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Synthetic Genes for Antimicrobial Peptides

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Abstract

The goal of this project was to clone and express the antimicrobial peptide protegrin 1 (PG-1). Initially a yeast system was chosen but was discarded due to technical difficulties. Invitrogen's bacterial T7 expression system was chosen next to express the peptide. PG-1 expression was verified by anti-his immunoblot and then the peptide was purified by IMAC. Its activity was verified using a *Bacillus subtillis* radial diffusion assay.

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Acknowledgements

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1 Introduction

Bacterial infections have been a problem for macroorganisms for as long as they have existed on the earth. Humans are not an exception to this concern. As science has progressed many means to combat bacteria have been developed. One of these means is antibiotics. These compounds are able to attack bacteria on the molecular level and defeat them directly. Many higher organisms, however, have built-in antibiotics known as antimicrobial peptides. These proteins act as endogenous antibiotics and protect the host animal from a wide range of bacterial pathogens (Cho et al 1998).

This masters thesis focused on the development of an artificial gene for an antimicrobial peptide and subsequent expression of the gene in host cells. Antimicrobial peptides can be found in most animals, and in the vast majority of cases an animal has several different kinds of antimicrobial peptides. Alpha defensins, human antimicrobial peptides, function in neutrophils where they aid the cell in killing bacteria and fungi once the neutrophil has ingested the pathogens (Lehrer and Ganz, 1999). Human beta defensins have also been shown to act as chemo attractants for memory T-cells in addition to damaging microbial membranes (Yang et al 1999). Another very broad class of antimicrobial peptides, the cathelicidins, are also found in the neutrophils of various animals. These antimicrobial peptides are synthesized at the C-terminal end of a highly conserved cathelin domain. The cathelin domain serves to keep the antimicrobial peptide in an inactive storage form. This domain is widespread among mammals, which suggests it has considerable value. Once the cathelin bound peptide has been synthesized, it is processed by neutrophil elastase and the peptide is released from the cathelin domain. This cleavage converts it to its active form (Shi and Ganz 1998). One cathelicidin, LL-37, has been

localized to epithelial cells of the human airway, and to serous and mucous cells of the submucousal glands. LL-37 has shown activity against *Pseudomonas aeruginosa*, a bacterium that causes chronic lung infections (Turner et al. 1998).

Another class of antimicrobial peptide is the protegrins, which are the focus of this thesis. These peptides were originally found in porcine leukocytes, and are 16 – 18 amino acid residues in length. They are a member of the cathelicidin family. Protegrins show broad-spectrum activity and have been proven active against *Candida albicans, Listeria monocytogenes, Staphylococcus epidermidis, and Bacillus subtilis* along with several other strains of bacteria (Yasin et al. 1996; Qu et al. 1997; Steinberg et al. 1997; Cho et al. 1998; Shi and Ganz 1998; Turner et al. 1998).

Antimicrobial peptides in general are being widely studied right now in order to determine their potential utility in medicine. The ability to produce synthetic analogues for use in treatment of bacterial infections is also being investigated. If antimicrobial peptides can be created using a cloned gene then it would be possible to produce them in sufficient quantities at a reasonable cost. The high cost of a fair number of drugs is due to the fact that they must be isolated directly from their natural environment. In some cases this can prove to be very difficult. Studies have already been done examining the effectiveness of murine analogues of some antimicrobial peptides for combating bacterial infections in mice. These proteins have the potential to become an alternative to the over used antibiotics that have recently proved less effective as the number of resistant bacterial strains increases.

2 Background

2.1 Previous Antimicrobial Cloning Work

Several other antimicrobial peptides have already been successfully cloned and expressed in bacterial systems. Expression as a fusion protein is necessary to protect the product from degradation by cellular proteases as well as to prevent the product from exerting its toxic effects on the host cell. Haught et al. (1998) successfully expressed the antimicrobial peptide P2 as a fusion protein with bovine prochymosin in *E. coli*. P2 is a twenty three amino acid antimicrobial peptide that was developed synthetically. When expressed, the fusion protein accounted for sixteen percent of the total cellular protein and formed inclusion bodies. The inclusion bodies were purified by centrifugation, and cyanogen bromide was used to cleave the methionine linker that joined the two fusion partners. Afterwards cation exchange HPLC followed by reversed phase HPLC were used for additional purification. The activity of the recombinant product was found to be identical to a chemically synthesized control.

Buforin II was expressed as tandem repeats fused to an acidic peptide in *E. coli*. This method was adopted in an attempt to increase yields and minimize harmful antimicrobial activity. The acidic protein was successful in neutralizing the basic charge of the peptide and in forming inclusion bodies. Inclusion bodies were recovered by centrifugation and sonication, and resolubilized then treated with cyanogens bromide to separate the multimers from each other and the fusion protein. Recombinant buforin II antimicrobial activity was compared with natural buforin II via radial diffusion assay. They were found to have identical antimicrobial activity. (Lee et al. 1997)

A final example of antimicrobial expression is the development of a system that could be used for expression of a variety of antimicrobial peptides. The system results in the peptide of

interest being produced as a fusion protein with the F4 polypeptide. F4 promotes the formation of inclusion bodies which is essential. Of numerous peptides produced with this system MSI-344 was isolated and purified. Cation exchange chromatography was used to isolate the fusion protein which was then cleaved with hydroxylamine to separate MSI-344 and the F4 peptide. Recombinant MSI-344 compared favorably with synthetic controls when assayed for activity. (Lee et al. 2000)

2.2 Protegrins

Protegrins are a small family of recently identified antimicrobial peptides, originally isolated from porcine neutrophils. After synthesis, they are initially stored as precursors that remain inactive until hydrolyzed extracellularly by neutrophil elastase (Shi and Ganz 1998). To date, five naturally occurring protegrins have been found, PG-1 to PG-5 (Steinberg et al. 1997). These peptides are cysteine-rich and show a significant amount of similarity among their peptide chains. All are cationic and amphipathic, containing both polar and nonpolar amino acids (Steinberg et al. 1997; Cho et al. 1998). Protegrins are between 16 and 18 amino acid residues in length. This structure folds to form two antiparallel β–sheets connected by a hairpin turn which is stabilized by the presence of two cysteine disulfide bonds that typically occur between C6-C15 and C8-C13 (Steinberg et al. 1997; Roumestand et al. 1998; Cho et al. 1998). One study showed that these disulfide bonds are a key factor in protegrin microbicidal activity and a significant decrease was observed when one or both of these bonds were removed (Qu et al. 1997).

2.2.1 The PG-1 Protein

The most widely studied of the natural protegrins is PG-1, which contains 18 amino acids and exhibits a broad range of antimicrobial activity. Figure 3.1 shows the structure of this peptide. This peptide has been assayed for activity against many representative strains of both gram positive and gram-negative bacteria, as well as some antibiotic-resistant strains. It has also shown some antifungal and even antiviral activity (Yasin et al. 1996; Qu et al. 1997; Steinberg et al. 1997; Cho et al. 1998; Shi and Ganz 1998; Turner et al. 1998). Under different experimental conditions, PG-1 has exhibited excellent antimicrobial activity *in vitro* against a variety of bacteria such as *Escherichia coli, Listeria monocytogenes, Proteus vulgaris,* and *Bacillus subtilis. B. subtilis* was used in this work for assessing activity. Typically, relatively low concentrations of the peptide are sufficient, since it has curious potency compared to certain other antimicrobial peptides (Yasin et al. 1996; Qu et al. 1997; Turner et al. 1998). In one experiment, PG-1 was tested *in vivo* on mice that were inoculated with various types of bacteria. Steinberg et al. (1997) found that the mortality rate of the mice was significantly decreased after treatment with PG-1 relative to untreated mice.

While not a serious public health problem yet, some strains of resistant bacteria have evolved. The mechanisms of resistance involved range from a decrease in the net negative charge of the membrane to make it less electrically attractive, to physical pumping to remove the peptides (Peschel 2002). Currently a synthetic analogue of PG-1 is undergoing Phase III clinical trials for FDA approval. The compound being developed by Intrabiotics Pharmaceuticals, known as IB-367, is an oral rinse for use by cancer patients suffering from mucosistis. The FDA has granted Fast-Track designation to the compound (www.intrabiotics.com).

2.2.2 Mechanism of Antimicrobial Activity

The exact mechanism by which protegrins inactivate various microbes has not yet been found but it is believed to operate by the toroidal pore model. This means that groups of PG-1 peptides come together and form a ring like structure to isolate a segment of the cell membrane. Once isolated, the two leaflets of the membrane fold in and connect to each other resulting in an unregulated pore. Using radiolabeled lipids and radiolabeled PG-1 peptides in a synthetic bilayer and tracking shifts in their positions by NMR it was confirmed that PG-1 inserts across the membrane with its long axis parallel to the lipids. Looking at Figure 3.1 it can be seen that at either end of the hairpin structure there is a concentration of cationic arginine residues while in the center there are hydrophobic amino acids (Leu, Tyr, Phe, Val). This makes sense considering that the ends of the peptide would be interacting with the negatively charged phosphate head groups while the middle portion would be in contact with the hydrophobic fatty acid tails. It is uncertain at this point if there are any earlier stages of multimerization before the formation of the isolating ring structure. Further research is still required but this is currently the favored model for PG-1's method of action (Yamaguchi et al. 2002).

2.3 Bacillus subtilis

The organism used for assaying the PG-1 peptide expressed from the artificial gene is *Bacillus subtilis.* This bacterium is gram-positive and commonly found in nature, typically soil and straw samples. It is aerobic and has the capability to form spores. Bacteria in the genus *Bacillus* are characterized by their short, thick, rod-like structure. They are motile due to the fact that they possess flagella. This bacterium is considered a mesophile, since its growth is favored in the moderate temperature range, with optima between 20 and 45 degrees Celsius. *B. subtilis* is a common bacterium for laboratory use because it is relatively easy to grow and typically not

considered a pathogen (Prescott et al. 1999). For these reasons, this bacterium was chosen for use in this thesis to assay PG-1.

2.4 Pichia pastoris

The yeast *Pichia pastoris* was the expression system initially chosen. It is a commonly used system for the expression of proteins and more than 100 have successfully been produced by it (Higgins and Cregg 1998). One of the many reasons that *P. pastoris* is used is that it genotypically resembles *Saccharomyces cerevisae*, which is a very well characterized system. This makes *P. pastoris* very easy to work with. It is also quite inexpensive relative to other expression systems. This type of yeast is easy to transform and grow in the laboratory. *P. pastoris* utilizes glucose as a primary carbon source, which is inexpensive and easy to obtain. Methanol can be used as an alternate carbon source due to the alcohol oxidase (AOX1) gene. In cloning the alcohol oxidase promoter is used as the driving force behind cloned protein expression. Adding methanol to a culture induces expression of whatever gene is downstream from the AOX1 promoter. The cultures are capable of growing to high cell density that will improve the amount of protein being expressed. The cultures are also quite resilient. These reasons are why *P. pastoris* was initially chosen to express the synthetic PG-1 gene.

2.5 T7 Expression System

The pRSET vector is based on the T7 promoter expression system. Expression of the target genes cloned into the pRSET vector is induced by providing a source of T7 DNA polymerase in the host cell. This is accomplished by using the *E. coli* host BL21(DE3)pLysS which contains a chromosomal copy of the T7 DNA polymerase gene. The T7 DNA polymerase gene is under the control of the lacUV5 promoter which is induced by IPTG. T7 RNA

polymerase is expressed upon induction and converts almost all of the host cell's resources to transcribe the gene of interest which is downstream from the T7 promoter (www.invitrogen.com). This system was later used due to difficulty with the *P. pastoris* system.

3 Materials and Methods

3.1 Design of the Synthetic PG-1 Gene

The amino acid sequence of the antimicrobial protein PG-1 (GenBank accession number AAB27599.1) can be seen in Figure 3.1.

