Isolation and Amplification of the strK gene of Streptomyces griseus: A look at a specific phosphatase

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by

Danielle Marie Evers

May 2004

Dedication

Thank you Mom, Dad, John, Kathy, Mark, Jodi, Greg, Mark, Meg, Bobby. You have all contributed to who I am today and I will carry you always in my heart, no matter where my path may lead from here.

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Abstract

My project focused on the study of streptomycin-6-phosphatase, an enzyme that has shown specificity for the substrate streptomycin-6- phosphate. The study included attempts at isolation, purification, and amplification of *strK*, the gene encoding this phosphatase. These efforts were made in order to come closer to gaining an understanding of the specificity of this enzyme especially in comparison to alkaline phosphatase, a well-documented unspecific phosphate with notable similarity of sequence and structure.

The major question which developed into the study undertaken this year was: "How does a specific phosphatase compare to the unspecific alkaline phosphatase (AP)?" This is a longterm project that could take years to come close to elucidating an answer to. My approach to this large question, under direction of Dr. Evan Kantrowitz, was to begin with streptomycin-6-phosphatase, the product of the *strK* gene in *Streptomyces*, and embark on a study that would lead to greater understanding of this specific phosphatase.

This undertaking was pursued based upon previous publications identifying conservation of amino acid sequence between the two phosphatases. This similarity was most poignantly found at sites found in AP noted as contained in the active site and in taking part in metal binding. From this information, the question for my study became,

“How much can I learn about *strK* gene product during my time of study so that progress is made toward shedding light on the complex question posed above?”.

Acknowledgements

"We don't accomplish anything in this world alone ... and whatever happens is the result of the whole tapestry of one's life and all the weavings of individual threads from one to another that creates something..." Sandra Day O'Connor

Above all else I want to express my deepest love and gratitude to my family and friends. To my family, I would not be at this point without you and I would never be able to step out and take on the world without knowing you are behind me. To my friends, sometimes you are "Wanna be rebels but didn't have a clue" and sometimes you are talking to the "Man in the Mirror"; nothing but love to those who know.

Over the past four years I have found a family at Boston College; one that has added to my life in so many ways and has led to the project presented within this document. My gratitude extends to all those who have contributed to my education. Whether or not I went willingly into the realm of academia; there have been numerous people who have brought me to the water and taught me to drink. I thank Dr. Timothy Duket, who taught me what it means to really think and discover what may be hidden in a text and how amazing it can be when you find it. I thank Dr. Scott Miller who rejuvenated my passion for science and taught me that sitting in front of a mirror manipulating molecular models is not something to be ashamed of.

Above all else I would like to extend my gratitude to Dr. Evan Kantrowitz who has provided me the opportunity to extend my studies beyond where I thought possible and who has continual challenged me and pushed me beyond what I even expected of myself. Additionally, I would like to thank the entire Kantrowitz group and the Chemistry Department of Boston College especially: Jim Cardia, Cheryl Wojciechowski, Robert Boulanger, Dr. Steven Bruner, Todd Lyons, and Robin Skory. I want to also

extend thanks to Dr. Wolfgang Piepersberg who provided a *Streptomyces griseus* strain and pKMWKI plasmid, containing the gene of study in this document.

My education has become something that is not only my own and something that I have acquired so that I may share it with the world as best I can. I only hope I can live up to the magnificence of all those who have contributed to it. I go forward with all of you as a part of me; I look forward to the journey we will undertake together.

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Chapter 1: Introduction

1.1 General background for project.

In order to gain a thorough knowledge of biological processes it is important to have an understanding of proteins and enzymes. Alkaline phosphatase (AP) is a multi-subunit enzyme extremely important in mammals. This enzyme has been implicated in diseases and has been found important for cell division in normal and transformed cells. AP is an enzyme found in prokaryotes, archaea and eukaryotes. Study of this enzyme is most often conducted on AP in the bacteria, *Escherichia coli* (*E. coli*), due to the wealth of knowledge surrounding the study of *E. coli* and the relatively high yield and purity of AP isolated from this organism. Further benefit of using this organism for study includes that the *E. coli* enzyme is heat stable and easy to produce as a free enzyme or as genetically fused to a proteic partner [1]. In addition, the similarity of the AP enzyme in *E. coli* to that of other prokaryotic APs and those of eukaryotic organisms make it an ideal target of study. AP has generally become the model system for study of metalloenzymes and for both these reasons, a study of alkaline phosphatase in *E. coli* is important and provides the possibility of extensively benefiting biological research.

The study of *E. coli*, alkaline phosphatase is important in that is gives evidence to the action of a wide variety of metalloenzymes and the subsequent pathways which they effect. The understanding of the action of AP improves the understanding of enzymes that pass through similar phosphoenzyme intermediates such as Ser/Thr phosphatases and other phosphatases and sulfatases.

An enzyme which is similar to *E. coli* AP in its metal binding requirement is the protein product of the *strK* gene in *Streptomyces griseus (S. griseus).* This phosphatase is not widely studied but believed to be a phosphatase of the same family of AP with

specificity for streptomycin-6-phosphate hydrolysis and, to a lesser degree, that of streptomycin-3”-phosphate [2]. Recently, studies on *S. griseus* have arisen as molecular biologists who have extensive knowledge and experience with *E. coli* have become interested in this organism.

1.2 Alkaline phosphatase structure and mechanism.

E .coli AP is a serine phosphatase that catalyzes the non-specific hydrolysis of phosphomonoesters, releasing inorganic phosphate and alcohol. The enzyme has a dimeric quaternary structure with each monomer containing binding sites for two Zn^{+2} and one Mg^{+2}. Each polypeptide chain is 449 amino acids in length and contains an active site which is not shared across the interface. The active site on each chain is located 32Å from its counterpart on the opposite chain. Dimerization of *E. coli* AP occurs only in the presence of metals, with 26 hydrogen binding interactions occurring between residues along the interface [3]. **Figure 1.1** gives a structural depiction of the alkaline phosphatase dimer.

The catalytic mechanism of *E. coli* AP has been elucidated through biochemical and structural determinations. Most recently, evidence contributing to the assignment of a mechanism for AP has been obtained through X-ray crystallography. A depiction of the active site as determined by crystallographic methods is shown in **Figure 1.2**. At the start of the reaction, three water molecules enter the active site as the Ser102 hydroxyl hydrogen bonds with a Mg-coordinated hydroxide ion. The phosphate containing substrate (ROP) binds to the free enzyme, forming a non-covalent complex (E•ROP). The Ser moiety deprotonates, forming a Mg-coordinated water and readying Ser for

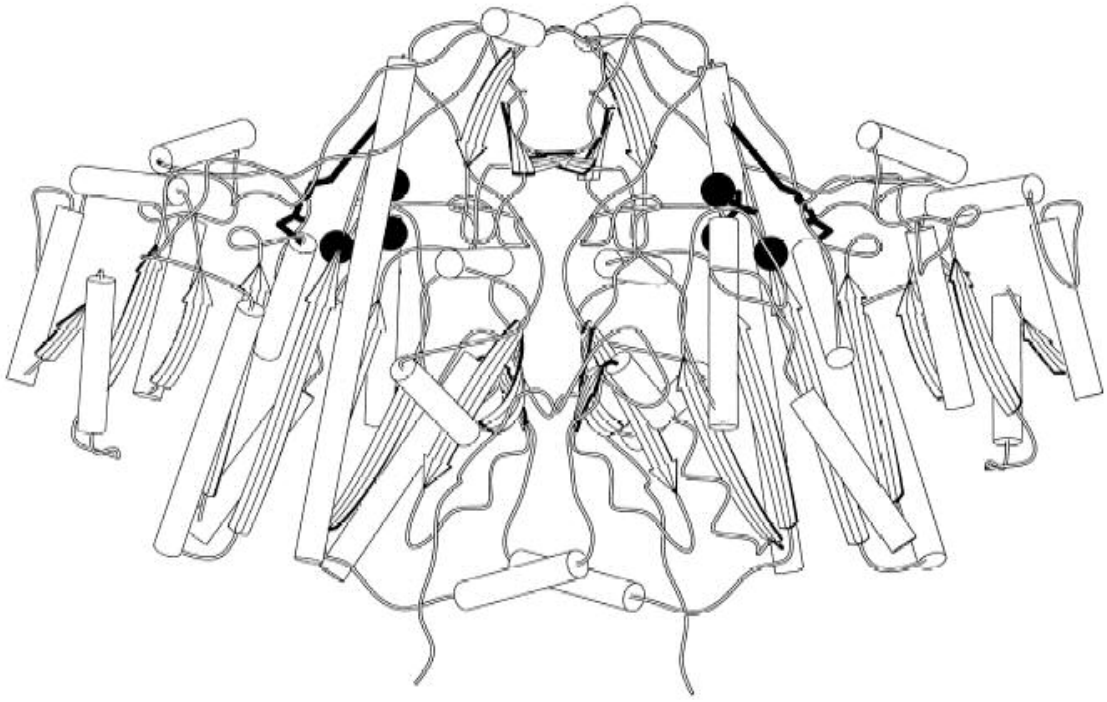


Figure 1.1
Structural representation of alkaline phosphatase dimer. Beta sheets are shown by ribbons in the correct direction of the sheet. Alpha helices are represented by cylindrical tubes. Metal ions in the active site of each monomer are depicted as solid spheres [4].

