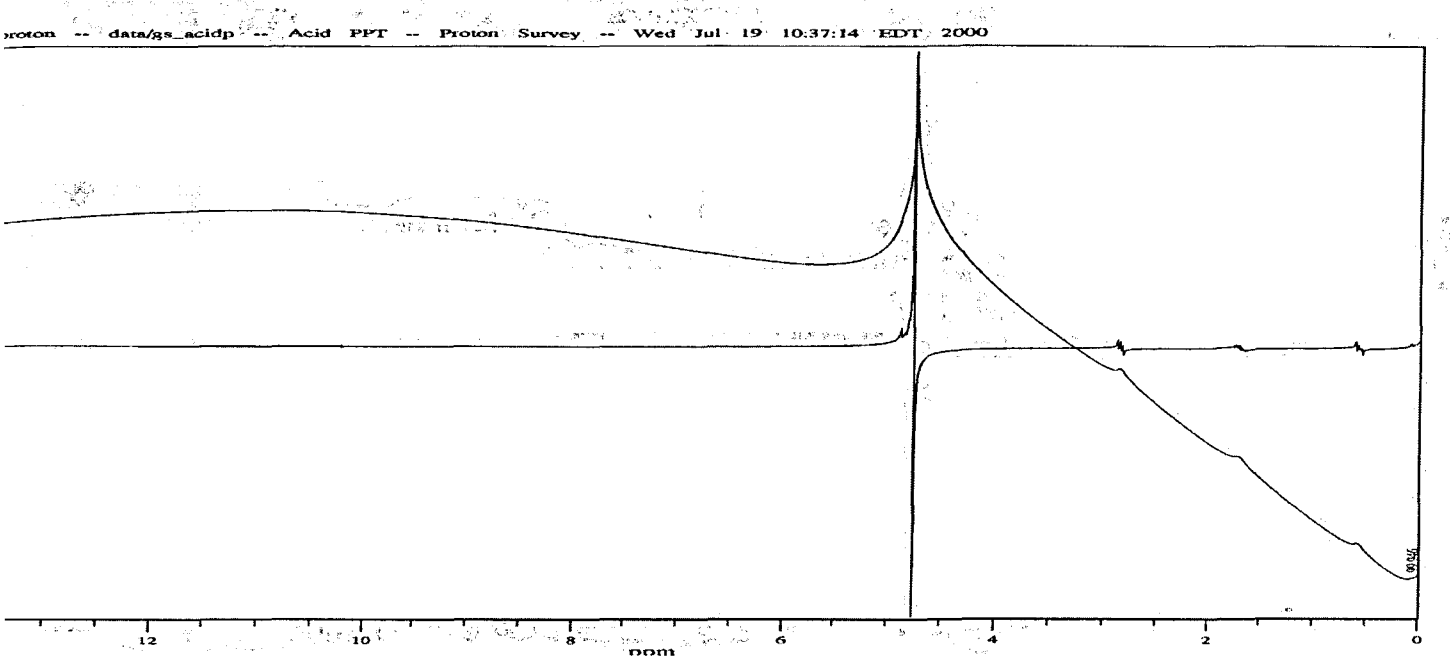
Figure 11: Spots were resolved (from left to right); WT day 7, WT day 14, *pks* B- day 7, *pks* B day 14, in a chloroform mobile phase and resolved with short wave UV. Note the presence of a spot that increases in concentration in the WT from day 7 to 14. This spot is absent in both mutant samples.

Now that we had obtained samples from the WT that contained one spot that the disruptions did not, we were ready to move onto some spectroscopic analysis. The samples for days 7 and 14 for the wildtype strain were combined and the solvent was evaporated under reduced pressure to yield approximately .2mg of brown crystals. The crystals were heated in a 100°C oven in a .5 ml head space MS tube, and 1ul of head space air was collected and injected into a Hewlett-Pakard model 5890 series MS with a GC inlet system. No spectrum was produced due to the low volatility of the crystals.

The remaining brown crystals were dissolved in D2O and analyzed in a GE 300MHz NMR system locked to the deuterium resonance of the solvent D2O. These experiments produced the following ^{1}H and ^{13}C spectra (Fig 12).

A.





ire 12: A: 300MHz 1D carbon spectrum of unknown crystals. Signals were recoreded at 15, 20 and 55 ppm. B: 300MHz 1D on spectrum of unknown crystals. Signals recorded were at .2, 1.5, and 2.8ppm.