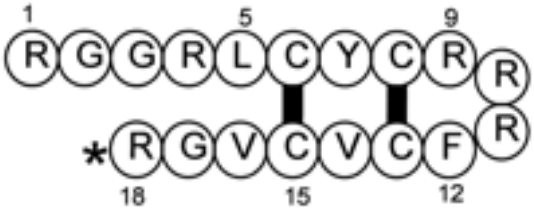

Figure 3-1 Structure of PG-1. This figure shows the amino acid sequence and the secondary structure of the PG-1 protein. The solid bars between cysteine (C) residues represent disulfide bonds. The asterisk indicates amidation at the C-terminus of the protein.

From the amino acid sequence of PG-1, a DNA sequence was designed and optimized for expression in *Pichia pastoris.* This involved determining the codon bias of this organism. The *pim1* gene (GenBank accession number AJ006686) of *P. pastoris* was examined for this purpose. This particular gene is over one thousand seven hundred bases long and provides an adequate sample of the codon usage patterns of *P. pastoris*. Each occurrence of the relevant codons corresponding to the amino acids in PG-1 in *pim1* was noted. The most frequently occurring codons were considered to be the preference of *P. pastoris* for the specific amino acids in the protein PG-1 and were used in the design of the nucleotide sequence (see Table 3-1). The entire sequence of the proposed synthetic gene can be seen in Figure 3.2, in both single- and double-stranded form.

A.
5'–AGAGGGGGGAGATTATGCTATTGCCGGCGGCGGTTTTGCGTGTGCGTGGGGAGATGA-3'

B.
5' – AGAGGGGGGAGATTATGCTATTGCCGGCGGCGGTTTTGCGTGTGCGTGGGGAGATGA– 3'
3' - TCTCCCCCCTCTAATACGATAACGGCCGCCGCCAAAACGCACACGCACCCCTCTACT– 5'

Figure 3-2 Nucleotide sequence of the synthetic PG-1 gene. Part A shows the single stranded form of the coding region for the protein. Part B shows the complete double stranded form of the nucleotide sequence.

The protein-coding region of the gene was designed in the form of two overlapping oligonucleotides, as shown in Figure 3.3.

5' – AGAGGGGGGAGATTATGCTATTGCCGGCGGCGGTTTTGCGTGTGCGTGGGGAGATGA – 3'
3' - TCTCCCCCCTCTAATACGATAACGGCCGCCGCCAAAACGCACACGCACCCCTCTACT – 5'

Figure 3-3 Overlapping oligonucleotides. The green highlighted region shows the oligonucleotides that were commercially synthesized.

Table 3-1 Codon Bias of Pichia Pastoris. The codons for the relevant amino acids corresponding to the protein PG-1 were counted in the pim1 gene and the most frequently occuring codons have been highlighted in yellow.

Amino acid	Codon	Number of Occurences
Phe	TTT	14
	TTC	8
Leu	CTT	9
	CTC	4
	CTA	4
	CTG	7
	TTA	9
	TTG	13
Val	GTT	8
	GTC	5
	GTA	2
	GTG	9
Tyr	TAT	15
	TAC	4
Cys	TGT	4
	TGC	3
Arg	CGT	1
	CGC	2
	AGA	11
	AGG	2
	CGA	5
	CGG	2
Gly	GGT	8
	GGC	4
	GGA	7
	GGG	2

The overlap was intentionally designed to be a GC rich region that allowed for added stability of the two nucleotides during their elongation. The actual DNA sequences corresponding to the highlighted regions in Figure 3.3 were ordered from Sigma Genosys (The Woodlands, TX).

3.2 Generation of the Archival Plasmid

The two custom designed oligonucleotides were mixed in equimolar amounts along with 2 mM dNTPs and H_2O, and elongated using the Klenow fragment of DNA polymerase I (New England BioLabs, Beverly, MA). The elongation reaction mix was incubated at room temperature for approximately one hour. This resulted in the complete blunt ended double stranded sequence for the synthetic PG-1 gene, *spg1*. Next, the plasmid pBluescript (Stratagene, La Jolla, CA) was treated with 20 units of the restriction enzyme *Eco*RV (New England BioLabs, Beverly, MA) and incubated at 37^{o} Celcius for one hour to produce a blunt ended cloning site in the multiple cloning site (MCS) of the plasmid. This digestion left the 5' ends of the restriction site in the vector phosphorylated so it was unnecessary to phosphorylate the synthetic oligonucleotides.

The enzyme T4 DNA ligase (New England BioLabs, Beverly, MA) was used to ligate the insert into the blunt *Eco*RV cloning site in pBluescript. The ligation reaction was conducted following a ligation protocol adapted from *Laboratory DNA Science* (Bloom et al. 1996), which can be found in Appendix A. The previously made elongation reaction was mixed with the *Eco*RV-digested pBluescript and 10X buffer for T4 ligase. Four hundred units of the enzyme were added and the reaction mix was incubated at room temperature for three hours.

The recombinant plasmid was transformed into TOPO Top 10F' chemically competent *E. coli* cells obtained from Invitrogen (Carlsbad, CA), the protocol for which is in Appendix A.

The cells were plated on LB agar plates with ampicillin (0.1 mg/ml), IPTG (0.5 mM), and X-gal (4 ug/ml). The plasmid, pBluescript, contains the alpha fragment of the lac Z gene as well as a gene that confers resistance to the antibiotic ampicillin. These features allowed for easy detection of plasmid uptake by the host cells and selection of recombinants. The bacterial transformants containing the recombinant construct grew as white colonies. Although the insert is only fifty-seven bases it still succeeded in disrupting the alpha fragment of the lac Z gene, due to an in frame stop codon at the 3' end. In this instance, the stop codon was very helpful because inserts smaller than approximately 200 bases usually fail to completely disrupt the function of the alpha fragment, making recombinant selection difficult. Liquid cultures of randomly selected white colonies containing 5 ml L broth (5 g Bacto Tryptone, 2.5 g Yeast Extract, 0.25 g NaCl for 500 ml) and 5 µl ampillicin (100 mg/ml) were prepared and incubated overnight at 37^{o} Celcius. The plasmids were isolated from the cells using standard plasmid minipreparation techniques, the protocol for which can be found in Appendix A. Samples of the isolated plasmids were run on a 1% agarose gel.

3.2.1 Confirmation of *spg1* Insert

Once isolated, the plasmids were analyzed for the presence of an insert into the MCS. This was done by performing a polymerase chain reaction (PCR) across the MCS using 50 pmol each of the M13 Forward and M13 Reverse primers from Sigma Genosys (The Woodlands, TX), which flank the multiple cloning site of pBlueScript. Two and one-half units of Taq DNA Polymerase (Promega, Madison, WI) were used to catalyze the reaction. See Appendix A for the complete PCR protocol. Samples of the PCR reaction products were run on a 1.5% gel and visualized by ethidium bromide staining, see Figure 4.2.

3.2.2 Sequencing of Insert

Once the recombinant plasmid was confirmed to contain an insert, it was sequenced to ensure that it was still intact and contained the correct sequence. This was done using ABI sequencing technology. See Appendix A for exact protocol.

3.3 Extension of *spg1* Insert

3.3.1 Design of Longprimer and Shortprimer

Once the presence of the insert was successfully confirmed, the next step in the process was to perform PCR on the archival plasmid using specially designed primers. Primers were designed to add restriction sites and secretion signals to the ends of the amplicon and can be seen in Figure 3.4. They were ordered from Sigma Genosys (The Woodlands, TX). The "longprimer" would add an XhoI restriction site and a Kex 2 secretion signal to the 5' end of the amplification product. The "shortprimer" would add an EcoRI site to the 3' end of the amplicon. PCR was performed using 50pmol of each primer in addition to 2.5 units of Taq DNA polymerase (Promega, Madison, WI).

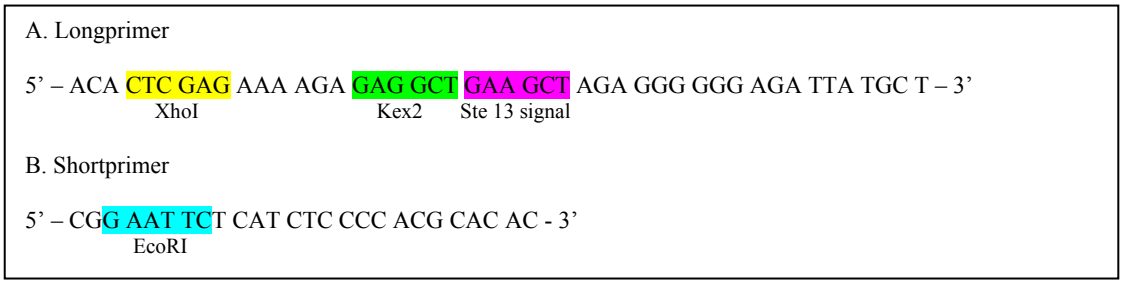

Figure 3-4 Extension Primers. Part A shows the sequence of the longprimer designed to extend the 5' end of the coding region of spg1 and add an XhoI restriction site and a Kex2 secretion signal (yellow and green highlighted regions, respectively). Part B shows the sequence of the shortprimer designed to extend the 3' end of spg1 and add an EcoRI restriction site (blue highlighted region).

3.3.2 PCR Reactions with Extension Primers

A PCR reaction was performed on the archival plasmid, pAM5 using 50 pmol each of the extension primers and 2.5 units of Taq polymerase. A dummy reaction was also set up using the same reagents except substituting water for the polymerase. The reaction products were then run on a 1.5% gel. The full-length extension product that would have resulted from a successful PCR with longprimer and shortprimer can be seen in Figure 3.5.

5' – ACACTCGAGAAAAGAGAGGCTGAAGCT AGAGGGGGGAGATT ATGCTAT TGCCGGCGGCGGTT TTGCGTGTGCGTGGGGAGATGA – 3'
3' – TGTGAGCTC TT TTCTC TCCGAC TTC GATC TCCC CCC TCT AATACGATAACGGCCGCCGCCAAAACGCACACGCACCCCTCTAC T – 5'

Figure 3-5 Full length Extension Product. This is the sequence of the extended spg1 gene containing the appropriate restriction sites and secretion signal.

3.4 PCR with Combination Primers

To examine the function of the longprimer and shortprimer individually, each was combined with one of the M13 primers. The longprimer was mixed with the M13 Reverse and the shortprimer with M13 Forward. pAM5 and 50 pmol each of longprimer and M13 Reverse primer were mixed in a PCR reaction with 2.5 units of Taq polymerase (Promega Madison, WI). The same was done with the shortprimer and the M13 Forward primer. The following PCR reactions were also performed: pAM5, 50 pmol each of longprimer and short primer, and 2.5 units of Taq polymerase; pAM5, 50 pmol each of M13 Forward and M13 Reverse primers, and 2.5 units of Taq polymerase; pBluescript, 50 pmol each of M13 Forward and M13 Reverse primers, and 2.5 units of Taq polymerase. Samples from each of the PCR reaction products were run on a 1.5% agarose gel and can be seen in Figure 4.5.

3.5 Attempted Construction of the *P. pastoris* Expression Vector

Had a PCR product been obtained from the reaction with the long and short primers the next step would be to put this product into the pPIC9 expression vector. Hypothetically the longprimer/shortprimer PCR product and the pPIC9 vector would have been separately double digested with the appropriate restriction enzymes and then mixed and joined together with T4 ligase. The result of this would have been the directional cloning of the insert into the multiple cloning site.