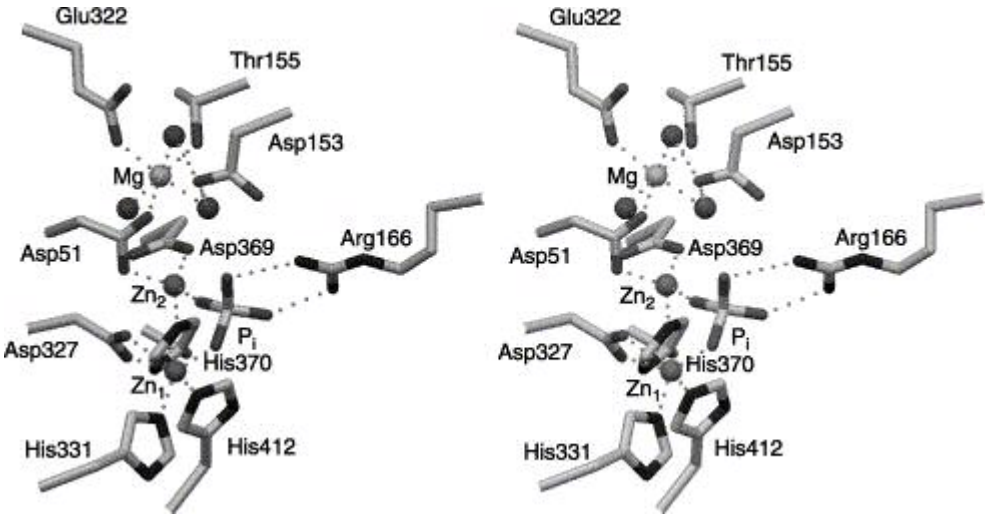


Figure 1.2
Stereoview of the active site of the *E. coli* alkaline phosphatase enzyme. This representation was made using the program SETOR with coordinates from the protein data bank file 1ED8. For clarity, waters bound to the Mg^{2+} are not labeled [5].

nucleophilic attack. A zinc ion (Zn_2) coordinates to the deprotonated state of Ser102 which engages in a nucleophilic attack on the phosphate group of the substrate in complex (E•ROP). This results in a phosphoseryl intermediate (E-P), which has been observed directly through crystallographic methods. Zn_1 plays its role by orienting the bridging oxygen and assisting the alcohol leaving group (RO^-). Following, a hydroxide nucleophile attacks the phosphorous, releasing the phosphoseryl intermediate and resulting in the non-covalent enzyme-P_i complex (E•P_i). Finally, the phosphate group is released as Ser is protonated. Mg-coordinated water is implicated in directly donating the hydrogen to reprotonate Ser102, leaving the enzyme in a free state [6]. This mechanism follows as shown in **Figure 1.3**.

Crystals have been used not only to give the structure of the active site of AP, providing evidence of mechanism and position of atoms as would be necessary for catalysis, but alternate methods have also been used to obtain crystals which capture stages of the mechanism of this phosphatase. The E-P covalent complex has been determined through the structure of a crystal of the wild-type enzyme in a flow cell and, alternatively, by determining the structure of mutant AP (H331Q) in the presence of excess P_i (**Figure 1.4**). The crystal produced by site-directed mutagenesis of AP at residue 331 allowed visualization of the activated water molecule positioned for attack on the phosphoseryl intermediate furthering the understanding of the mechanism by providing evidence at a molecular level.

The role of the magnesium atom has been determined by Stec *et al.* [7], through discovery that Ser102 is oriented in a position to be activated by zinc only when magnesium is present in the active site. In addition, a water molecule bound to the

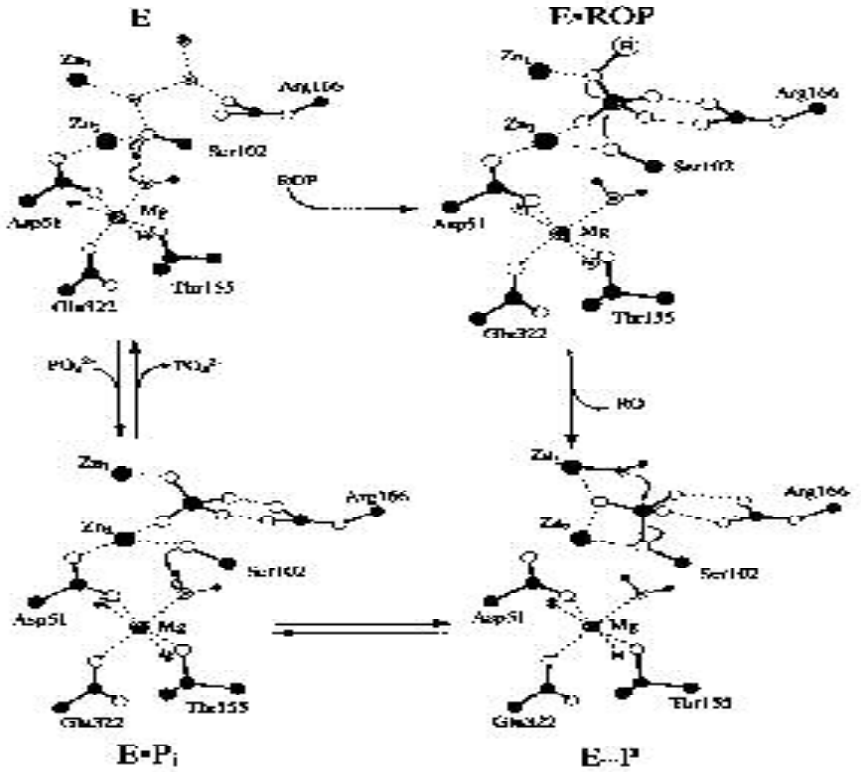


Figure 1.3
Representation of the catalytic mechanism of *E.coli* alkaline phosphatase (see text for details). Hydrogens are not shown. [6]

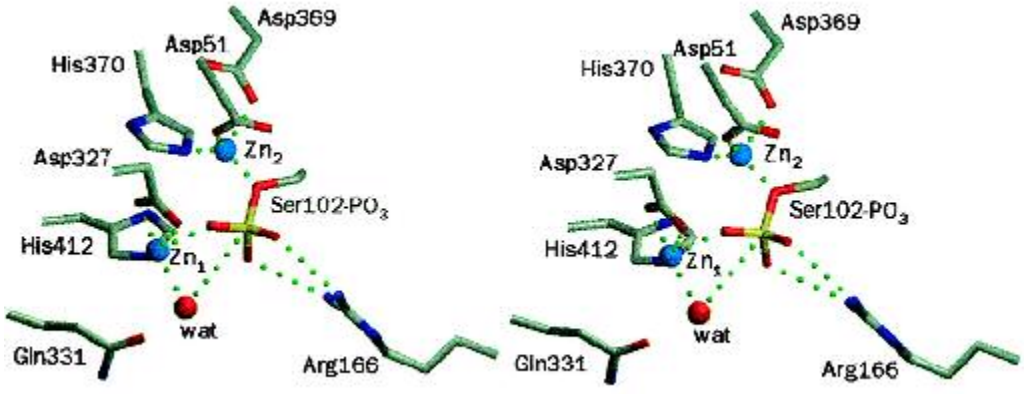


Figure 1.4
*Stereoview of the covalent E-P intermediate. The intermediate (*Ser102-PO_3*) is captured in the H331Q structure as mentioned in the text. The activated water molecule is coordinated to Zn_1 as described. This structure was produced through use of the Protein Data Bank file 1HJK [8].*

magnesium has been determined as amphoteric, acting as a base in the generation of the Ser102 nucleophile, and as an acid in the regeneration of the Ser102 hydroxyl group by donating a hydrogen ion.

With two intermediates, the rate-limiting step is determined based upon the acidity of the environment of the reaction. In alkaline pH conditions, the dissociation of the E-P complex is rate limiting, while the rate of reaction is controlled by the hydrolysis of the phosphoryl-enzyme complex at acidic pH values [1].

AP, in general, differs between organisms in its metal binding requirements and conditions for maximal activity. However, this enzyme in any organism works through the conserved mechanism described above. Mammalian enzymes are about 20-30-fold more active than the *E. coli* enzyme and the AP for *Thermotoga maritime* (*T. maritima*) requires cobalt ion rather than zinc ion for maximal activity. This requirement has been linked to two residues that differ from those near the *E. coli* AP Mg binding site, D153 and K328, corresponding to histidine and trytophan in *T. maritima* AP, respectively. It has, therefore, been predicted that one or both of these residues is responsible for the altered metal binding of the AP enzyme for this organism. Study of *E. coli* AP allows researchers to gain knowledge of the activity of AP in this organism while site-directed mutagenesis allows study of amino acid changes that are found in AP in other species. In this way, use of *E .coli* AP gives evidence not only of AP in this one organism but allows study of AP and, most often, its metal binding affinity in a number of other organisms.