The data collected in this series of NMR experiments is inconclusive. The most likely cause of such weak signals is the small sample size we used, however we would have expected at least some signals upfield from the ones we obtained. To confirm this, we compared our data with the NMR analysis of Lichenysin A, a non-ribosomal peptide similar in structure to our putative structure (Fig 13).

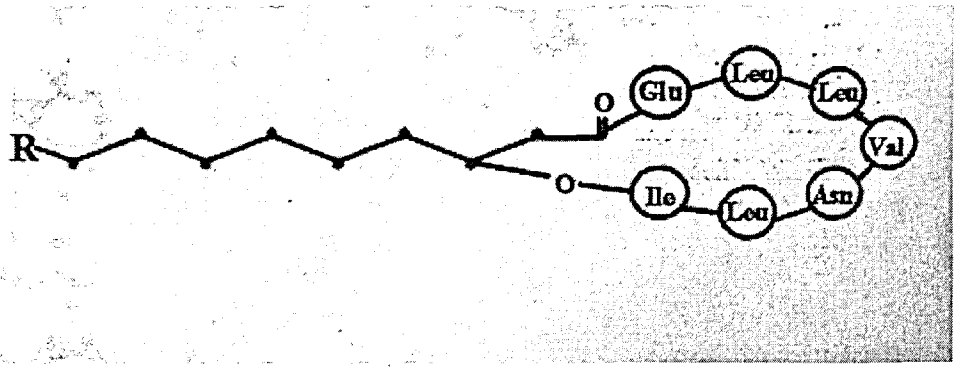


Figure 13: Structure of Lichenysin A, a non-ribosomal peptide similar to our proposed structure (20).

Lichenysin A produces strong signals at 9.8ppm, 7.4ppm, 4.38ppm, and 2.01ppm. We would have expected to at least see some stronger peaks upfield of the deuterium signal produced at 4.8. In addition, investigators were able to obtain a GC-MS of Lichenysin A on the same machine described here. Collectively, these results suggested that our purification scheme did not successfully purify any non-ribosomal peptide.

To gain further knowledge into any function of our purified components, concentrated samples of the dissolved pellets were spotted onto plates of *Staphylococcus* to monitor for any growth inhibition. There was no inhibition of growth at any concentration (data not included); no antimicrobial activity was observed.

Whether we had purified correctly or not, it was our contention that *Agrobacterium* was expressing the *pks* genes at low levels to begin with, so we deemed it appropriate to approach the problem in a new way. Because we knew the sequences of the genes and the nucleotides between the operons, we decided to attempt to clone out each operon and assemble them under controlable promoters in *E. coli.* This way we could attempt to constitutively express our genes and attempt to collect high concentrations of product. We used an Expand Long Template PCR System (Roche) and attempted to clone out each operon for ligations and transformations. We monitored for fragments of the appropriate size for our operons (12.8kb, 8.3kb, 21.8kb). We tried several times at various Tm's but failed to obtain any clones (data not included).

Currently we are making efforts to construct a mutant with the entire *pks* gene sequence removed. We have obtained a ClonePlex-AK Library construction kit (Lucigen) which is normally used to clone two DNA fragments independently into the same vector. The pLEXX-AK vector used contains two dispersed *Hinc*II cloning sites, each flanked by two different antibiotic resistance genes (Fig 14). We used sequences flanking the cluster of *pks* genes to design primers to clone the entire region into the pLEXX-AK vector, leaving the genomic DNA without the *pks* genes and inserting the entire region into the plasmid. This would result in a mutant ready for all sorts of functional assays, as well as a potentially recoverable plasmid containing the entire region of biosynthetic genes.