3.6 Construction of pRSET Expression Vector

Two custom designed oligonucleotides utilizing the naturally occurring PG-1 gene sequence were mixed in equimolar amounts in an annealing solution of 100 mM Tris and 150 mM NaCl at pH 7.4 and allowed to incubate at room temperature for ten minutes. This resulted in the complete double stranded sequence for the synthetic PG-1 gene with the necessary restriction overhangs. Next, the plasmid pRSET A (Invitrogen, Carlsbad, CA) was treated with 20 units each of the restriction enzymes *Eco*RI and *Xho*I (New England BioLabs, Beverly, MA) and incubated at 37^{o} Celcius for one hour. This digestion left the 5' ends of the restriction site in the vector phosphorylated so it was unnecessary to phosphorylate the synthetic oligonucleotides. The sequence of the two synthetic genes is as follows:

5'-TCGAGAGGGGAGGTCGCCTGTGCTATTGTAGGCGTAGGTTCTGCGTCTGTGT
CGGACGAGGATGAG-3'

5'-AATTCTCATCCTCGTCCGACACAGACGCAGAACCTACGCCTACAATAGCACA
GGCGACCTCCCCTC-3'

The enzyme T4 DNA ligase (New England BioLabs, Beverly, MA) was used to ligate the insert into the doubly digested pRSET A vector. The ligation reaction was conducted following

a ligation protocol adapted from *Laboratory DNA Science* (Bloom et al. 1996), which can be found in Appendix A. The previously made annealing reaction was mixed with the double digested pRSET A and 10X buffer for T4 ligase. Four hundred units of the enzyme were added and the reaction mix was incubated at room temperature for three hours.

The recombinant plasmid was transformed into TOPO Top 10F' chemically competent *E. coli* cells obtained from Invitrogen (Carlsbad, CA), the protocol for which is in Appendix A. The cells were plated on LB agar plates with ampicillin (0.1 mg/mL) and allowed to incubate overnight at 37 degrees Celcius.

3.6.1 Confirmation of Insert

After isolation, the plasmids were analyzed for the presence of an insert into the MCS. This was done by performing a polymerase chain reaction (PCR) across the MCS using 50 pmol each of the T7 Promoter and T7 Reverse primers from Sigma Genosys (The Woodlands, TX), which flank the multiple cloning site of pRSET A. Two and one half units of Taq DNA Polymerase (Promega Madison, WI) were used to catalyze the reaction. See Appendix A for the complete PCR protocol.

3.6.2 Sequencing of pRSET A Insert

Once the recombinant plasmid was confirmed to contain an insert, it was sequenced to ensure that it was still intact and contained the correct sequence. The sample was sent to University of Massachusetts Medical School to be sequenced.

3.7 Expression and Purification

Plasmids with the correct insert were isolated and then transformed as described above into codon competent BL21:DE3. Four different plasmids were isolated and transformed to

minimize the chance of having an unexpressive plasmid. A single colony was taken and inoculated into a 5 ml culture containing 50 ug/ml ampicillin and 35 ug/ml chloramphenicol and grown overnight at 37 degrees Celcius. These initial cultures were then added to either 25 ml or 100 ml of LB broth with antibiotics and grown to an OD_{600} between 0.4 and 0.6 at which point IPTG was added to a final concentration of 1 mM to induce expression. The cultures were incubated for one hour post induction to allow adequate time for expression. Harvesting began by centrifuging the cultures at 10,000 x g for fifteen minutes. The pellet was then resuspended in 50 mM sodium phosphate buffer at pH 7.0 with 300 mM NaCl, 2 ml buffer for every 25 ml of culture, and sonicated with four one-minute passes at power setting four on a Sonifer Cell Disruptor (model W185) sonicator. Afterwards the suspension was centrifuged at setting six in an IEC clinical centrifuge to pellet insoluble material. The insoluble material was washed twice with a five percent solution of Triton X-100. Further centrifugation pelleted any remaining insoluble material which was then resuspended in 50mM sodium phosphate buffer at pH 7.0 with 300 mM NaCl and 6M guanidine HCl. The supernatant from the initial cell harvest and the resolubilized material were subjected to batch flow chromatography. An aliquot of Clontech Talon Immobilized Metal Affinity Chromatography (IMAC) resin was equilibrated in the appropriate buffer and then 50 µl of resuspended resin was added to each tube and incubated for one hour. Afterwards the tubes were centrifuged on setting two on an IEC centrifuge to pellet the resin and then washed twice with the sodium phosphate buffer. The supernatant was removed and SDS-PAGE sample buffer was added to the resin to elute any bound protein. These samples were then run out on a SDS-PAGE gel.

After confirmation of protein expression in fresh cultures by anti-his tag Western blot an alternative purification scheme was adopted. All new samples believed to have protein were

pooled and sonicated. Insoluble material was spun down and resuspended in 50 mM sodium phosphate buffer at pH 7.0 with 300 mM NaCl and 6M guanidine HCl. No detergent wash was performed. The soluble and insoluble fractions were each put through a mixed batch/gravity-feed chromatography protocol with 0.5 ml Clontech Talon IMAC resin and eluted with 10 mM sodium phosphate buffer with 300 mM NaCl at pH 5.0. Batch chromatography was performed initially with one hour incubation and then this resin was loaded onto a mini column for elution. The resulting fractions were adjusted back to pH 7.0 with sodium hydroxide then assayed for antimicrobial activity via radial diffusion assay.

3.8 SDS-PAGE gel analysis

Once isolated the above samples were run on a non-reducing SDS-PAGE gradient gel to look for evidence of expression. The resolving gel was cast with a 10 – 20% gradient of acrylamide with methylene bis-acrylamide and the stacking gel was five percent. The acrylamide used was 40%T:5%C. 20 µl of each sample was loaded onto the gel, which was run at 50V for two hours. Staining was performed with Coomassie blue for at least eight hours. Destaining was done with repeated incubations in a solution of ten percent glacial acetic acid and ten percent methanol.

3.9 RNA Isolation and Analysis

RNA analysis and RT-PCR were used to attempt to determine if the protein was being expressed at minimal levels. Total cellular RNA was isolated using Invitrogen's Trizol reagent. This product is a mixture of phenol and guanidine thiocyanate for cell lysis and RNA extraction. The manufacturer's protocol was followed without variation and will be described briefly here. A 5ml aliquot of cells was centrifuged at 10,000 x g and the pellet resuspended in 1 ml of Trizol

reagent. After a brief incubation 0.2 ml of chloroform was added and the samples vigorously shaken then centrifuged to enhance phase separation. The aqueous phase was then removed and mixed with isopropanol, incubated, and centrifuged to precipitate the RNA. Seventy-five percent ethanol was used to wash the pellet once, after which it was resuspended in DEPC-treated RNAse free water.

Ambion's RETROScript kit was used to perform RT-PCR on the RNA isolates. The RT-PCR was performed according to the manufacturer's protocol without modification. Reverse transcription (RT) primer one was internal to the PG-1 gene: 5'-CGCCTACAATAGCACAGGC-3'. RT primer 2 was positioned at the end of the putative mRNA: 5'-CGGTGGCAGCAGCCAACTCAG-3'. The PCR primer sequence was: 5'-GACCACAACGGTTTCCCTCT -3'.

RNA samples were found to have contaminating DNA. This was discovered by performing PCR following the recipe in Section 3.2.1 with primers that amplified across an unexpressed region of pRSET A. The primer sequences are as follows: 5'-CGCTCAAGTCAGAGGTGGCG-3', 5'-CGTGCACACAGCCCAGC-3'

Removal of the contaminating DNA was attempted using acid phenol extraction followed by the DNA-*free* DNase kit from Ambion. The procedure for the DNA-*free* kit was performed according the manufacturers protocol without variation. DNA removal did not appear to be successful and will be discussed in Sections 4.7 and 5.5.

3.10 Immunoblotting

After running the culture samples on an SDS-PAGE gel the proteins were then transferred to PVDF membrane using a Transphor semi-dry transfer unit. The membrane was rinsed in 50 mM Tris, 0.2 M NaCl buffer (Buffer 229) at pH 7.4 for ten minutes. Blocking was done for

twenty minutes using the same buffer as above with five percent non-fat dry milk added. One-fifth vols of a 1:100 dilution, in blocking buffer, of the primary anti-his antibody (Santa Cruz Biotech, Cat No. sc-803) was added to the blocking solution and allowed to incubate for two hours at room temperature. The membrane was then rinsed with Buffer 229 w/0.05% Tween 20, washed for five minutes, and then again for twenty minutes. A second blocking was performed for twenty minutes in Buffer 229 w/ .05% Tween and 5% milk after which 10 µl of secondary antibody (anti-rabbit HRP conjugate: Cell Signaling Tech) was added to the solution and incubated for one hour at room temperature. After this incubation the rinse and wash mentioned above were repeated. The membrane was then soaked with the chemiluminescent substrate for one minute and then exposed to X-ray film.

3.11 Radial Diffusion Assay

The radial diffusion assay was used to assay the biological activity of antimicrobial samples. Ten milliliters of 10 mM sodium phosphate buffer at pH 7.4 was mixed with 0.1 g agarose and 0.3 mg of tryptic soy broth powder. This mixture was heated and allowed to cool to ~ 43 degrees Celcius at which time 200 µl from a log phase *B. subtilis* culture was added to the gel. The mixture was poured onto a petri plate and allowed to solidify. Wells were then punched into the agar and 10 µl of a 2.5 mg/ml solution of PG-1 and culture samples were put into their appropriate wells. The plate was then allowed to incubate for three hours after which an overlay gel consisting of 10 ml 10 mM pH 7.4 phosphate buffer, 0.1 g agarose, and 0.6 mg tryptic soy broth powder was cast. As described above, it was heated and allowed to cool then poured over the underlay gel.

4 Results

4.1 Isolation of pAM5

The archival plasmid was named pAM5. Agarose gel electrophoresis of the uncut plasmid minipreparations confirmed the presence of a plasmid as seen in Figure 4.1.

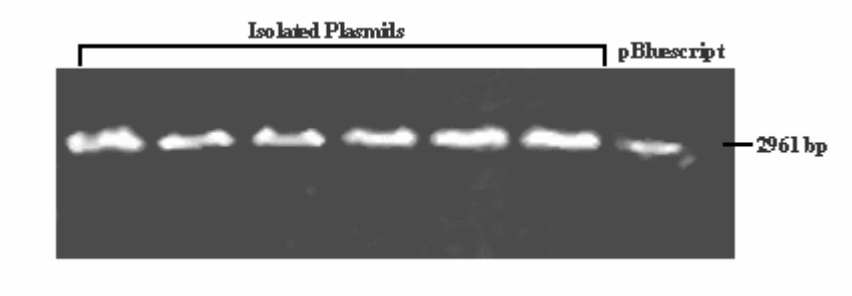

Figure 4-1 Plasmid minipreparation gel. Lane 7 (from left) is the control lane containing pure uncut pBluescript. Lanes 1-6 (from left) contain samples of plasmid isolated from various colonies of transformed cells. The gel clearly shows these plasmids are running equally with the pBluescript control.

4.2 Confirming the Presence of the Insert and Proper Sequence

Polymerase chain reaction (PCR) was performed on the hybrid plasmid for confirmation of an insert. There was a detectable size difference between the amplification products of the miniprep samples and the control, unaltered pBlueScript (see Figure 4.2). The insert was then sequenced using ABI sequencing technology (see Appendix A for procedure). This technique was also used to screen for recombinant plasmids when constructing the pRSET A expression vector (data not shown).

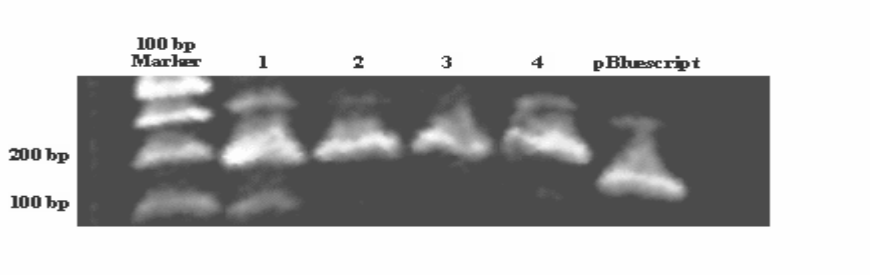

Figure 4-2 PCR Across the MCS. The above gel photo shows a PCR of the multiple cloning site (MCS) of recombinant pBluescript plasmids (lanes 1-4) and unaltered pBluescript (lane 6 from left) using M13 forward and reverse primers. Lane 1 is a linear size marker. This gel indicates that the recombinant plasmids contain an insert (~200bp) because of the noticeable size difference between them and unaltered pBluescript (~150bp).