1.3 Alkaline Phosphatase as model metalloenzyme

AP is used as a model for a superfamily of metalloenzymes that has been identified. Sequence analysis of multiple enzymes has identified a group of proteins with similar metal-binding sites and predicted structural fold. Included in this group are phosphopentomutase, phosphonate monoesterase, Streptomycin-6-phosphatase, alkaline phosphatase, and several sulfatases [9]. These enzymes have been found to contain a set of conserved amino acid residues that are implicated as important for catalysis. As much is known about *E. coli* AP, it is often used as a model to give evidence for the less studied and less understood enzymes.

One of the most poignant similarities among these enzymes that has been suggested is a shared phosphoserine intermediate stabilized by associated divalent cations, as is found in AP. Conservation of sequence extends beyond a single serine residue and alignment is shown as reported by Galperin *et al.* [9] while many of the specific residues highlighted are those known to be involved in phosphate binding in AP. Most specifically, all the amino acid residues that interact with Zn_1 (Asp-327, His-331, and His-412) and Zn_2 (Asp-51, Asp-369, and His-370) are conserved throughout this family of enzymes. Alternately, the Mg binding sites were found to contain substituted residues in the other enzymes and, therefore, have different metal binding requirements at this site. Despite the difference in metal binding requirements of the enzymes and their differing specific substrates, they all act on phosphocarbohydrates. In addition, AP has been found to be able to react under conditions with the divalent cations some of the other enzymes require for activity. The overall outcome of the study that has been

completed to date has been to suggest similarity and open the door to further investigation.

Streptomycin-6-phosphatase is an enzyme of this family that cleaves the phosphate group from phosphocarbohydrates, as does AP, but interest in this enzyme has arisen because it has been found to exert specificity. This specific phosphatase is found in *Streptomycetes* and is involved in the streptomycin biosynthesis pathway. The furthering of research of AP is especially important for an enzyme such as streptomyces-6-phosphatase as it is an enzyme for which the scientific community lacks great understanding.

1.4 Streptomycin-6-phosphatase general information and specificity.

Streptomyces are a mycelial gram-positive soil bacteria that involve a complex life-cycle including spore formation. *Streptomcyes* DNA has a characteristically high G-C content (69-73 %) [10]. The specific gene product of study is streptomycin-6-phosphatase, a metalloenzyme which exhibits specificity

Streptomycin-6-phosphatase is a product of the *strK* gene in *Streptomyces.* This gene is part of a cluster whose products function in the streptomycin (SM) biosynthesis pathway. This approximately 5 kb DNA segment is located downstream of *strI*. The involvement of *strK* and other genes of the cluster (*strFGHI*) were determined as acting in the SM biosynthesis pathway as demonstrated by expression in *Streptomyces lividans* and further evidence given through expression of a homologous gene cluster in *Streptomyces glaucescens*. It has been determined, as a generalization, that the full complement of genes necessary for a particular biosynthetic pathway are often found

within a single cluster, as is evidenced by this example. In addition, clusters have been found to contain regulatory and resistance genes through study of gene clusters for antibiotic production pathways [2].

Previously published research has identified streptomycin-6-phosphatase, the *strK* gene product, as an excreted phosphatase as determined through its sequence similarity to *E. coli* AP gene (*phoA*). Conserved sequences include all portions of the *PhoA* protein known to be involved in catalytic activity and cofactor binding. The differences noted were located in the amino-terminus, comprising the signal peptides when translated [2]. What was unknown initially was the specificity of this phosphatase which was suggested as working in the SM biosynthesis pathway.

Mansouri and Piepersberg's work [2] involved activity assays on the present phosphatase in order to determine its action and possible specificity. Through determination of activity of complete medium, a SM-6-phosphate hydrolyzing enzyme was found to be present. This activity was narrowed to action near the end of the organisms vegetative growth phase and preceded SM production. As noted earlier in this report, this activity was also found when only SM-3''-phosphate was present as a substrate, but with slower rates of hydrolysis.

These determinations were found through cloning and expression of *strK* in *S.lividans* with assays conducted on the supernatant from cell growth. The plasmid is created by insertion of full length *strK* gene into the *streptomycete* vector pIJ702 after cleavage out of the initial vector with SphI and SstI, resulting in plasmid pKMWK1. This placed the *strK* gene under the *mel* promoter with the plasmid also containing a BamH1 site that can be used to make linear the circular DNA.

1.5 Streptomycin-6-phosphatase Project

The current undertaking has been brought about by the information and research cited previously in this report. There is clearly a need for greater understanding of streptomycin-6-phosphatase as a specific phosphatase with utility in the streptomycin biosynthesis pathway. The degree of relatedness between this enzyme and alkaline phosphates raises the level of importance as so much is understood about AP as a nonspecific phosphatase. Evidence of the function of an enzyme which follows a similar mechanism, or in the least, has similar composition at several important residues, but which displays clear specificity would be useful for the further understanding of AP and related enzymes. This fact makes the understanding of streptomycin-6-phosphastase as sought through this study, one with clear support for the beginning of such an undertaking and also one whose results could have numerous useful implications.

Chapter 2: Materials and Methods

2.1 Study of Alkaline Phosphatase

A. Materials

YT media (8 g Tryptone, 5 g Yeast extract and 5 g sodium chloride per 1 L of solution), ampicillin, Tris (hydroxymethyl) aminomethane (Tris), ethylene diamine tetraacetic acid (EDTA), *p*-nitrophenylphosphate (PNPP), SDS, acrylamide, ammonium persulfate (APS), TEMED were used for the study of alkaline phosphatase.

B. Strains and plasmids

E .coli strain, EK087 (Δ(*phoA-proC*), *phoR*, *tsx*::Tn5, Δl*ac*, *galK*, *galU leu*, *str*^{r}), plasmid containing *phoA,* pEK154 (DNA plasmid containing *phoA* gene of 5101 base pairs and known restriction sites) were used for transformation during study of AP.

C. Methods

Methods have been designed and tested over time for the study of *E. coli* enzymes and specifically, AP. Of interest are methods used for the expression of *phoA* gene which gives the protein product AP. Also of importance are the methods used for sequencing AP and for analysis of enzyme activity. In the research laboratory of Dr. Evan Kantrowitz, from which the basis of knowledge of AP in this paper was derived, the growth and purification of AP involves *E. coli* strain EK087 transformed with plasmid pEK154 (**Figure 2.1)**. All information on AP contained in the proceedings of this work has been published by the laboratory of Dr. Evan Kantrowitz. Shown in **Figure 2.2** is a representative agarose gel showing overexpression of this enzyme at one step in AP purification.

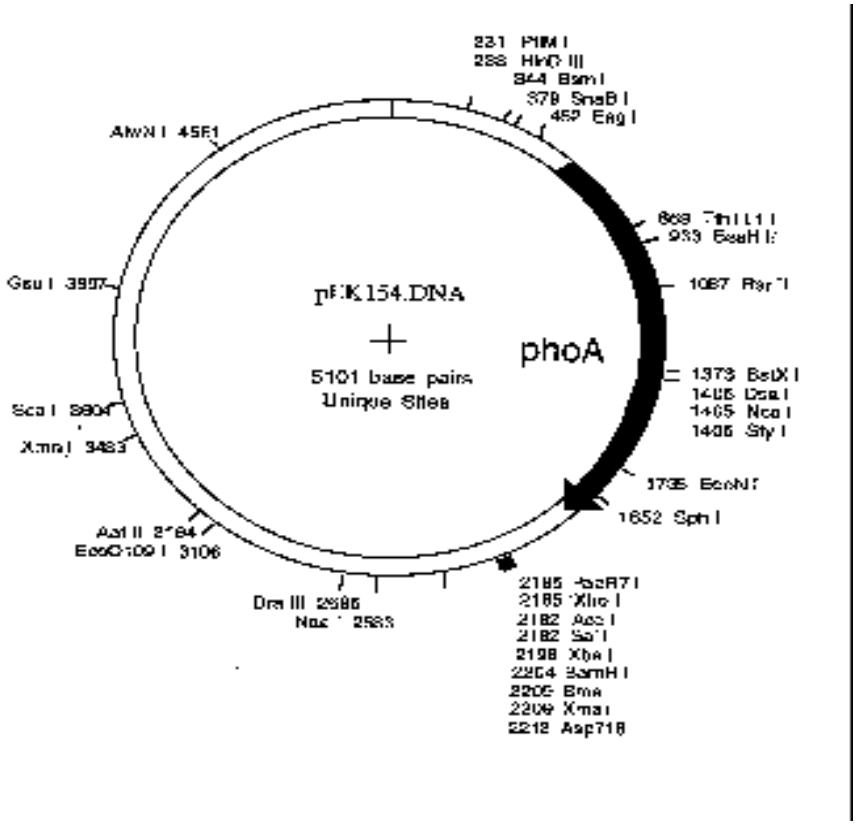


Figure 2.1
Visual representation of pEK154, plasmid containing *phoA* gene encoding AP.

QuickTime™ and a TIFF (LZW) decompressor are needed to see this picture.