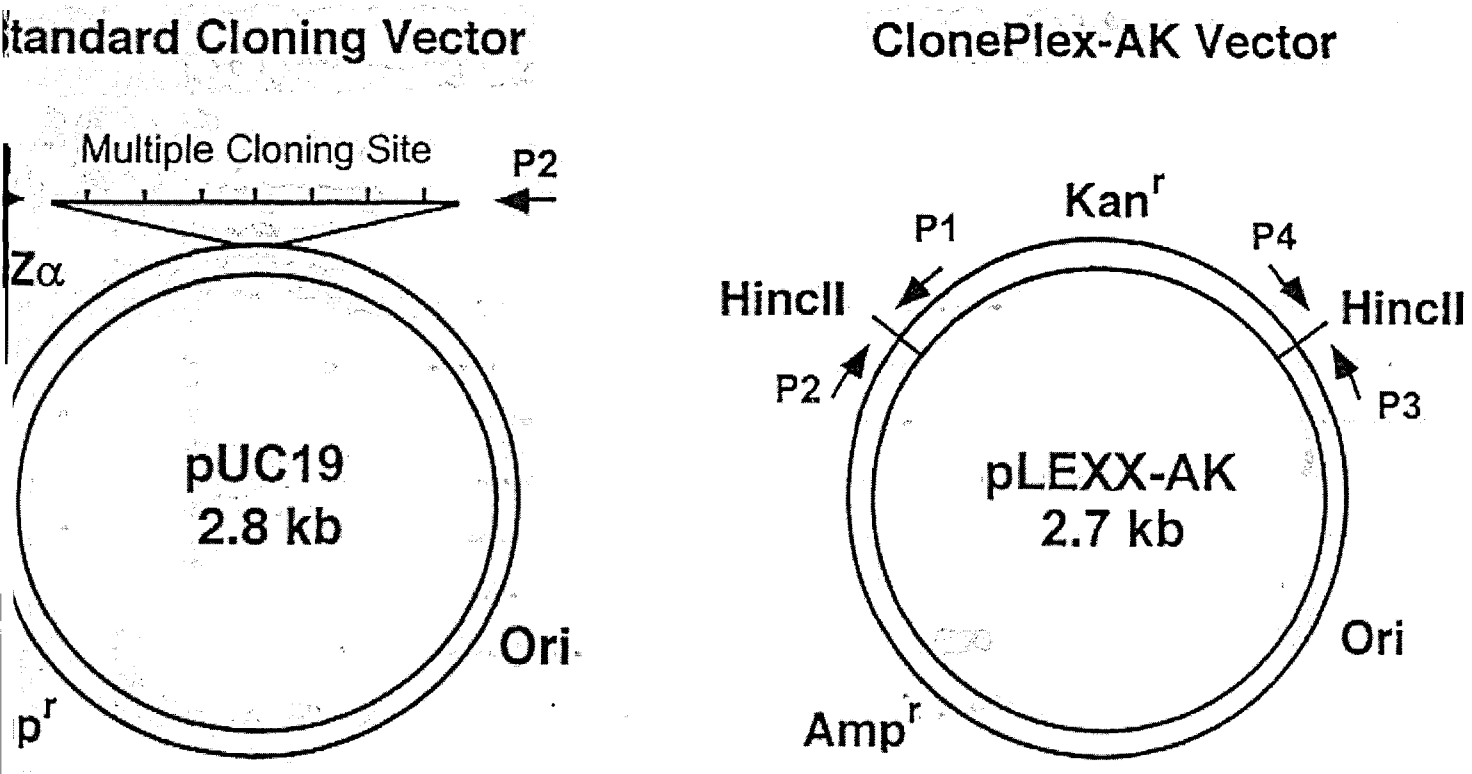


Figure 14: Schematic diagram illustrating the difference between conventional cloning vectors and the ClonePlex pLEXX-AK duplex cloning vector. P1-P4 indicate primer binding sites. Amp: ampicillin resistance gene; Kan: kanamycin resistance gene; *lac*Zα: alpha fragment of the β-galactosidase gene; Ori: origin of replication; *Hinc*II: cloning site for blunt end DNA fragments.

We have already obtained the primers necessary for the reaction and have converted the overhanging ends into phosphorylated blunt ends and ligated them to the vector. The vector is now ready for electroporation into *E. coli.*

It seems that these new genetic approaches will be the answer to discovering the function of the genes in question. Because the genes do not seem to be expressed under normal conditions, we must now direct our efforts to genetic manipulation. The fact that the expression of these genes is repressed may suggest a role in the function of the genes, and may also provide clues as to the evolutionary background of the putative polyketide synthetase genes themselves. While we are making progress on analyzing the genes via PCR, further efforts should be made into investigating the role of these genes *in vivo.* There must be some environmental cues that enhance the expression of these genes, although the conditions that stimulate other bacteria to make these compounds have failed to do so at high levels. Nevertheless, more rigorous chemical purification (HPLC or affinity chromatography) may be sufficient to obtain larger amounts of non-ribosomal peptide for analysis. It is also worth mentioning that FAB-MS experiments will provide clues as to the nature of the compound isolated from the wildtype strain; it is of interest because the mutant strain failed to produce it. It should be noted that no assays other than the *lac* Z disruption have been conducted to directly monitor gene expression, so future efforts should include a more reliable method to monitor gene expression. Until that time the role of the *pks* cluster in *Agrobacterium tumefaciens* remains unclear.

Materials and Methods

Microorganisms and culture conditions:

Agrobacterium tumefaciens strain A348 was used throughout this work. Stocks of bacteria were stored in frozen glycerol cultures at -70°C. The cultures were grown in M9 minimal media with sucrose as a carbon source. Bacterial growth in minimal media was monitored by measuring optical density at 600nm.