After the plasmid containing the correct sequence of the insert had been established, a freezer stock was prepared of the cells from which the plasmid was isolated. Figure 4.3 displays the sequence data.

EcoRV
M13 R: 5'- TTCGAT TCA TCT CCC CAC GCA CAC GCA AAA CCG CCG CCG GCA ATA GCA TAA TCT CCC CCC TCT ATCAAG –3'
EcoRV

EcoRV
M13 F: 5'- CTTGAT AGAGGG GGG AGA TTA TGC TAT TGC CGG CGG CGG TTT TGC GTG TGC GTG GGG AGA TGA ATCGAA –3'
EcoRV

Figure 4-3 Sequencing Data. The yellow highlighted regions designate the EcoRV restriction site. The data indicates that the plasmid sequenced contained the complete and correct sequence of the spg1 gene. The M13 reverse primer gives the sequence of the bottom strand while the forward primer gives the sequence of the top strand.

4.3 Results of PCR with Long and Short Primers

PCR with the long and short primers was unsuccessful. Although a strong band was visible in the LS lane, this was the only band seen. This made it impossible to determine if the bands represented an amplification product or left over primer. Given that this was the only band seen in this lane besides the vector band (not shown), and that it ran equidistant to the primer band in the dummy lane, the band was attributed to the long and short primers included in the reaction mix. Figure 4.4 shows an example of a gel from one of the PCR runs.

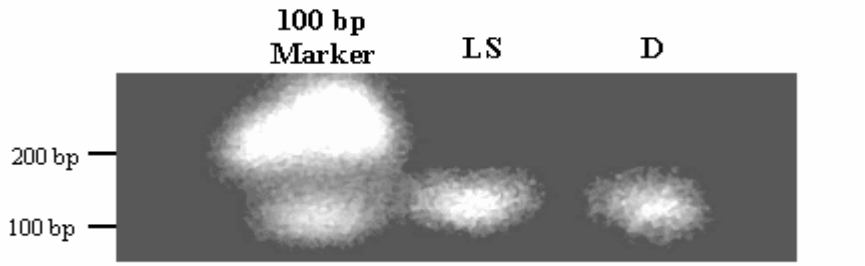

Figure 4-4 PCR using longprimer and shortprimer. Lane 2 (LS) shows the PCR reaction of the archival plasmid using longprimer and shortprimer. Lane 3 (D) shows the dummy PCR containing the same reagents as LS except for the substitution of the enzyme with water. No amplification product is seen in the LS lane; the band seen is a result of the primers used. There is no difference seen between the LS and the D lanes. The PCR product, if had been seen, would run at ~80bp.

4.4 Mixing of M13 Primers with Long and Short Primers

After initial difficulties with the long and short primers, another PCR was performed using those primers in combination with the M13 Forward and Reverse Primers. The gel in Figure 4.5 shows that the long and short primers do work individually to produce a product but for some reason fail to function when mixed together, as seen in the LS lane, reinforcing the results shown in Figure 4.4. Based on the results of the PCR described in Section 3.4 and seen in Figure 4.5 Lane 5 (FR), it was hypothesized that one or both of the M13 primers was hybridizing with the insert contained in the archival plasmid, pAM5. The sequences of the M13 Forward and Reverse primers and the *spg1* insert were analyzed for possible hybridization sites for the primers. The pairing between at least three bases on the 3' end of either primer with part of the insert was the basic criterion used in this analysis. It was found that the M13 Reverse primer could possibly base pair to one strand of insert in this fashion as shown in Figure 4.6.

To test the hypothesis that the M13 Reverse primer can hybridize to the *spg1* insert, a PCR reaction was set up that contained the archival plasmid pAM5, 100 pmol of the M13 Reverse primer, and 2.5 units of Taq polymerase. The results of this reaction can be seen in Section 4.5 in Figure 4.6.

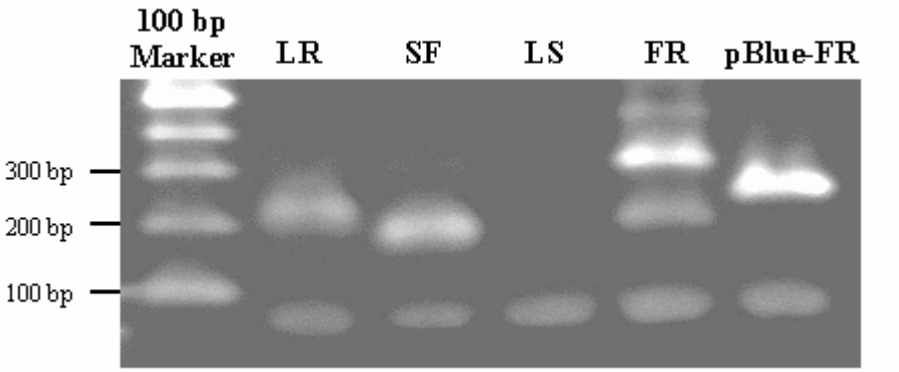

Figure 4-5 PCR with Mixed Primers. In order to test the function of the designed extension primers, longprimer and shortprimer, each were combined with another primer that flanks the MCS of the plasmid. LR indicates the reaction containing pAM5 and the longprimer and M13 Reverse primer. SF indicates the reaction containing pAM5 and the shortprimer and M13 Forward primer. LS indicates the reaction containing pAM5 and the longprimer and shortprimer. FR indicates the reaction containing pAM5 and the M13 Forward and Reverse primers. pBlue-FR indicates the reaction containing unaltered pBluescript and the M13 Forward and Reverse primers. Amplification products are seen in the LR and SF lanes, indicating that the primers are functional when used with other primers, but not when combined together, as indicated by the LS lane where no amplification product is seen. Multiple amplification products are seen in the FR lane.

4.5 Complications with the M13 Reverse Primer

From the reactions described in Section 3.4 and 3.5, the results seen in Figure 4.6 were obtained. Multiple bands were seen in the R lane of the gel indicating unexpected hybridization of the M13 Reverse primer to the insert region. The lower diagram in Figure 4.6 shows points of hybridization between the M13 Reverse primer and the *spg1* gene.

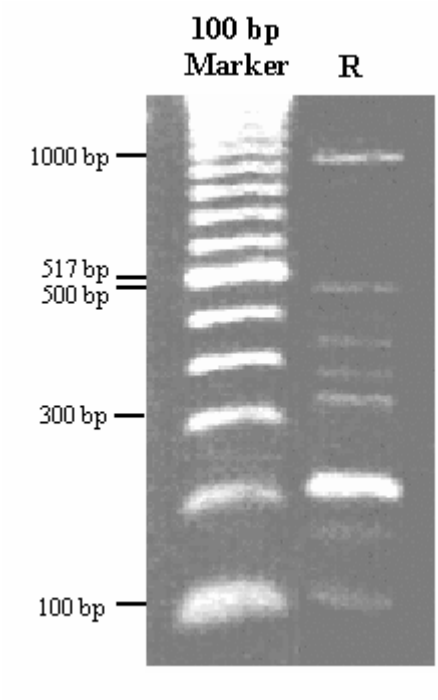

Figure 4-6 PCR using only M13 Reverse Primer. R indicates the reaction containing pAM5 and the M13 Reverse primer. Multiple bands are seen in the R lane suggesting that this primer has more than one priming site on the plasmid, including one in the insert region. This diagram below this caption shows the sequences of the M13 Reverse primer and the bottom strand of the spg1 coding region and the possible base pairing that could occur between them as indicated by the vertical lines.

M13 Reverse 5' –AGG AAA CAG CTA TGA CCA T G– 3'

spg1 Bottom strand 3' –TC T CCC CCC TCT AAT A C G ATA ACG GCC GCC GCC AAA ACG CAC ACG CAC CCC TCT- 5' ACT– 5'

4.6 SDS-PAGE Analysis

SDS-PAGE analysis of cultures prior to any processing revealed no evidence of expression. Figure 4.7 shows whole cell lysate from a 100 ml culture. Close examination reveals that all lanes are identical regardless of whether they were induced or not. If significant expression had occurred a prominent band should have been visible within the same size range as the Cytochrome C standard. Figure 4.8 shows the results of whole cell lysate gel analysis from a separate 25 ml culture grown following the standard protocol. Again, no significant difference can be seen between the lanes. Through the various purification procedures, SDS-PAGE analysis was never able to visualize any expression of the protein.

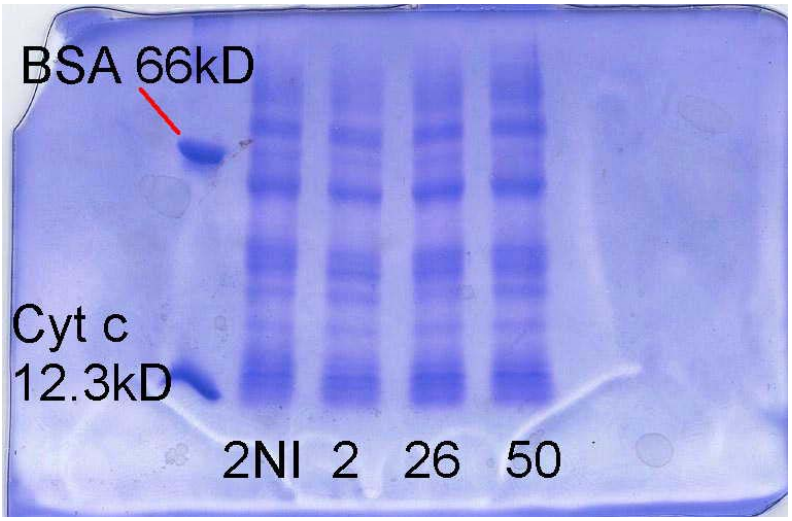

Figure 4-7 Gel showing the whole cell lysates. 2-NI is an uninduced PG-1 clone while 2, 26, and 50 were induced with 1mM IPTG. Expression of the target protein would have produced a prominent band with a MW of ~5.5kD running slightly below the Cytochrome C standard.

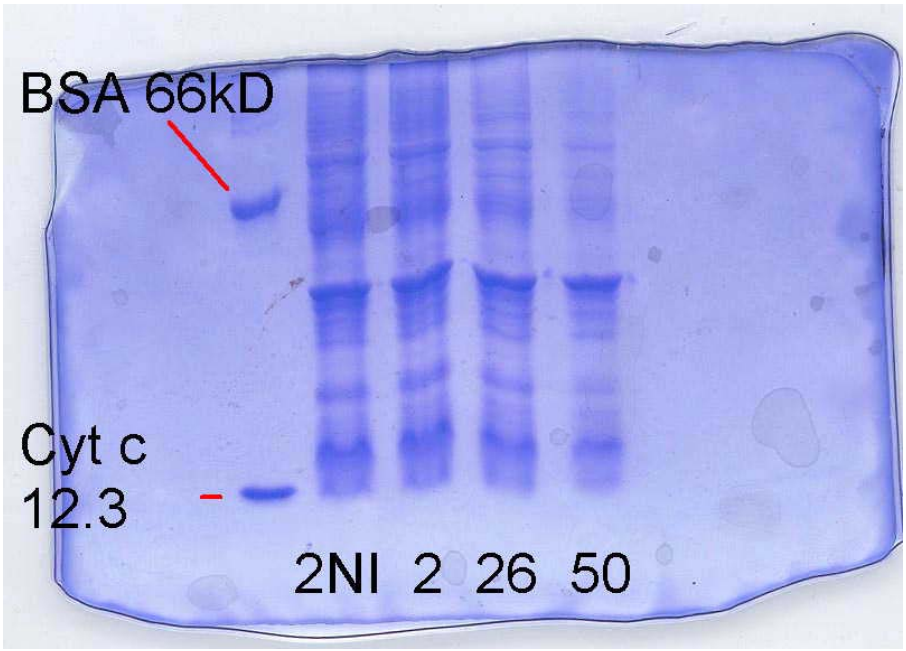

Figure 4-8 A second gel showing results from whole cell lysates of a 25ml culture. No significant difference can be seen between the uninduced (2NI) and induced lanes (2, 26, 50).