Lane:1 2 3 4 5 6 7

Figure 2.2
SDS gel containing purified *E.coli* AP after overexpression *phoA* containing plasmid (pEK154) transformed into *E.coli* strain EK087.
Lanes 1-6 represent protein harvested
Lane 7 contains wild type AP standard

The activity of AP was analyzed by activity assays involving *p*-nitrophenyl-phosphate (PNPP). The reaction mixture for the assay contains Tris at pH 8.0 and PNPP and the change in absorbance read at 410 nm over time is plotted to give specific activity of enzyme as shown in **Figure 2.3**.

In addition to kinetic data, structural data has been compiled for alkaline phosphatase. This data has widely been discovered from analysis of protein crystals. Any structural representation of AP provided in this work was created using Chimera from Protein Data Bank file 1ALK, unless otherwise noted.

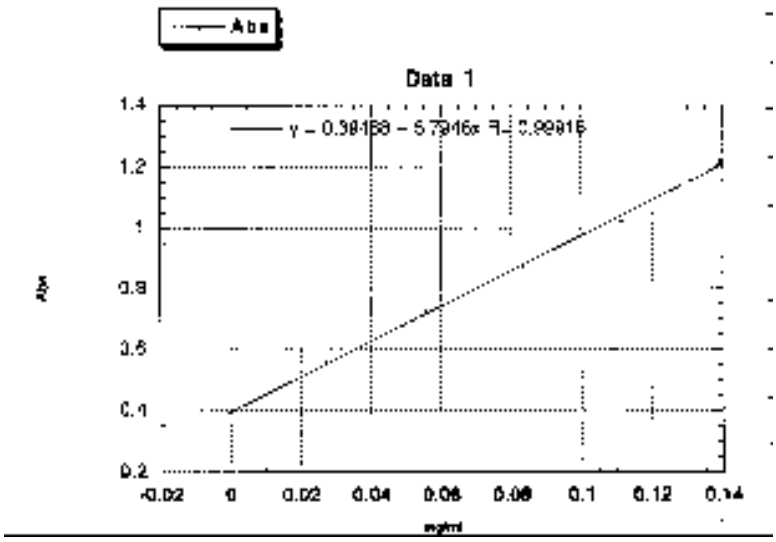


Figure 2.3
BioRad Protein determination using a standard curve as plotted in Kaleidegraph. The sample's absorbance was recorded for the same wavelength as the standard determinations. Concentration of the sample determined was reported as 1.11 mg/mL.

2.2 Study of the *strK* gene product

A. Materials

YED media (10 g yeast extract, 10 g glucose per L, pH 7.0 adjusted with 2 N NaOH) was used for cell growth. Achromopeptidase, dimethyl sulfoxide, anhydrous 99.9+% (DMSO), ethylene diaminetetraacetic acid (EDTA), lysozyme, phenol: chloroform: isoamyl alcohol, 25:24:1, ProteaseK, RNaseA, streptomycin, thiostreptin, Tris-Cl, and Triton X-100 were purchased from Sigma^{R}. Polymerase chain reaction (PCR) was carried out using GeneAmp^{R} core reagents kit supplied by Perkin Elmer while utilizing the GeneAmp PCR System 9700. Bactozol Bacterial Isolation Kit, and cetyl trimethyl ammonium bromide (CTAB) were also used for study.

B. Strains

Streptomyces griseus obtained from Dr. Wolfgang Piepersberg, pKMWK1 (subclone (pUC18) containing *strK* gene (bp 6240-7829) ligated to *streptomycete* vector pIJ702 after both were cleaved with SstI and SphI.

C. Methods

Study began with attempts at growth and isolation of *strK* from *S. griseus* strain ordered through ATCC. Cells were grown in YED media, at 27˚ C for 24 hours. The cells were harvested, prepared for amplification through isolation using the Bactozol Bacterial Isolation Kit, and subjected to polymerase chain reaction (PCR).

From review of work published by Wolfgang Piepersberg from Bergische Universitat in Wuppertal, Germany [2], it was found that his lab produced a plasmid containing *strK* and that Dr. Piepersberg's lab had the *Streptomyces griseus* strain for which the sequence was published. After correspondence with Dr. Piepersberg, he

shared the plasmid and the *S.griseus* strain for which a sequence was available for the purpose of research which follows. The plasmid obtained was created by Dr. Piepersberg's lab by cleavage of a subclone (pUC18) with SstI and SphI and ligation of the full-length *strK* gene (bp 6240-7829) to *streptomycete* vector pIJ702 which has also been cleaved with the same combination of restriction enzymes, resulting in plasmid pKMWK1 (**Figure 2.4)** [2].

Primers were developed by examining the sequence flanking the *strK* gene to be used in amplification by PCR. Before amplification of the gene of interest, the cells were grown, the DNA isolated, and the gene purified to a level making PCR possible. All growth of *streptomycetes* was in YED media and involved growth for 40-48 hours at 27˚ C. Various methods were used for harvesting the plasmid and DNA after cell growth.

Initially, plasmid purification was performed using Qiagen plasmid mini prep. After growth, the media containing the plasmid pKMWK1 was pelleted by centrifugation and the protocol was followed as provided by Qiagen. Methods developed by Maniatis [11] were also attempted. For cell lysis, two methods were tested: lysis by alkali and lysis by boiling. Both procedures were followed as published by Maniatis, through steps of cell lysis by chemical treatment for lysis by alkali and cell breakage by subjection to boiling water for the latter.

Total DNA isolation and purification was also attempted in an effort to isolate the *strK* gene. Initially, after growth of *Streptomyces griseus* cells for the 40-48 hour period

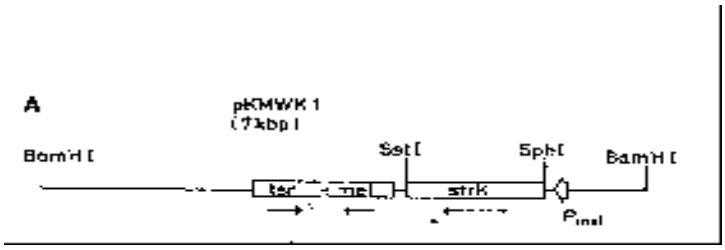


Figure 2.4
PKMWK1 plasmid developed by and reproduced here from Piepersberg's work [2].

as mentioned above, the cells were lysed and DNA isolation attempts followed with the Bactozol Bacterial Isolation Kit. This method utilizes lysis, lysate solubilization, DNA precipitation, and DNA wash steps as given by Bactozol. Other techniques used included a Maize DNA extraction as described by Mehling *et al.* [10] adopted from Wood and Hicks, a method involving use of cetyl trimethyl ammonium bromide (CTAB) with listed modifications [10], and a "High Yield preparation of genomic DNA from *Streptomyces"* [12].

The Maize DNA extraction involved a lysis buffer (25 mM Tris, 25 mM EDTA, 15 mg lysozyme, and addition of 50 μg/mL RnaseA). Treatment involved addition of this lysis buffer followed by incubation for one hour at 37˚ C. The next step involved addition of 500 μL of 5 M NaCl and vortexing and then cell lysis continued with 1.2 mL SDS added to the suspension and further incubation at 65˚C for 20 minutes. After 2.4 mL of 5 M potassium acetate was added, the solution was vortexed to mix, left on ice for 20 minutes and precipitate removed by centrifugation for 30 minutes at 6000 rpm. The supernatant volume was adjusted to 8 mL and DNA recovered by precipitation with isopropanol. The DNA precipitation with isopropanol was extended to at least an hour while stored at –20˚ C to ensure an optimal amount of DNA is recovered. The precipitate was subsequently dissolved in 700 μL 50 mM Tris/10 mM EDTA (pH 8.0), insoluble substances were spun off, and the aqueous phase was transferred to 1.5 mL microcentrifuge tube and 75 μL 3 M sodium acetate and 500 μL isopropanol were added and the solution centrifuged for 2 minutes. The precipitate was washed with cold 70% ethanol, dried and dissolved in 100 μL TE (10 mM Tris/1 mM EDTA, pH 8.0) [12].

The CTAB method was approached with given modifications as will also be noted in the proceeding. The CTAB method is followed in this isolation attempt according to that published by Wilson *et al.* [15] . This procedure involved the overnight growth of *S. griseus* under the same conditions as listed previously. A 1.5 mL aliquot was transferred to a sterile Eppendorf and centrifuged for 30 seconds to isolate the pellet which was then resuspended in 200 μL STET (50 mM Tris/HCl, pH 8.0, 50 mM EDTA, 0.1% (v/v) Triton X-100, 8% (w/v) sucrose). The next section of the procedure consisted of following the alterations listed by Mehling *et al*. [10], the first of which was to add increased lysozyme (10 mg/mL lysis buffer) and to use Proteinase K (200 μg/mL lysis buffer). This lysis reaction was incubated at 37˚ C for 60-120 minutes until the suspension cleared (nearly 90 minutes). According to the original protocol, the lysis solution was centrifuged for ten minutes, the globular precipitate was removed, 8 μL CTAB was added to the supernatant, vortexed and centrifuged for 5 minutes. The supernatant was discarded and the pellet resupended in 300 μL 1.2 M NaCl. Returning again to the modifications, following the resuspension, the DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1). In accordance with the original protocol, the recovered DNA was washed twice with 70% ethanol, centrifuging and discarding the supernatant each time. The second time care was taken to ensure the pellet was completely dry and then was resuspended in 20 μL TE buffer (10 mM Tris/HCl, pH 8.0, 1mM EDTA).