Construction of mutants:

Strain A348 cells were subjected to homologous recombination and two mutant strains were derived. A *lacZ* insertion mutation under ampicillin control was inserted into the *pks* B gene for the monitoring of gene expression, and a pRL119 plasmid was inserted into the *pks* B gene to create a disruption mutant.

Inhibition assays:

A348 cells were grown on M9 minimal plates for 7-14 days at 28°C. Plugs from these plates were transferred onto lawns of *E. coli* and *Staphylococcus,* and plates were incubated for 24h at 37°C to monitor for growth inhibition.

Compound isolation:

Bacterial cells were removed from M9 minimal media after 7-14 days by centrifugation (13,000 x g, 4°C, 15 min). The supernatant was subjected to acid precipitation by adding

concentrated HCl to achieve a final pH of 2.0 and allowing the precipitate to form at 4°C for 48h. The pellet was collected by centrifugation and washed several times with acidic water (pH 2.0 with concentrated HCl), dissolved in alkaline water (pH 8.0 with NaOH), and lyophilized overnight. The dried product was extracted with diethyl ether, solvent was removed with the aid of a rotary evaporator under reduced pressure, and the solid was washed with 3 volumes of hexane to remove alkanes, free fatty acids, and alcohols. The crude material was collected for further purification by the chromatographic procedures described below.

Thin layer and adsorption chromatography was carried out on silica gel plates and elution columns (B&J Inert SPE System, Burdick and Jackson, Muskegon, Mich.) with solvents of gradually increasing polarity: chloroform > acetone > chloroform : methanol (2:1) > methanol. The chloroform elutate demonstrated the greatest separation, so the compounds were separated in a silica gel column using this mobile phase. The product was dialyzed against sterile, distilled water overnight to remove the remaining cations and stored at -20°C.

Other TLC systems tested included chloroform-methanol-acetone-acetic acid 90:10:6:1 and chloroform-methanol-acetone 90:10:6.

GC-MS:

GC positive-ion electron impact was performed with a MS 5989A system (Hewlett-Packard). The ion source was maintained at 200°C. Injections were made (1μl) with a Hewlett-Packard autosampler in the on-column mode. The hydrogen carrier gas flow rate was 21cm/s. The oven temperature ranged from 100 to 280°C. the injector was maintained in the overtracking mode. The temperature of the detector was 325°C.

NMR:

All one dimentional NMR spectra were recorded on a GE 300MHz NMR spectrometer locked to the deuterium resonance of the solvent D2O, without spinning.

PCR:

In our first attempt to PCR each operon separately, the following primers were used:

Operon 1:

pksABCDE (should amplify 12778 bp)

AB-1 (38 bases) 5'-gcTcTAgATcAggAgTTTTTcAgATgcAgATcATAccg-3'

AB-2 (30 bases) 5'-gcAgATcTATggAcAcATcAgcccgccTcg-3'

Operon 2:

pksFGHII'I" (should amplify 8296 bp)

FG-1 (43 bases)

5'-ccgAATTcTAgAgccAAggggcggcTTATgAATTAcATcgAgc-3'

FG-2 (37 bases) 5'-cgAgcTcAAgcTTAAcAcgcAcgTTAcTTgTggAAcg-3'

Operon 3:

pksJLKMN (should amplify 21787 bp)

JK-1 (37 bases) 5'-ggggTAccAgcgAgAAcTgcccATgTccgAcTATAcc-3'

JK-2 (31 bases) 5'-gcTcTAgATcAggcTcATgcAgTTcTggTTg-3'

In our second attempt to PCR the entire region, the following sequences were used for primers:

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	643	25	61.73	48.00	6.00	2.00	
CTTTACGGTCTCCTCTATGATCAGC							
RIGHT PRIMER	1616	24	67.00	50.00	4.00	0.00	
CACTTGGCCTGTCTTTGATGGTTG							

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	211	24	60.98	45.83	4.00	0.00	
GTTACATTCCCAGTCCATGTCTTC							
RIGHT PRIMER	1217	25	60.61	44.00	4.00	3.00	
GTGTAGGTGAAGGGTTCTTTTAAGG							

All PCR experiments were carried out in a Perkin-Elmer DNA thermal cycler.

ClonePlex construction:

The repair of fragmented insert DNA, ligation to the pLEXX-AK vector, and transformation into competent *E. coli* cells was conducted according to the protocol described in the ClonePlex Library Construction Kit (Lucigen).

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