4.7 Analysis of RT-PCR

Use of RT-PCR ran into an unexpected problem in the form of contaminating DNA from an unexpected source. Although initial attempts showed bands in all lanes with the appropriate sizes, Figure 4.9, later experiments showed the presence of contaminating DNA. After this initial RT-PCR, work was done to remove any contaminating DNA.

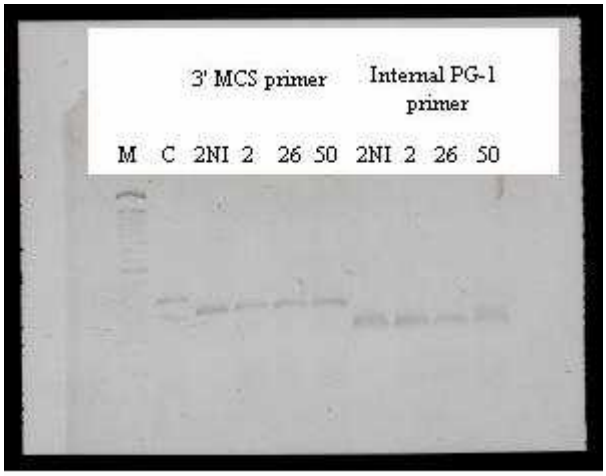

Figure 4-9 Initial attempt at RT-PCR. Overall the bands are where they should be. The 3'MCS product is slightly larger than the internal PG-1 product. This is because the latter use a primer internal to the PG-1 gene that is upstream from the terminal 3'MCS primer. There is also a signal of roughly equal intensity in the uninduced lanes. The 3'MCS product is running at ~200bp while the Internal PG-1 product is at ~150bp.

After two acid phenol extractions and three DNase treatments the contaminating signal was still present after PCR. Eventually it was found that the contamination was in the Taq polymerase stocks themselves. These are commercially available enzymes purchased from outside distributors. The significance of this will be explained in the discussion section. Figure 4.10 shows the results of PCR done on several commercial enzymes using primers across the unexpressed ORI region of the plasmid. Some commercial enzymes were found to be contaminated with bacterial DNA.

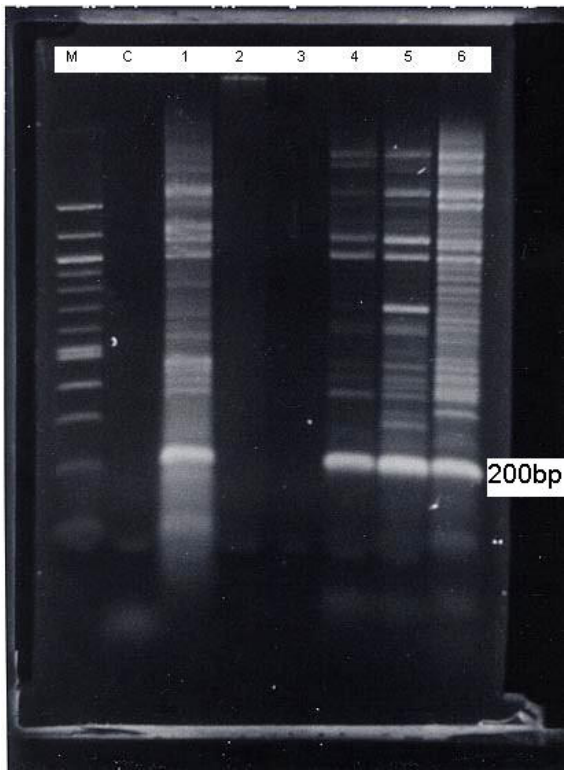

Figure 4-10 This figure shows a PCR reaction using the DNA primers described in section 3.10. None of the lanes have any intentionally added template. Lane C is a control which also lacks any form of polymerase. Lanes 1-6 are various commercially available DNA polymerases. While there are other visible bands it is clear that one signal predominates at 200bp. Lanes 2 and 3 show no PCR products of any kind.

4.8 Immunoblot Analysis

After gel analysis and RT-PCR proved unsuccessful, immunoblotting using anti-his antibody was used to look for evidence of expression. The procedure was performed as outlined in methods. Figure 4.11 shows a blot using whole cell lysates from a 25 ml culture series. The bottom-most row of bands on this gel clearly shows a difference in intensity between the uninduced lane 3, and lanes 4, 5, and 6 that were induced. The bands of interest are running in the expected size range of approximately 5.5kD.

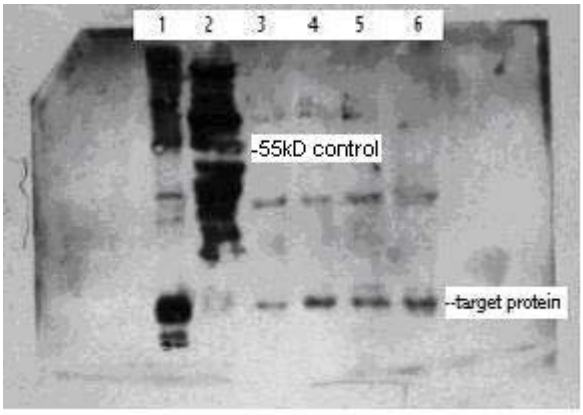

Figure 4-11 Immunoblot for His-Tagged Proteins on 25 ml cultures. Lane 1 is a his-tagged 88kD control protein. Lane 2 is whole cell lysate from a culture which is known to express a his tagged protein at ~55kD. Lane 3 is an uninduced PG-1 clone. Lanes 4-6 are induced PG-1 clones. Comparing lanes 4-6 to lane 3 in the size range of interest (~5.5kD) clearly shows that the intensity of the induced lanes is greater than that of the uninduced.

Figure 4.12 also shows a similar result to Figure 4.11. The layout of the lanes is identical.

Figure 4.12 resulted from an immunoblot performed on whole cell extracts from a 100 ml culture.

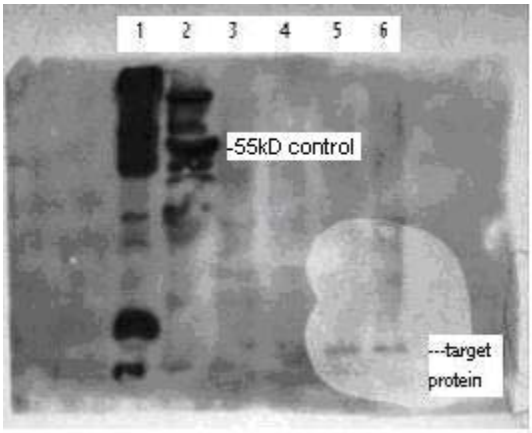

Figure 4-12 Immunoblot for His-Tagged Proteins on 100 ml cultures. Lane 1 is a his-tagged 88kD control protein. Lane 2 is whole cell lysate from a culture which is known to express a his-tagged protein at ~55kD. Lane 3 is an uninduced PG-1 clone. Lanes 4-6 are induced PG-1 clones. Comparing lanes 4-6 to lane 3 in the size range of interest (<10kD) again shows bands in he induced lanes (especially 5 & 6) and almost nothing in the uninduced lane.

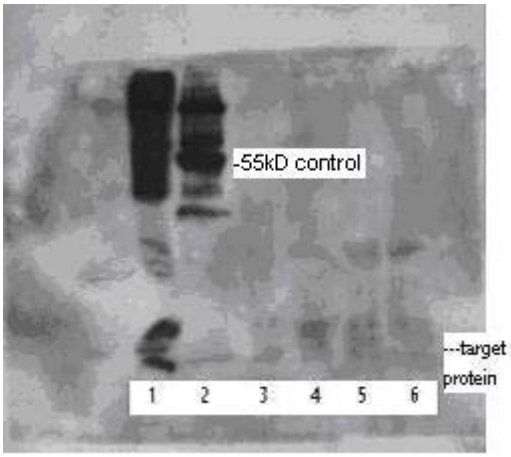

Figure 4-13 Immunoblot for His-Tagged Proteins on other 25 ml cultures. Lane 1 is a his-tagged 88kD control protein. Lane 2 is whole cell lysate from a culture which is known to express a his-tagged protein at ~55 kD. Lane 3 is an uninduced PG-1 clone. Lanes 4-6 are induced PG-1 clones. Comparing lanes 4-6 to lane 3 in the size range of interest (<10kD) shows bands present in the induced cultures that are not present in the uninduced lane. While the multiple banding seen here is somewhat undesirable it still supports the hypothesis that there is a protein in the induced cultures that is present in greater quantities than in the uninduced lane.

Figure 4.13 is a blot from a second 25 ml culture set up identically to the previous two. Overall the three blots show solid evidence that induction is occurring and that a his-tagged protein of approximately the correct size, 5.5 kD, is being produced.

4.9 Radial Diffusion Assays

Radial diffusion assays were performed on lysates from 25ml cultures in order to look for biological activity. Figure 4.14 shows a radial diffusion assay performed as outlined in Methods except that culture wells were loaded with 10 µl of lysate four times in order to increase the protein concentration. Clear areas radiating out from a well indicate antimicrobial activity as with the PG-1 positive control obtained from Dr. Robert Lehrer of UCLA. Whole cell lysates from PG-1 clones showed no antimicrobial activity even with sample stacking.

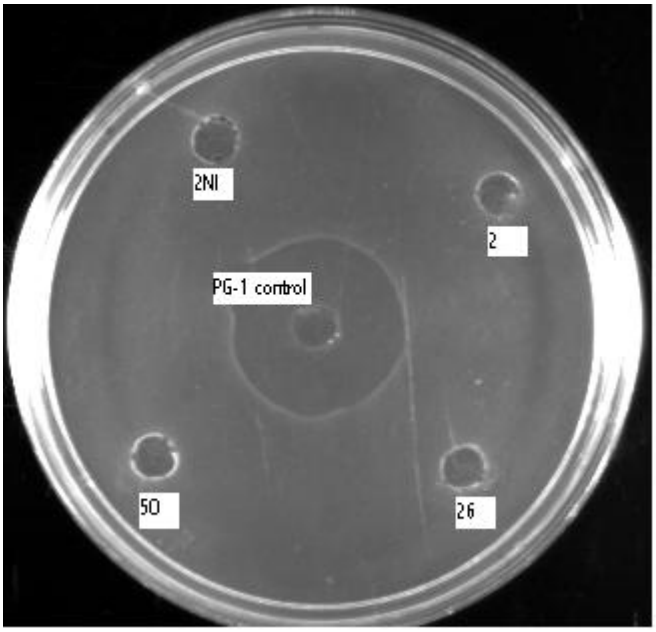

Figure 4-14 Radial diffusion assay. The central well is loaded with a pure PG-1 sample at 2.5 mg/ml. Well 2NI contains lysate from an uninduced PG-1 clone. Wells 2, 26, and 50 contain lysate from induced PG-1 clones. Each culture well received a total of 40 µl of lysate.

Figure 4.15 shows a radial diffusion assay using a serial dilution of pure PG-1 stock. The well at the twelve o'clock position contains PG-1 at a concentration of 2.5 mg/ml. The concentration decreases by a factor of ten with each well going clockwise. The plate shows that the assays lower limit for PG-1 detection is about 2.5 µg/ml (well number 4).