The DNA preparation technique published by Nikodinovic *et al.* [14] claims high DNA yield, with successful PCR amplification performed on DNA isolated in this manner (**Figure 2.5**). This procedure involves growth of *S. griseus* as previously

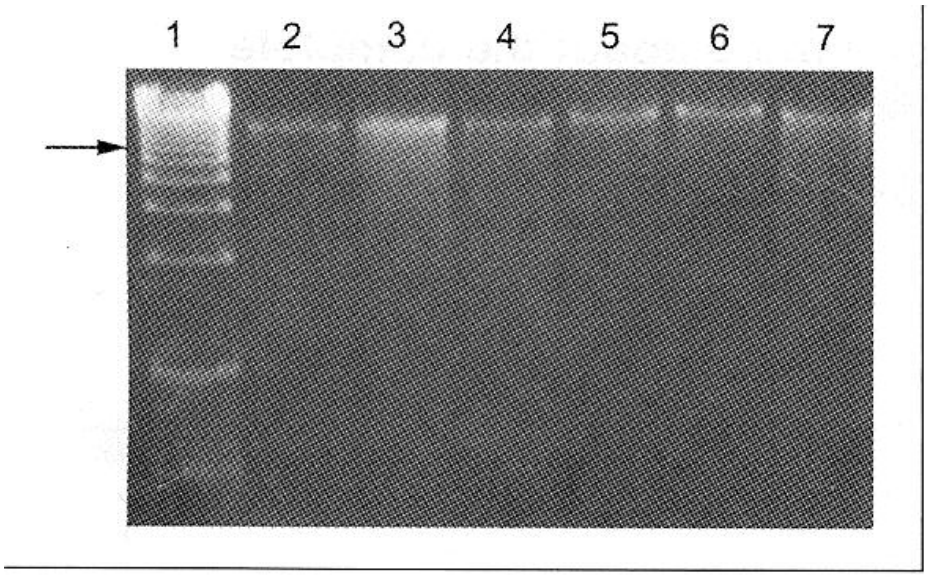


Figure 2.5
Picture of SDS gel published by BioTechniques demonstrating the yield and purification of *Streptomyces* DNA isolated by the above procedure [12].

Lane 1 represents a DNA ladder
Lanes 2-7 contain *Streptomyces* chromosomal DNA

described followed by two washings of 1.5 mL aliquot with 10% (w/v) sucrose. The washed DNA was resuspended in 10 mL lysis buffer (0.3 M sucrose, 25 mM EDTA, 25 mM Tris-HCl, pH 7.5, containing 2U RNase) in 50mL Falcon tube™. Added as crystalline solids were 10 mg lysozyme and 5 mg achromopeptidase (Sigma, St. Louis, MO, USA) followed by incubation at 37 ˚C for 20 minutes. Ten percent (w/v) SDS (1 mL) and 5 mg proteinase K (Sigma) were added with further incubation for 1.5 hours, this time at 55˚ C. All steps during extraction with chloroform were performed in glassware to prevent erosion of plastic containers. Following addition of 5 M NaCL (3.6 mL), and chloroform (15 mL) , the sample was rotated end-over-end for 20 minutes by hand at a speed of approximately 6 rotations per minute. The sample was centrifuged for 20 minutes at 5000x g and the top, aqueous layer was transferred to a clean Falcon tube™ with a pipet. The DNA was precipitated with addition of one volume of isopropanol and spooled on a closed tip Pasteur pipet for transfer to a mirocentrifuge tube. The recovered DNA was washed with 70% (w/v) ethanol. The DNA was air dried and then resuspended in a 50 μL buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA) prewarmed at 60˚ C.

Several alterations to this procedure were made as problems arose. The first alteration involved an increase in the number of chloroform extraction steps to clean the DNA to the highest degree. Another early modification was the addition of storage of the sample in –20° C refrigerator during the DNA precipitation step to recover the greatest amount of material. The benefits of this addition were found by following the procedure as written and then storing the precipitation sample at -20° C for fifteen minutes. After this storage, an additional amount of DNA was spooled, providing evidence that the

initial precipitation was incomplete. Additional testing of this alteration in procedure was followed through storage of the precipitation sample for fifteen minutes at -20° C prior to removing the DNA, spooling of the DNA, and then further storage in cold conditions. No further DNA was spooled after this second storage, showing that all possible DNA was recovered after fifteen minutes. The next alteration required careful spooling of the re-precipitated DNA for transfer of the DNA for washing with 70% ethanol so that none of the sample was lost.

Following attempts at isolation of the DNA were efforts at digesting the DNA to be able to purify and amplify the *strK* gene. Restriction digests were performed with SphI, SspI, and BamHI. The first two were used as they have sites flanking *strK* in *Streptomyces*. BamHI was used in attempts at digesting the plasmid as pUC1, into which the DNA segment containing *strK* is entered to create plasmid pKMWK1. This single site allows for restriction to give linear DNA that can be determined on a gel when run with circular plasmid, as the linear DNA does not travel as far as circular which runs as supercoiled. Restriction digests were performed according to New England Biolabs Protocol with use of the assigned buffers for the restriction enzymes used. Each digest consisted of 2, 4, or 6 μL of DNA, 2 μL of restriction enzyme and enough restriction buffer to give total volume of 50 μL.

Polymerase chain reaction (PCR) was then used as an attempt to amplify the DNA to obtain many copies of the gene of interest. This was carried out adhering to the protocol provided while using the GeneAmp PCR Sytstem 9700. Deviation from this protocol was introduced after unsuccessful attempts at amplification. These alterations followed after communication with Dr. Steven Bruner and research of other experimental

procedures involving *Streptomyces* DNA [10], both of which mentioned inclusion of 10% dimethyl sulfoxide (DMSO). The reason for the addition is not mentioned, but both researches have claimed success with DMSO present and Dr. Bruner also made mention of 10% glycerol in the PCR reaction sample. Both additions were made in several combinations, to test the greatest number of samples that could possibly produce the desired results. Additionally, upon recommendation by Dr. Steven Bruner, nested primers were used rather than primers with a restriction site already introduced. For this, a sequence was developed with 100% complementary to the *strK* gene including the few bases preceding the coding region, but lacking a region that does not bind to the DNA (See **Figure 2.6**).

Figure 2.6

Representation of:
Top-original primer with SacI restriction site
Bottom-nested primer with 100% complimentarity

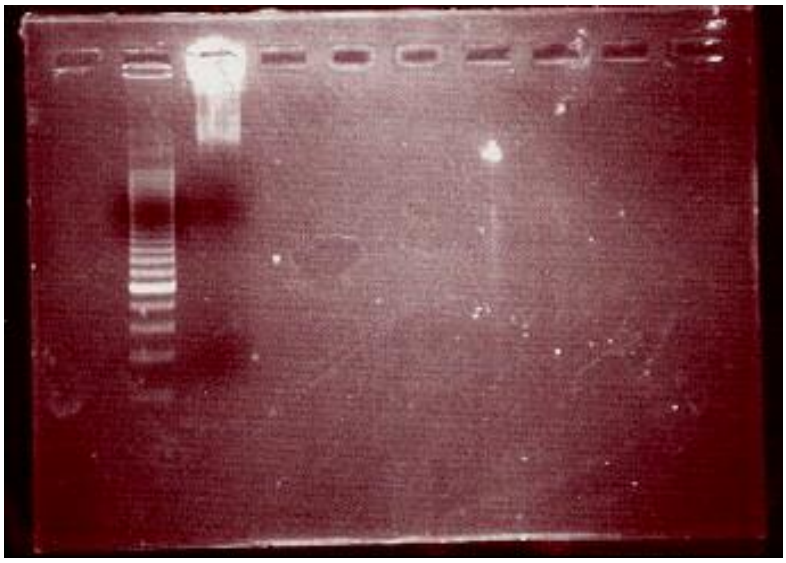
Chapter 3: Results

The study of *strK* gene began through a focus on *Streptomyces griseus* ordered through ATCC. Development of correct PCR primers for this strain became difficult due to lack of sequence knowledge and, so, alternatives were sought after. Following communication with Dr. Wolfgang Peipersberg of Bergische Universitat, Federal Republic of Germany, Dr. Piepersberg provided a strain of *S. griseus* for which he had published the sequence and the pKMWK1 plasmid containing the *strK* gene [2].

Through the study of these two sources of *strK* DNA, the many difficulties in dealing with *Streptomyces* were revealed. The remainder of this exploration was refocused as an attempt to deduce a method of obtaining the gene of interest. This task followed through multiple DNA isolation and plasmid preparation procedures; the results of which are to follow.

First, in dealing with attempts at plasmid purification, methods followed from Qiagen Miniprep, lysis by alkali, lysis by boiling, and Qiagen Midi Prep. The plasmid was traced after purification by agarose gel electrophoresis as seen in **Figure 3.1**, however, DNA amplification techniques were unsuccessful and other venues were pursued. The above efforts resulted in the inability to obtain unsheared DNA clean enough to undergo successful amplification by PCR. This was initially found with Qiaprep, but held true for both lysis methods introduced by Maniatis [11], and Qiagen Midi Prep.

The results of these trials continually showed DNA was obtained, but when amplification by PCR was attempted single bands were not produced when subjected to agarose gel electrophoresis. In addition, there was evidence that the DNA was undergoing shearing, possibly due to ribonucleases remaining after plasmid purification.



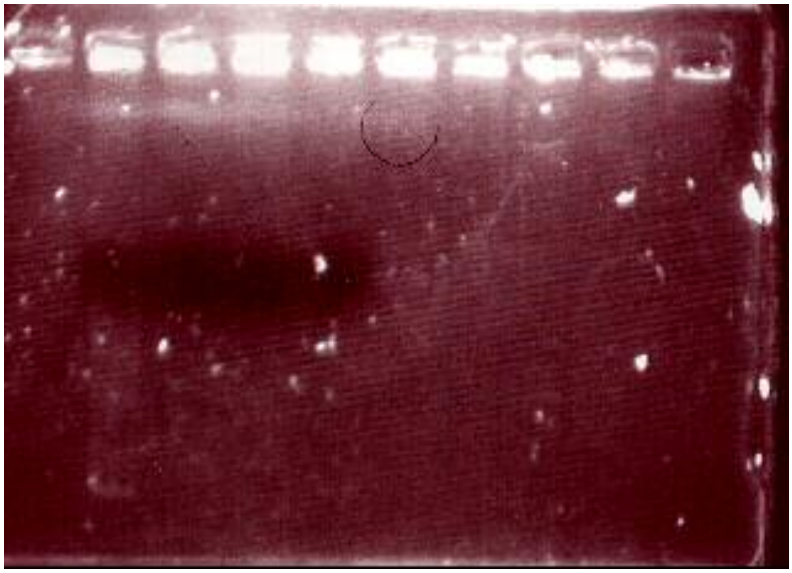
Lane: 2 3

Figure 3.1

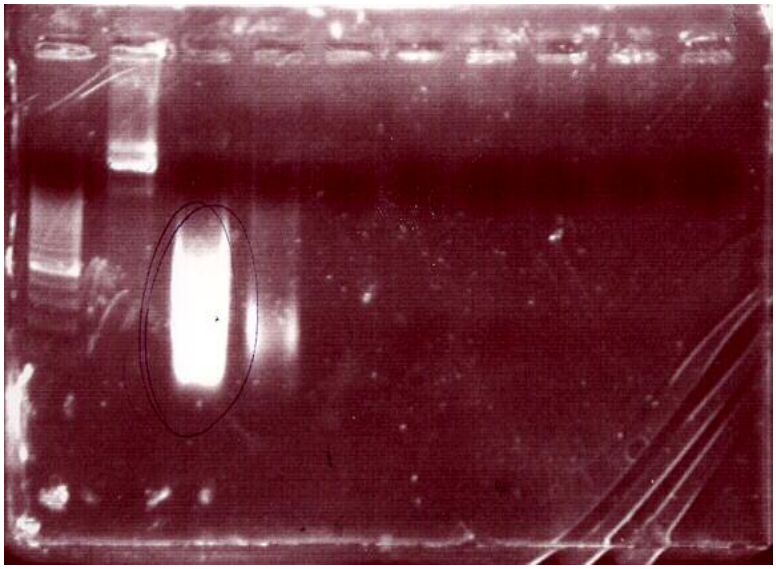
Plasmid purification as tracked by gel electrophoresis. Lane 3 represents isolated plasmid pKMWKI.

As shown in **Figure 3.2**, DNA was obtained and visualized by gel electrophoresis but when subjected to gel electrophoresis the following day, degredation of the DNA was apparent. The fact that the DNA in the top gel did not run because of suspected cell lysis remnants and the DNA in the second gel was apparently sheared after a twenty four-hour period, together gives evidence of the presence of nucleases remaining in the sample.

Undertaken simultaneously were attempts at total DNA isolation from *Streptomyces*. This was focused on finding a method that would enable purification of the total complement of DNA so that *strK* could be targeted and amplified. These purification attempts were followed by digestion with restriction enzymes for gel purification. All that was undertaken was in an effort to retain the purest stretch of *strK* DNA to subject to amplification attempts by PCR.



Lane: 1 2 3 4 5 6 7 8



Lane: 3 4

Figure 3.2

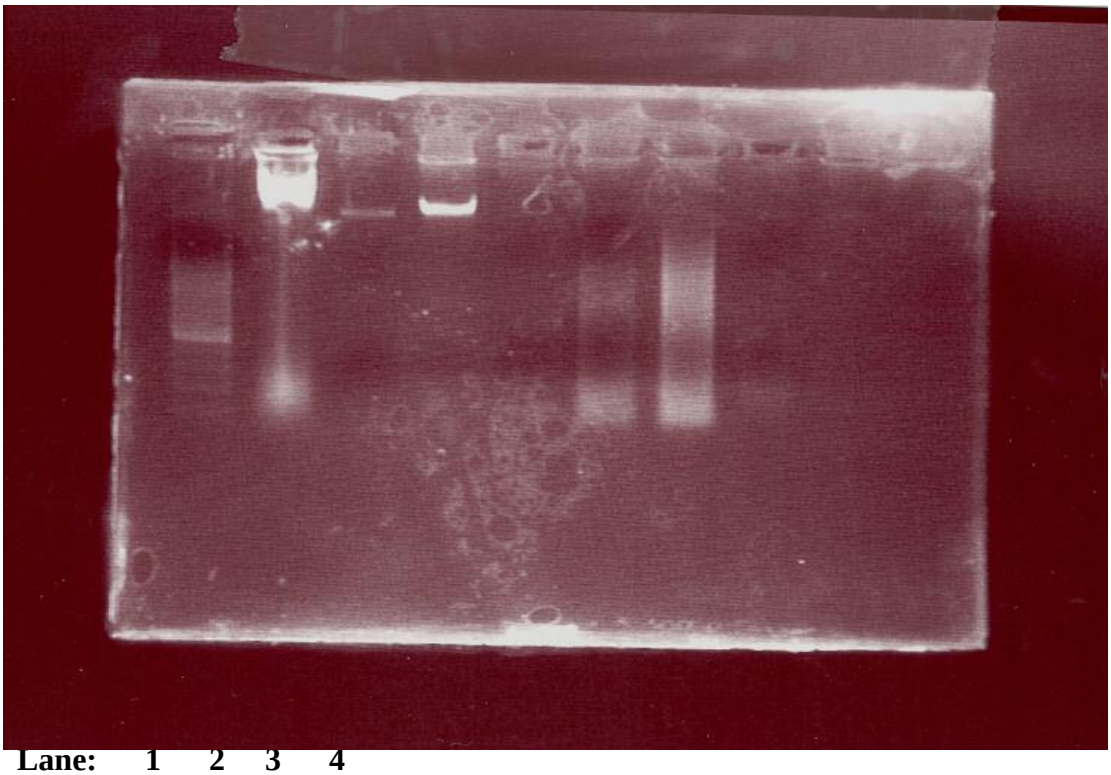
The top agarose gel shows samples of plasmid purified by lysis by boiling . The DNA did not migrate from the wells, showing that the plasmid was not completely isolated and other contaminants were present.
The bottom agarose gel was run a day later with lane 3 representing the same sample run that was observed in lane 5 in the top gel. What is made apparent by the juxtaposition of these two gel pictures is that the DNA was degraded when stored overnight.

These DNA isolation procedures included purification using the Bactazol Kit, maize DNA extraction, cetyl trimethyl ammonium bromide treatment (CTAB), phenol/chloroform extraction, and a *Streptomyces*-specific method adapted from Nikodinovic *et al*. [14], all of which have been described with only the provided notable alterations in **Chapter 2: Materials and Methods**. The seemingly most promising method of obtaining DNA, however, was found through use of the technique published to specifically target *Streotomyces* DNA [12]. This method was designed based on the fact that *Streptomyces*

are difficult to lyse, and the DNA is easy to shear and degrade. For this *Streptomyces* DNA isolation method, to overcome the difficulty in lysis, achromopeptidase is added in addition to SDS and lysozyme. The use of achromopeptidase was spurred by its success with other gram-positive cell lyses and that when used with lysozyme, cells become more susceptible to SDS lysis. This increase in cell lysis hopefully leads to an increase in amount of DNA for purification resulting in a higher concentration of pure DNA for subsequent study. The method is also rapid in comparison to other techniques used to accomplish similar goals so that exposure time to the many endonucleases *Streptomyces* are reported as containing is reduced. In addition to this decrease in time for the isolation method comes a decrease in the number of handling steps that is so important in that it can decrease the effects of shearing. This method also had to be altered however, as outlined in **Chapter 2: Materials and Methods,** as problems arose. DNA isolated with this method, including listed alterations, is shown in **Figure 3.3**.