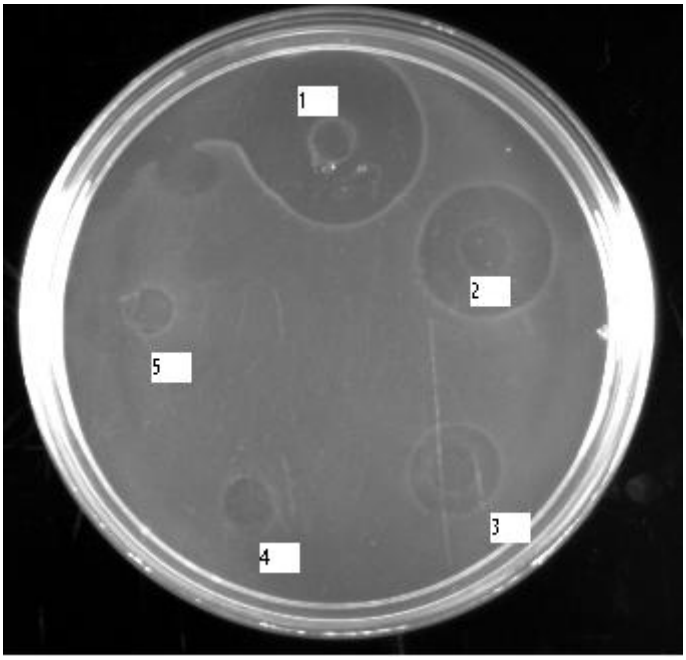

Figure 4-15 Radial diffusion assay with serial dilutions of control PG-1. Well 1 = PG-1 at 2.5 mg/ml. Well 2 = 0.25 mg/ml. Well 3 = 0.025 mg/ml. Well 4 = 2.5 µg/ml. Well 5 = 0.25 µg/ml. The dilution series shows that the lower limit of the assays sensitivity seems to be about 2.5 µg/ml. Below this concentration the assay may not be able to detect the presence of PG-1.

Radial diffusion assays using PG-1 purified by IMAC from culture extracts did show signs of antimicrobial activity (Figs. 4.16 and 4.17). As above, each well was loaded four times with 10 µl of of sample to increase the protein concentration. The diameter of the clear zone seems to indicate that the protein concentration in the well was slightly above 2.5 µg/ml. See Figures 4.16 and 4.17.

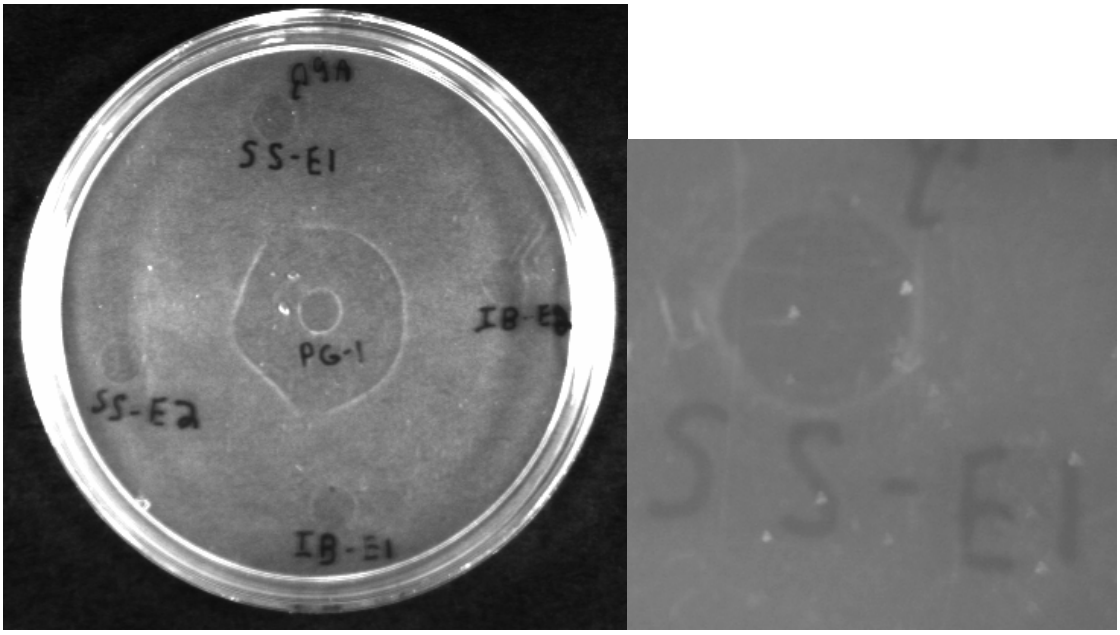

Figure 4-16 Radial diffusion assay with culture lysates purified by IMAC. SS-E1, SS-E2, IB-E1, and IB-E2 are elution fractions. The left hand picture shows the entire plate while the right hand picture is a close up of an elution fraction SS-E1 which produced a visible clear zone. The central well is loaded with pure PG-1 control at a concentration of 2.5 mg/ml.

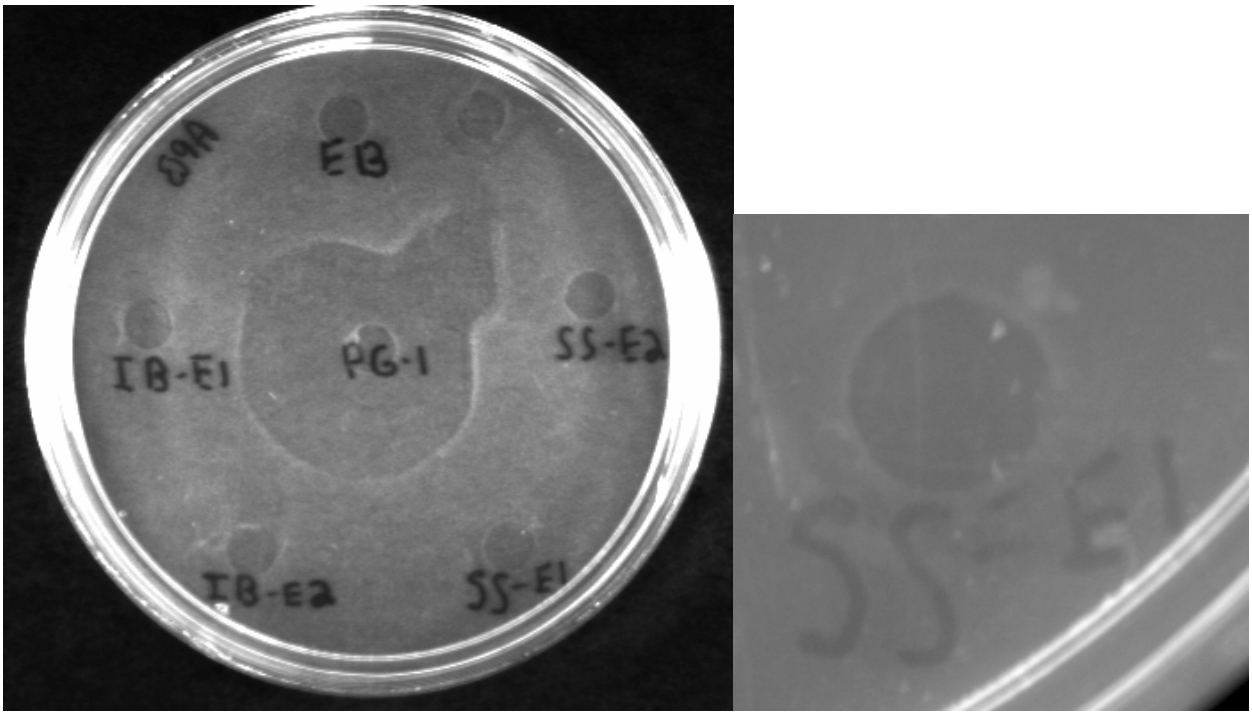

Figure 4-17 A second radial diffusion assay laid out similarly to Figure 4.16 Notice the elution buffer (see Section 3.7) well (EB) which shows no signs of a clear zone. This gives further indication that PG-1 in the lysates is responsible for the clear zones.

5 Discussion

5.1 Plasmid Minipreparation Analysis

Plasmid minipreparations were run out on 1% agarose gel against a control lane with unaltered pBluescript. Figure 4.1 clearly shows that the isolated plasmids are running equidistant with the control lane. Our insert of only fifty-seven bases would not produce a noticeable difference in the migration of the entire plasmid, so this was not a factor to consider when confirming the presence of the plasmid. Thus, we would expect the isolated recombinant plasmids to migrate at the same rate as pBluescript. This also means that this gel is not useful for confirming the presence of the actual insert.

5.2 Analysis of the MCS of pAM5 and Confirmation of the Sequence

PCR across the MCS of pBluescript was used to confirm the presence of an insert. This method was used because fifty-seven bases would not produce an observable size difference or affect migration in a 2961 base pair plasmid. In a plasmid that does not contain an insert, PCR across the MCS using the M13 forward and reverse primers gives a PCR product of 227 bases. The presence of the insert increases the size of the fragment to 284 bases, which is a twenty five percent increase in the size of the fragment. An increase of this magnitude is easily detectable on a two- percent agarose gel as shown in Figure 4.2. The slower band in each lane represents a PCR fragment that contains the insert of interest. From this gel it was possible to determine the best candidate for sequencing.

Sequencing of the plasmid from which the strongest PCR signal was obtained, again confirming the presence of the insert and showed that its sequence was completely intact. This plasmid was named pAM5, and a freezer stock of these cells was made and stored.

5.3 Analysis of *P. pastoris* Expression Vector Construction Attempt

5.3.1 PCR Using Designed Extension Primers

Based on the results of various PCR reactions (see Figures 4.4 and 4.5), it could not be concluded that an amplification product was generated from the extension primers and the *spg1* insert. Even though when individually paired with an M13 counterpart (see Section 3.4) amplification was seen, the longprimer and shortprimer appeared to be defective when used together. Repeated PCRs using the longprimer and shortprimer failed to produce amplification products and, therefore, these particular primers should not be used in future attempts.

5.3.2 Ligation into Expression Vector

The ligation into the expression vector was unsuccessful because the vector we were given was not the vector outlined in Section 3.6 and in the Invitrogen catalogue. It actually had one *Eco*RI site between two *Xho*I sites. Double digestion of this vector produced a MCS, which had two XhoI compatible ends as opposed to one *Eco*RI end and one *Xho*I end. Extensive time was spent attempting to determine the cause of our problems with ligation that eventually lead to the discovery of the vectors true design.

5.3.3 Hybridization of M13 Reverse Primer

Another unforeseen problem that arose was that the M13 reverse primer was believed to be hybridizing with the insert in the archival plasmid, pAM5, in addition to its native priming site. In Figure 4.5, multiple amplification products are seen in the FR lane. This reaction mix

contained pAM5 and the M13 Forward and Reverse primers. The control for this reaction is shown in the pBlue-FR lane, which contains the same primers as the FR reaction but using unaltered pBluescript as the template instead of the archival plasmid. The only difference between the plasmids used in those reactions is the presence of the *spg1* insert. The multiple amplification products seen in the FR lane of Figure 4.6 therefore suggest that one or both of the primers may have hybridized with our insert, amplifying alternate regions of the plasmid. Analysis of the insert and primer sequences showed that the M13 Reverse primer could possibly do just that. This was confirmed by the PCR using only the M13 Reverse primer, shown in Figure 4.6. As a result of this, and possibly other hybridizations of this primer to the insert region, multiple amplification products are seen.

5.4 PG-1 Expression

While not expressed in abundant quantities, the immunoblots show that the his-tagged PG-1 protein is present. There are a variety of possible explanations why the expression was weak. The most likely one is the fact that PG-1 is a toxic protein. Although it is being produced as a his-tagged fusion protein this may not fully control its toxic effects. Depending on how the protein is folded it still may be able to insert itself across the membrane and exert some toxic effects. Radial diffusion assays were found to be functional down to 2.5 µg/ml of protein. Whole cell lysates showed no signs of PG-1 activity when assayed. IMAC was used to attempt to isolate his-tagged PG-1.