Restriction digests were conducted on isolated DNA to aid in purification. The restriction enzymes were chosen using the sequence data known for *S. griseus*. This knowledge is limited to the specific coding regions, with little information provided extending far beyond the genes. Initially, BamHI and SspI with SphI were used to digest the plamid. SspI and SphI are the enzymes used to insert the *strK* gene into pKMWKI [2]. The plasmid was also noted to contain a BamHI site where cleavage would give linear DNA from the circular plasmid. Both of these digests were unable to give appreciable amounts of cut DNA that could be further used by gel purification and subjecting to subsequent PCR amplification attempts as evidenced by agarose gel electrophoresis.

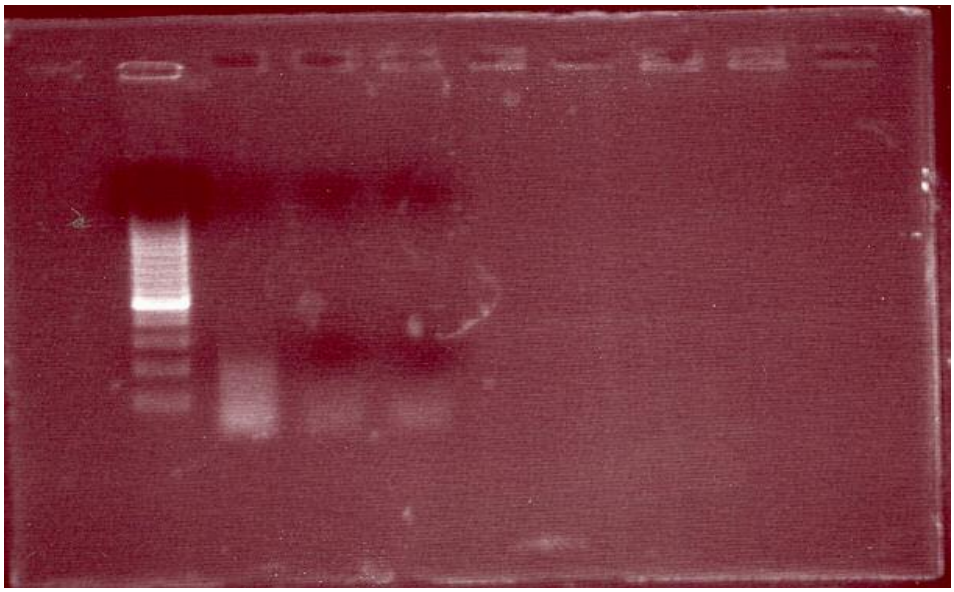
The PCR attempts mentioned followed protocol as outlined in **Chapter 2: Materials and Methods**. In all of the attempts, there was no clear band visible at the expected length of 1349 base pairs when the PCR product was subjected to agarose gel electrophoresis. A depiction of this can be found in **Figure 3.4** where lanes 2, 3 and 4 represent PCR product where clear distinct bands representing the amplified *strK* gene should be visible. Alterations of PCR conditions and primers used followed. Addition of ten percent each dimethyl sulfoxide (DMSO) and glycerol were added with gene amplification still not realized. These inconclusive results led to many alterations to the approach of this project and the conclusions reached will be addressed in **Chapter 4: Discussion**.



Lane: 1 2 3 4

Figure 3.3

Agarose gel depiction of DNA isolated using Streptomyces*-specific method. Lane 4 shows a 1:25 dilution of isolated sample.*



Lane: 1 2 3 4

Figure 3.4

PCR product was run in lanes 2, 3, 4 of this agarose gel. No PCR product is evident.

Chapter 4: Discussion

The study of enzymes is conducted based upon many different motivations and is directed toward many different applications. As of yet, the study of *E. coli* AP has been most often focused to obtain as much information about the requirements and conditions for activity, the mechanism by which the enzyme performs catalysis, and the effect of mutations on the activity of the enzyme. These studies have been to shed light on similar metalloenzymes, and to give evidence to the effects of AP in other organisms, especially eukaryotes and, specifically, mammals. This has been driven by the implications AP has been found to have on the control of cell cycle.

The study embarked upon for this project was focused on taking a closer look at another phosphatase; one which seemed to exhibit a conservation of sequence and had similar folding pattern to AP, but which has been shown to have specificity for its substrate. While alkaline phosphatase will cleave the phosphate group off many ligands in the cell, the *strK* protein product has been shown to have significant activity only in the presence of streptomycin-6-phosphate and, to a lesser degree, streptomycin-3"-phosphate. This raises many questions regarding the degree of similarity between AP and this enzyme and the implications of the differences that may lead to this discrepancy in specificity.

From available publications it seems that these questions have not been widely addressed and so, the focus of the project presented herein became to start the process of this investigation and leave the door open to future study. As mentioned in the **Abstract**, the focus of the research upon which this document is based developed into a study of the DNA isolation, purification, and amplification of the *strK* gene that encodes streptomycin-6-phosphatase. This would be conducted in concurrence with additional

research of previously published information on the two enzymes and comparison of structure from sequence alignment.

Through a study of streptomycin-6-phosphatase from *S. griseus,* it has been found that there are many obstacles when dealing with DNA from this organism. Many of the procedures for study of *E. coli,* with which many biochemists may be familiar, do not produce results when applied to *S. griseus*. The best approach to dealing with this organism became to attempt as many methods as possible in series until an appreciable result was obtained. Through this, careful note was taken of what provided the best results for incorporation into future methods, and which did not suit the organism of study so that certain procedures could be discarded. From this, the options were narrowed and the modification of additional procedures with similar steps allowed for a more focused attempt.

Possible causes of the inability to amplify the DNA sequence of interest involved predicted shearing of DNA. There was evidence as described in **Chapter 3: Results** of shearing of DNA after isolation. Additionally, incomplete digestion with restriction enzymes would hint at the fact that the DNA is not clean enough for the enzymes to fully cut the DNA. If this were the reason, it would give explanation to the lack of PCR product.

Through this study, it was found that *streptomyces* DNA was easily sheared and degraded, and that cell lysis was more difficult than would be assumed for other, especially gram negative, species. This lead to specific steps including the addition of chemicals to cell lysis and the addition of steps aimed at best purifying the DNA. Through this process, it seemed that the best base method was that directed toward high

yield of streptomyces DNA [12], with additions from previous methods made as discussed in **Chapter 3: Results**. DNA was obtained from these experiments and although no PCR was successfully completed, the steps taken will allow further research to begin from above ground zero, as the project continues.

The alterations in the PCR method have also, as of yet, been unsuccessful in amplification. The next step for the immediate future would be to attempt the PCR with these new conditions on DNA from a new growth and purification in hopes that PCR product would be generated.

What was discovered through this study was a high degree of conservation of sequence and arrangement for AP and streptomycin-6-phosphatase and, therefore, predicted mechanism of catalysis. Shown in **Figure 4.1** is a sequence alignment of the DNA encoding *E. coli* AP and *S. griseus* streptomycin-6-phosphatase. Most notable is the conservation of sequence between the two enzymes at places identified in AP as metal binding sites. The locations of identical amino acid include residues: 51, 322, 327, 331, 369, and 412. One of the zinc ions (Zn_1), necessary for the optimal mechanism of AP, is coordinated into the enzyme through His-412, one of the conserved residues. This zinc ion is responsible for association with the bridging oxygen of the substrate and for facilitating the departure of the alcohol leaving group. The conserved amino acids of Asp-51 and Asp-327 coordinate the Zn_2 ion which associates with the hydroxide of Ser-102 affecting the residue's ionization state. This interaction activates Ser-102 as a nucleophile for attack on the phosphate group of the substrate, giving the phosphoseryl intermediate. Another amino acid similar between the two enzymes is Glu-322 which is responsible for coordinating Mg^{+2} into the active site. The magnesium ion is coordinated

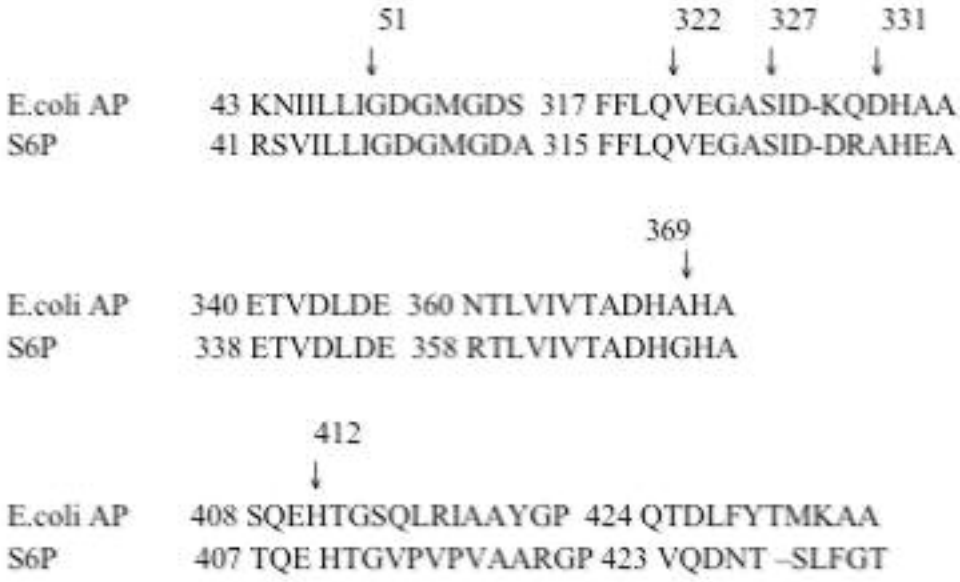


Figure 4.1
Sequence alignment of *E.coli* alkaline phosphatase and streptomycin-6-phosphatase. Numbered locations represent conservation at residues implicated in metal binding for AP.

to a water molecule which displays acidic properties. The water molecule acts to reprotonate Ser-102 for inorganic phosphate release. Therefore, the high degree of conservation of sequence between *E. coli* AP and streptomycin-6-phosphatase becomes even more significant with the fact that most of the residues imperative to metal binding are included in the sequence similarity. The importance of these metals in the formation of the active site for AP is shown in **Figure 4.2** in the depiction of these residues in AP coordinated to the respective metal ions.

Any researcher's project is only a small piece of the big puzzle. In their lifetime, they build on what has come before them, add what they can, and leave the door open to the future. This project was approached with this in mind; the scope of the study being larger than what could be addressed in a year long exploration serving as the culmination of four years of undergraduate study. The questions asked in this study were focused as a beginning to what would be an ongoing exploration for the future. What has been found will only serve to encourage further exploration as many issues are left unsettled. What is most important is that the questions that began this project are still relevant. Future work will focus on more attempts at obtaining the *strK* gene product so that the comparison of the AP and streptomycin–6-phosphatase can move out of the realm of theoretical exploration to direct study in the lab. The expanse of implications of gaining more information about alkaline phosphatase is still undefined and is an exciting and meaningful area for future research.

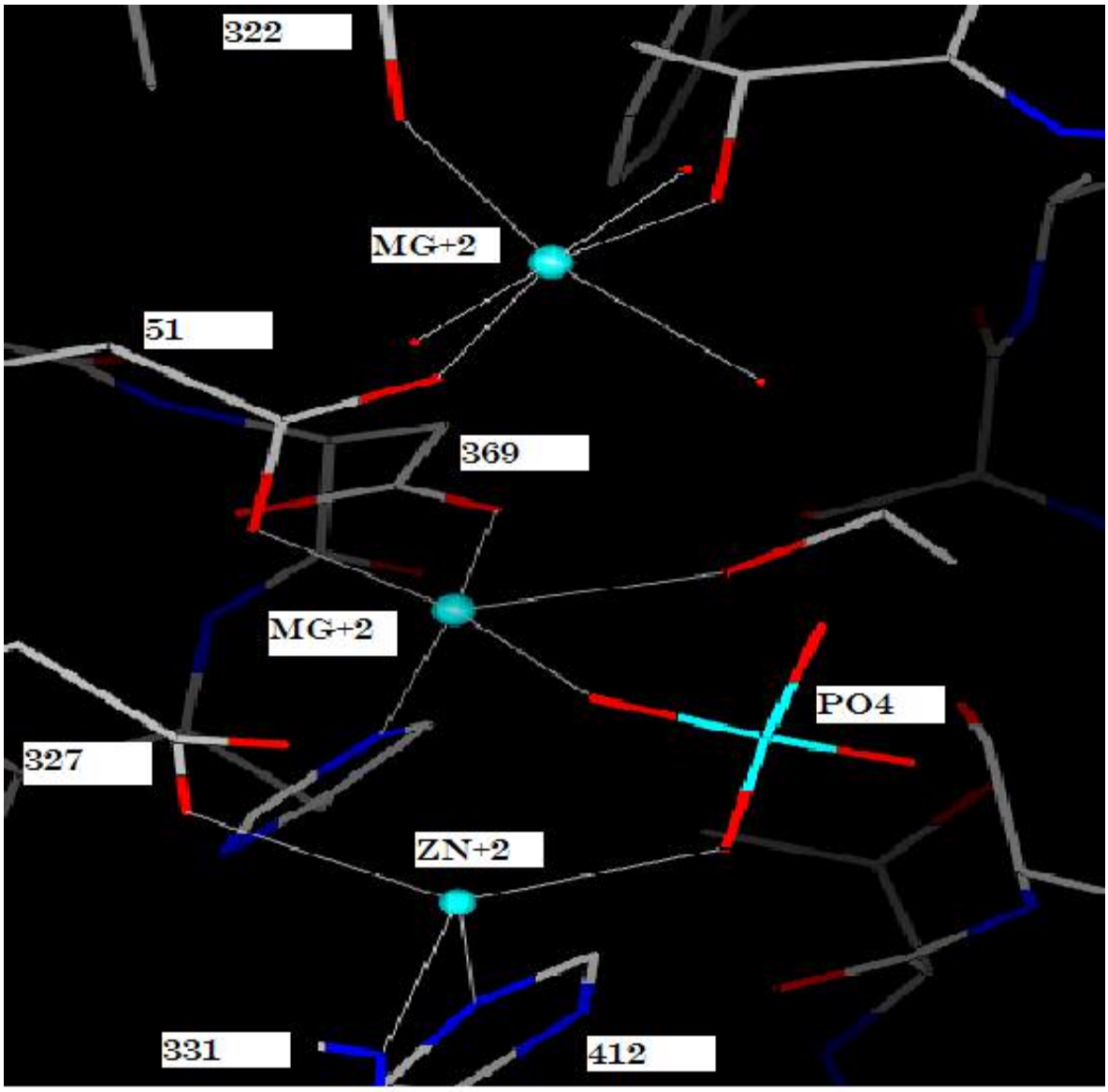


Figure 4.2 Depiction of AP active site generated with Chimera through use of *E.coli* AP PDB file (1ALK). The labeled amino acids are residues involved in the metal binding of AP that are conserved in streptomycin-6-phosphatase.

Chapter 5: References

1. Du, M.H., et al., *Artificial Evolution of an Enzyme Active Site: Structural Studies of Three Highly Active Mutants of Escherichia coli Alkaline Phosphatase.* J Mol Biol, 2002. **316**(4): p. 941-53.

2. Kambiz Mansouri, W.P., *Genetics of streptomycin production in Streptomyces griseus: nucleotide sequence of five genes, strFGHIK, including a phosphatase gene*, in *Mol Gen Genet*. 1991. p. 459-469.

3. Robert R. Boulanger, J., Evan R. Kantrowitz. *Characterization of a Monomeric Escherichia coli Alkaline Phosphatase Formed upon a Single Amino Acid Substitution*. in *J. Biol Chem*. 2003.

4. Murphy, J.E., T.T. Tibbitts, and E.R. Kantrowitz, *Mutations at positions 153 and 328 in Escherichia coli alkaline phosphatase provide insight towards the structure and function of mammalian and yeast alkaline phosphatases.* J. Mol. Biol., 1995. **253**: p. 604-617.

5. Wojciechowski, C.L. and E.R. Kantrowitz, *Glutamic acid residues as metal ligands in the active site of Escherichia coli alkaline phosphatase.* Biochim. Biophys. Acta, 2003. **1649**: p. 68-73.

6. EE Kim, H.W., *Reaction Mechanism of Alkaline Phosphatase based on Crystal Structure. Two-metal Ion Catalysis*, in *J. Mol. Biol.* 1991. p. 449-464.

7. Stec, B., K.M. Holtz, and E.R. Kantrowitz, *A revised mechanism of the alkaline phosphatase reaction involving three metal ions.* J. Mol. Biol., 2000. **299**: p. 1303-1311.

8. Holtz, K.M., B. Stec, and E.R. Kantrowitz, *A model of the transition state in the alkaline phosphatase reaction.* J. Biol. Chem., 1999. **274**: p. 8351-8354.

9. Galperin, M., Bairoch, A., Koonin, EV, *A superfamily of metalloenzymes unifies phosphapentomutase and cofactor-independent phosphoglycerate mutase with alkaline phosphatase adn sulfatases.* Protein Sci., 1998. **7**: p. 1829-35.

10. Annette Mehling, U.F.W., Wolfgang Piepersberg, *Application of random amplified polymorphic DNA (RAPD) assays in identifying regions of actinomycete genomes*, in *FEMS Microbiol. Lett.* 1995. p. 119-126.

11. Maniatis, T., E.F. Fritsch, and J. Sambrook, *Molecular Cloning: A Laboratory Manual*. 1982, Cold Spring Harbor: Cold Spring Harbor Laboratory. p. 368-369.

12. Jasmina Nikodinovic, K.D.B., Jo-Anne Chuck, *High Yield preparation of genomic DNA from Streptomyces*, in *Biotechniques*. 2003. p. 932-936.

13. Wilson, K., *Preparation of genomic DNA from bacteria*, in *Current Protocols in Molecular Biology*, R.B. F.A. Ausubel, R.E. Kingston, D.D> Moore, J.A> SMith, J.G. Seidman, K, Struhl, Editor. 1990, Greene Publishing and Wiley Interscience: New York, NY. p. Chapter 2.4.