Radial diffusion assays performed on these samples show signs of antimicrobial activity. Based on the diameter of the clear zone it appears that the concentration of PG-1 in the well was approximately 2.5 µg/ml. The fact that the wells were loaded four times over means that the actual concentration of PG-1 in the elution fraction may have been roughly 0.625 µg/ml.

5.5 RT-PCR

One interesting discovery made during this thesis is that a fair number of commercially available Taq DNA polymerases are contaminated with plasmid DNA. Most likely this DNA is left over from the purification process. This was realized while attempting to use RT-PCR to search for plasmid expression. As a control for contaminating DNA in the RNA preparations it is necessary to have primers that amplify across an unexpressed region. The only available region for this on a plasmid is the origin of replication. After two separate acid phenol extractions and three DNase treatments contaminating DNA was still present. It was suggested the homemade Taq polymerase, cloned in our own lab, used may have plasmid contamination. A commercial Taq was used and the signal was still detected. Eventually PCR was performed with all components added except any form of a template. The questionable signal was again detected. This leads to the conclusion that at least some commercial Taq polymerases have plasmid contamination. Usually this is not an issue as the researcher is using primers specific to the gene of interest or the MCS of the plasmid. In both cases these are unique; however, there is no reason to believe that many plasmids might not have the same origin of replication. The ultimate question from all of this is where is the contaminating DNA signal coming from? Is it plasmid contamination in the RNA prep or the plasmid in the polymerase prep. The constant presence of this signal makes it difficult to state whether any signal detected during actual RT-PCR results from RNA or contaminating DNA. Treating the polymerase itself with DNase worked with controls that were spiked with a template. However this treated polymerase was unable to generate a signal when used with the controls in the Ambion RT-PCR kit.

6 Conclusions

Overall this appears to have been a successful thesis. The desired antimicrobial peptide was expressed and displayed antimicrobial activity. Although PG-1 was expressed by the bacteria it was in very small quantities. This could be due to any of the factors outlined above. The work done here represents the first successful cloning of the PG-1 peptide in a bacterial system and supports the feasibility of producing antimicrobial peptides by cloning. It also further supports the fusion protein approach while highlighting that the choice of fusion partner is an important consideration. Future work involving optimization of expression may be able to improve the yield of this system to the point where it may be detectable by simple SDS-PAGE analysis. Using a plasmid construct with the PG-1 gene repeated such that each successful transcription/translation event results in multiple PG-1 peptides linked together with cleavage sites between them may also be a viable option. As mentioned in the Background, tandem repeats were used to good effect in the expression of recombinant buforin II (Lee et al. 1997). A different fusion tag partner may also increase expression. It seems that the his-tag complex used here only did an average job of limiting PG-1's antimicrobial activity considering the non-cleaved fusion peptide was active. The F4 protein mentioned previously may be better suited to inclusion body formation and overall stability. Expression in alternative systems may also be explored if a cloning approach is still desired. A mammalian system would seem most favorable. Due to differences in mammalian membrane structure it is likely that many of the toxic effects of PG-1 would not be an issue. Engineering the protein so that it is exported out of the cell after production may also be possible with a mammalian system. This would greatly simply purification. Given the small size of the protein, synthetic manufacturing can and has been used to produce the peptide. Intrabiotic's protegrin analogue is synthetically manufactured

and is in Phase III FDA trials as stated above. The only drawback to this system is the production of some environmentally hazardous compounds, particularly triflouroacetic acid.

Antimicrobial peptides have great potential in fighting bacterial, viral, and fungal infections. With the number of antibiotic resistant bacterial strains increasing it is getting increasingly difficult to treat infections using conventional means. So far no evidence has been seen of bacterial resistance to PG-1. Hopefully this will remain true for some time as true resistance would, in the case of PG-1, require substantial restructuring of the bacterial membrane and cell wall. While difficult, this restructuring is possible as there are many strains of bacteria that currently can resist antimicrobial peptides by membrane alterations which reduce negative electrical charge. With further research these peptides have the potential to become a significant tool in controlling the spread of microbial infections.

Appendix A: Laboratory Protocols

A-1 Agarose Gel Electrophoresis

BAGSHAW LAB PROTOCOLS

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a simple and effective method for separating DNA fragments in the range of 0.5-25 kilobase pairs. DNA is a polyanion because of the phosphate groups, and thus will migrate toward the anode in an electric field. The charge-to-mass ratio is the same for all DNA's, so the fragments separate according to size (length) because of the seiving effect of the agarose gel: the smaller the fragment, the faster it migrates. By including marker DNA fragments of known length (e.g. restriction digests of lambda DNA), you can measure the length of unknown fragments (see "Recombinant DNA Coloring Book").

The principal variable in agarose gel electrophoresis is the percentage of agarose (g/100 ml) in the gel. A 1.0% gel is more porous than a 1.5% gel, and all DNA fragments will migrate faster in a 1.0% gel than in a 1.5% gel. The migration rate, and thus the time needed to complete a run, can also be changed by changing the voltage, and this has some effect on resolution.

Materials

- Electrophoresis buffer (TBE)
- Ethidium bromide solution
- Electrophoresis-grade agarose
- DNA molecular weight markers
- 55°C water bath or incubator
- Horizontal gel electrophoresis apparatus
- Gel casting tray and comb (slot former)
- DC power supply
- Two-cycle semi-log graph paper

1. Weigh out the agarose and measure (graduated cylinder) the TBE buffer. How much of each you will need depends on which electrophoresis apparatus you are using and what percent gel you want to make. For example, to make a 1% gel you would use 0.5 g of agarose in 50 ml of buffer, or 2 g of agarose in 200 ml of buffer. Place the buffer in a flask or screw-cap bottle, add the agarose and swirl. Put the flask (with a foil cover or slip-on cap) or the lightly (not tightly) capped bottle in a bioling water bath. A Rival Hot-Pot is very convenient. Check the flaskor bottle and swirl it occassionally. When the agarose is properly melted the solution will be crystal clear.

An alternative way to melt the agarose is to "nuke" it in a microwave oven. Remember: do not put aluminum foil in a microwave. Use a plastic cap on a flask or a screw cap bottle. Start with

one minute on high power, then check the bottle. If the agarose is not yet melted, "nuke" it some more in 20 second increments. Don't let the solution boil over.

After the agarose solution has been prepared, it must be cooled to about 55^{o} before it can be poured. The most convenient way to do this is to leave it in a water bath or incubator for a few hours, even overnight. If you're in a hurry, let the flask or bottle stand on the bench until it is

cool enough. Put a thermometer in the solution, and swirl it occassionally. If you're <u>really</u> in a hurry, run cold water on the outside of the flask or bottle while you swirl it.

2. While the agarose solution is cooling, prepare the gel tray. Place the casting tray, with the gel tray inside it, on a level surface (one of the black plastic platforms with the four threaded legs and built-in levelling bubble is best) and position the comb (slot former) so that the teeth are about 1mm above the bottom of the gel tray and 0.5 cm from one end. When the agarose solution has cooled, pour it into the gel tray. Allow at least one half hour for the gel to set and cool. As it sets, the gel will become translucent.

3. Carefully pull the comb straight up out of the gel. Remember that the partitions between wells must not be broken. Now remove the gel tray from the casting tray. This can be difficult, especially with the small gels, because the gel that formed outside the gel tray tends to form a seal. It often helps to take a spatula and run it between the outside edge of the gel tray and the casting tray.

4. Fill the electrophoresis apparatus until the buffer covers the support where the gel tray will rest. Lower the gel tray into the apparatus, leaving no bubbles under it. If necessary, add more buffer so that the gel is covered by about 1 cm.

5. Carefully pipet your samples into the wells of the gel, layering them smoothly under the buffer. Be very careful not to puncture the bottom of the well or damage the partitions between wells. It is not necessary to put the pipet tip far down into the well. Put the lid on the apparatus, plug it into the DC power supply, and run it at 12 volts overnight. Be sure the lid is oriented so that the DNA migrates toward the anode (red). Remember: **RUN TO THE RED.**

6. Here's an alternative way to load the gel and start the run. This is especially useful when you need to get the maximum amount of sample into the well, and also when you are running precious samples that you can not afford to lose. Put enough buffer in the electrophoresis apparatus so that the buffer comes up to the ends of the gel but **does not cover the gel.** The wells at this point should be completely empty. Load your DNA samples into the wells, and if there are any empty wells, fill them with buffer. Turn on the power supply and let the gel run for about 1/2 hour. At this point the DNA has migrated into the gel, if only a fraction of a centimeter. Now cover the gel with buffer and resume the run.

GEL STAINING AND ANALYSIS

The method of staining most commonly used for DNA uses ethidium bromide (EB), a dye that intercalates between the planar base pairs of double-stranded DNA. EB fluoresces when irradiated with ultraviolet light; The fluorescence is weak for free EB but very intense when the dye is bound to DNA. **CAUTION**: EB is one of the few toxic substances you will use in this lab. It is a known mutagen and thus a potential carcinogen. Always wear gloves when carrying gels or solutions containing EB, and dispose of solutions only in the designated waste container.

1. After unplugging the DC power supply, carefully lift the gel, still in its tray, out of the electrophoresis apparatus and place it in a plastic box. Cover the gel with 0.5 mg/ml EB and stain the gel for at least 30 minutes with occasional gentle shaking. It is even better to leave the gel in the stain solution while you're doing other things, then photograph it when convenient.

2. Pour off the solution into the designated waste container and cover the gel with distilled water. You can photograph your gel now or later. **Note:** This destaining step is not strictly necessary, but it will reduce background and enhance contrast. This can be important when you are running genomic DNA digests, or when you are trying to see faint bands.

3. Carefully place your gel on the UV light box. Place a clear plastic ruler along one side of the gel so that the edge of the ruler lies on top of or beside the gel and the zero mark is at the edge of the well.

4. Photograph the gel and develop the film. Using the image of the ruler on the photo, measure the distance migrated (in cm) for each DNA band.

5. From the data on restriction sites in your marker DNA, determine the exact length of each fragment in your marker samples. Alternatively, if you are using a commercially prepared marker, the product data sheet should give you the fragment lengths. Using semi-log graph paper, plot fragment length on the log scale vs. migration distance on the linear scale and draw the best-fit curve. All the marker data (e.g. l/Eco RI and l/Hind III should fit a single standard curve. If they don't, you did something wrong.

6. From the migration distance of bands in lanes containing fragments of unknown size, determine the length of each fragment.

10 X TBE buffer, 1 l

Tris	107.8 g
Boric acid	55.0 g
EDTA	7.4 g

Dissolve and adjust to 1 l, no need to check pH

Ethidium bromide stain solution

Fill 2 l foil-wrapped bottle with water.
Add 100ml ethidium bromide, 5 mg/ml

A-2 Cycle Sequencing with ABI Ready Reaction

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CYCLE SEQUENCING WITH ABI READY REACTION

Plasmid DNA Purification. The quality of the plasmid DNA template is critical to the success of DNA sequencing. For automated sequencing on the ABI 310 Genetic Analyzer using the ABI Ready Reaction chemistry, the DNA must be in water, not in TE bufer, and must be at a concentration of at least 0.1 μg/μl. We prefer the Qiaprep plasmid mini-prep kit from Qiagen. Grow a 5 mL culture of cells, spin down the whole lot, and isolate plasmid DNA according to the kit protocol. Be sure to elute the DNA in water. Run a 2 μl sample overnight on a gel, preferably with a standard of known concentration, to estimate the DNA concentration. If there is any evidence of host cell DNA or RNA contamination, reject the prep and do over.

Cycle Sequencing

1. For each reaction, combine the following in a 0.5 mL PCR tube:

   8 μl ABI Ready Reaction mix
   4 μl primer, 1.0 pmol/ μl
   1-8 μl plasmid DNA
   sterile reagent grade water to 20 μl

2. Cover each reaction mix with a drop of mineral oil and spin briefly to collect everything at the bottom of the tube.

3. Cycle program: 96^{o}, 15 seconds; 50^{o}, 15 seconds; 60^{o}, 4 minutes; 25 cycles. On our MJ Research cycler this program is named ABI.

Product Purification

1. Carefully transfer the reaction mix to one of the ABI sample tubes. These look like PCR tubes but have no lids. It is important not to transfer any mineral oil. With the plunger of the micropipettor depressed, push the tip of the tip to the bottom of the tube, and release the plunger slowly. After withdrawing the tip from the reaciton tue, wipe the outside with a Kimwipe to remove mineral oil, then transfer the reaction.

2. To each reaction mix add 75 μl of 70% ethanol containing 0.5 mM $MgCl_2$. Mix thoroughly and let stand 15 minutes at room temperature.

3. Centrifuge tubes 15-20 minutes in a microcentrifuge. Mark and orient the tubes so that you will know where the pellet should be. Carefully pipet off the supernatant without disturbing

the invisible pellet. Immediately wikc off the last tiny drop of liquid with a Kimwipe tip (one corner of a Kimwipe twirled into a fine tip). Proceed to the next sample.

4. Dry all the samples in the 55^{o} incubator for at least 15 minutes. If you are not going to run the samples immediately, stop here, place a rubber septum in each tube, and store the samples in the refrigerator.

Preparing samples for the run

1. To each tube add 20 µl of Template Suppression Reagent. Replace (or place) the rubber septum and vortex each sample vigorously to dissolve the extension products.

2. Denature the reaction products by placing the tubes in a boiling water bath, or in the MJR cycler set on "Denature," for three minutes. Chill in an ice water bath.

3. Spin the samples briefly to collect everything at the bottom of the tube. The samples are now ready to be loaded into the instrument.

A-3 Ligation

Ligation Protocol

Adapted from Laboratory DNA Science (Bloom et al. 1996)

1. Ligation reaction mix:

   3ul digested pBluescript,
   3ul elongation mixture,
   2ul 10X reaction buffer,
   11ul H_2O
   1ul T4 ligase.

2. Combined all of these ingredients into a single Eppendorf tube and mixed. The mixture was then incubated at room temperature for three hours.

A-4 Plasmid Mini-Preps

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PLASMID MINI-PREPS

This protocol is useful when you need a small amount of a large number of different plasmids, for example to verify the sizes in a cloning or re-construction experiment. It is exactly like the large-scale prep but on a small scale. You can easily do two or three dozen plasmid mini-preps in a day.

Day before: For each plasmid mini-prep, inoculate 5 ml of L + Amp medium with a single colony from a recent plate. Shake overnight at 37^{o}; the rotating platform in the incubator cabinet is convenient for this. (**Note:** all plasmids constructed with pBluescript, and most of the others we use, have the Amp^{R} marker. A few have the Tet^{R} instead; in that case substitute L + Tet.)

Prep day:

1. For each mini-prep, pellet the cells from the overnight culture by centrifugation. Ten minutes in the bench-top centrifuge, a.k.a."mushroom", at setting 5 will do nicely. Pour off the supernatant into the liquid biohazard waste container, drain the tube thoroughly and wipe the remaining supernatant from the inside of the tube with a Kimwipe. Remember to dispose of the Kimwipe in the solid biohazard waste container

2. Resuspend the cells in each centrifuge tube in 0.2 ml of 25 mM Tris/ 50 mM EDTA, pH 8.0. Vortexing is OK at this step. Transfer each suspension to a 1.5 ml microcentrifuge tube.

3. Add 0.4 ml of freshly prepared 0.2 M NaOH/1% SDS and mix by gently rocking the tubes. **Do not vortex!** The extract at this step becomes very viscous, then clears slightly. Set on ice for 10 minutes.

4. Add 0.3 ml of cold 5 M potassium acetate solution (3 M KOAc and 2 M acetic acid, pH 4.8), mix thoroughly but gently, and leave on ice for 5 minutes.

5. Centrifuge for 15 minutes, then transfer the supernatant to a clean 1.5 ml microcentrifuge tube. Be very careful not to transfer any of the precipitate.

6. Add 0.5 ml of isopropanol, mix thoroughly and let stand at room temperature for 2 minutes or longer. (Note: This is the first point at which, if necessary or convenient, you can interrupt the protocol and resume the next day. Leave the plasmid prep in isopropanol at room temperature, then resume the protocol with the centrifugation step.)

7. Centrifuge for 10 minutes, then carefully remove and discard the supernatant. Dissolve the precipitate in 50 μl of TE buffer.

8. Add 25 µl of 7.5 M ammonium acetate and leave on ice for 20 minutes or more. A convenient alternative is to add the ammonium acetate and leave the tubes on ice or in the refrigerator while you do something else, or until after lunch, or the next day. Whatever.

9. Centrifuge for 10 minutes and transfer the supernatant to a clean microcentrifuge tube. Add 200 ml of ethanol and let stand at room temperature 2 minutes or longer.

10. Centrifuge for 10 minutes, then carefully remove and discard the supernatant. Air dry the pellet and dissolve it in 50 µl of TE buffer containing 10 µg/ml RNase A. Store in the refrigerator. Run 5 µl on a 1% agarose gel to check the quantity and quality of the plasmid DNA.

1.0 M Tris, pH 8.0

Dissolve 12.1 g Tris base in distilled water. Adjust pH to 8.0 with concentrated HCl, and volume to 100 ml with distilled water.

0.5 M EDTA, pH 8.0

Dissolve 18.6 g disodium EDTA dihydrate and 2.6 g NaOH in distilled water, and adjust volume to 100 ml.

25 mM Tris/50 mM EDTA, pH 8.0

1.0 M Tris, pH 8.0	2.5 ml
0.5 M EDTA, pH 8.0	10 ml
Water to 100 ml, autoclave	

0.2 M Na OH, 1% SDS

Sodium dodecyl sulfate (SDS), 2 g, water to 100 ml
NaOH, 1.6 g, water to 100 ml
Mix equal volumes before use.

TE buffer

1 M Tris, pH 8.0	1.0 ml
0.5 M EDTA, pH 8.0	0.2 ml
Water to 100 ml, autoclave	

5 M potassium acetate

Potassium acetate	29.44 g
Glacial acetic acid	11.5 ml
Water to 100 ml	

A-5 Restriction Enzyme Digestion

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RESTRICTION ENZYME DIGESTION

The protocol described here is appropriate for digesting plasmid or lambda DNA for mapping, subcloning, making probes, *etc*. We have a different protocol for digestions of genomic DNA.

Cleavage of DNA into a set of discrete fragments by digestion with a restriction endonuclease is the most fundamental operation in recombinant DNA work. The reaction is accomplished simply by incubating the DNA and enzyme together in a small volume under appropriate buffer conditions. Like all enzymes, each restriction endonuclease requires a specific pH, ionic concentration and temperature for optimal activity. However, most of these enzymes will work well under one of a small set of conditions. Most suppliers of restriction enzymes also provide a "10X buffer", which contains all the necessary buffers and salts at pricisely 10 times the right concentration for the enzyme reaction. Therefore, in order to make a reaction mix of any desired volume, you will use 1/10 that volume of 10X buffer. It is **absolutely essential** that you use the right buffer for each enzyme, so when you are using several different enzymes you must pay attention to which buffer you use in each reaction. The restriction enzymes we use most are from New England Biolabs, and the accompanying 10X buffers have names like "NEBuffer EcoRI" or "NEBuffer 2". We store the 10X buffer together with the enzyme, so you should always be able to find it.

Restriction enzymes will lose activity if they are mistreated, particularly if they are not kept cold. **NEVER** let restriction enzymes sit on the bench top while you set up the digestion reaction or do other things. The restriction enzyme is always the last component added to the reaction mix, so leave it in the freezer until everything else is ready. Take the enzyme from the freezer and immediately place it on ice. You don't need a big ice bucket for this; a small plastic container or styrofoam cup filled with crushed ice will suffice. After you have added the enzyme to your digestion reactions, immediately return the enzyme to the freezer.

The following is a generic protocol for a restriction enzyme digestion in a volume of 20 ml. This volume is appropriate when the entire sample will be used for a single agarose gel run. Sometimes you will need to prepare larger volumes so you have enough for two or more gel runs, subsequent digestion with a second enzyme, ligation, *etc.*, and to do that you would keep the proportions of all components constant. For example, if you wanted to make a reaction mix of 100 ml, you would use 10 ml of 10X buffer. However, you might not use 5 ml of enzyme. Most enzymes are at a concentration of 10 units/ml, and one unit will digest one mg of DNA in one hour. Using 50 units is probably a waste, and some of these enzymes are expensive.

Materials
DNA to be digested
10X restriction endonuclease buffers
Restriction endonucleases
Stop mix

1. Pipet the following into a clean 0.5 ml microcentrifuge tube:

X ml DNA (0.1 to 1 mg DNA in H_2O or TE buffer)
2 ml 10X restriction buffer
17 - X ml H_2O

Note: The value of x in this recipe depends on the concentration of the DNA. If the DNA is very concentrated you might use only 1 ml and add 16 ml of H_2O. If the DNA is dilute, you might need to use 17 ml of DNA and no water at all.

2. Add 1 ml of restriction endonuclease (1 to 5 units/mg DNA), mix the sample gently but thoroughly, and incubate the reaction mixture 1 hr. or more at the recommended temperature (in general, 37°C). It is often convenient to incubate for a longer time, e.g. set up the reactions in the morning, incubate all day and then run an agarose gel overnight. The volume of restriction endonuclease added should be no more than 1/10 the volume of the final reaction mixture, because glycerol in the enzyme storage buffer may interfere with the reaction.

The restriction enzymes are in a solution containing 50% glycerol, which is thus very dense and viscous. It is **absolutely critical** that the enzyme is thoroughly mixed into the reaction mixture. If mixing is incomplete, your restriction digestion will be incomplete, or won't work at all. This is the most common cause of failure among novice gene jockeys. Hold the tube up to a light and gently flick the tip with your finger. Even though the contents are colorless, you will see a swirl of refracted light, called a Schlieren pattern, that results from the mixing of solutions that have different refractive indices. When the Schlieren pattern disappears, your sample is mixed.

3. After incubation, stop the reaction by adding 5ml of stop mix. This contains the following: ficoll, a sucrose polymer for density; EDTA to chelate Mg^{++}; sodium dodecyl sulfate (SDS) to denature proteins; bromphenol blue, a tracking dye. Mix thoroughly and incubate 5 min. at 65°. The sample is now ready for electrophoresis. **Note**: After a restriction digestion and **before** you add stop mix (which contains SDS, a protein denaturant) **think** about what you're going to do with the digest. If the entire sample is going to be run on a gel, either now or later, go ahead and add the stop mix. But if you are going to use the DNA for ligation, further digestion or other enzymatic reaction, **do not** add stop mix to the whole reaction.

A-6 Transformation

TRANSFORMATION PROTOCOL
FOR TOP10F' *E. coli*
Adapted from Zero Blunt® TOPO® PCR Cloning Kit
for Sequencing Manual
(Invitrogen Carlsbad, CA)

1. Add 2 μL of the cloning reaction into a vial of TOP10F' One Shot® Chemically Competent *E. coli* and mix gently. Do not mix by pipetting up and down.

2. Incubate on ice for 5 to 30 minutes.

3. Heat-shock the cells for 30 seconds at 42^{o} C without shaking.

4. Immediately transfer the tubes to ice.

5. Add 250 μL of room temperature SOC medium.

6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37^{o} C for one hour.

7. Spread 10-50 μL from each transformation on a prewarmed selective plate and incubate overnight at 37^{o} C.

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