TEMPORAL ANALYSIS OF BACTERIOPHAGE FELIX O1 GENE EXPRESSION

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
in
Veterinary Medical Sciences

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November 8, 2002
Blacksburg, Virginia

Keywords: Bacteriophage, Felix O1, *Salmonella*

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(ABSTRACT)

Bacteriophage Felix O1, also known as enterobacteria phage O1, has been used to type *Salmonella* Typhi and is an excellent candidate for use in bioremedial and therapeutic applications. It has extremely high intra-species specificity and is strictly virulent in nature, unable to undergo lysogeny. To facilitate the development of the bacteriophage for use in these areas, the full sequence of the genome had been elucidated previously. In this work, identification and classification of functional coding sequences via reverse transcriptase-polymerase chain reaction was performed.

All of the 115 putative open reading frames (ORFs) studied were found to be functional. 53.0%, 9.6%, and 18.3% of the ORFs investigated were found to initiate expression early, middle or late in the lytic cycle, respectively. Expression of the remaining 19.1% ORFs was evident when the amount of total RNA was increased and when samples were taken at a later time point. Comparisons between bacteriophage Felix O1 and the phage with the most shared homologs, phage T4, revealed many similarities in times of gene expression.

ACKNOWLEDGMENTS

I would like to thank my committee members for their guidance, patience and encouragement during my graduate studies. Thanks to Dr. Nammalwar Sriranganathan for his fatherly guidance, helping me overcome more obstacles than I cared to face. Thank you Dr. F. William Pierson for pushing me to do the best work that I could do. Thanks to Dr. Willard Eyestone for always treating my project with enthusiasm and offering helpful suggestions.

Thanks to my friends and the staff at CMMID. Special thanks to Kay Carlson, for not only teaching me the basics of bacteriophage work, but also for her kindness and emotional support. Thanks to my good friend Mohamed (Naguieb) Seleem for his technical assistance and friendship.

I would also like to thank my family back home in Canada for the amazing support that they've given me over the course of my academic career. Thank you to my mother, Nancy Kenney, for her everlasting commitment to helping me obtain the goals I have set. Thanks to my father, John Borris and my sister, Deborah Borris for their encouragement over the years. Thank you to my grandparents, Gwen Dowe, Hilda Borris and Raymond Dowe for their assistance during my studies. Finally, I would like to thank my angel, Julie Spearman, for her patience, understanding and support.

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CHAPTER 1: PREVALENCE AND CONTROL OF SALMONELLA

Health and Economic Costs of *Salmonella* Infections

With the turn of the twenty-first century the bacterium, *Salmonella*, continues to cause disease on a global scale. *Salmonella* Typhi infection results in typhoid fever, a disease which was much more prominent in the United States in the late 19th and early 20th centuries claiming over 35,000 lives in 1900 alone (Guthrie, 1992); however, typhoid fever remains a significant cause for concern in developing nations with inadequate sanitation. Infection with non-typhoidal salmonellae causes salmonellosis, a separate yet nevertheless potentially fatal disease. The Centers for Disease Control and Prevention (CDC) estimate more than 1.4 million cases of salmonellosis occur annually in the United States with more than five hundred casualties as a result (Mead *et al.*, 1999). Salmonellosis was the most frequently laboratory diagnosed foodborne infection by The Foodborne Disease Active Surveillance Network (FoodNet) in 2001 (CDC, 2002). American economic losses, between 0.5 and 2.3 billion (1998) dollars annually (Frenzen *et al.*, 1999), provide an additional impetus to find new strategies to minimize the staggering health and economic effects of salmonellosis.

The most common source of salmonellosis today in developed regions of the world is the food supply including fresh meat, milk, vegetables, and table eggs. Improper handling at food processing centers or during preparation can lead to widespread *Salmonella* contamination and outbreaks of infection. While susceptibility to *Salmonella* can vary greatly with many contributing factors such as serotype and the immune status of the host (Arthur *et al.*, 2001), there have been reports of a single bacterium having the ability to cause disease (Hockin *et al.*, 1989). Rising occurrences of multi-drug resistant strains such as *S.* Typhimurium DT104 is cause for great concern. The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA), World Health Organization (WHO) and CDC are developing and implementing numerous strategies to survey and combat *Salmonella* infections including public education, incidence monitoring and improvements to inspection practices in food processing centers.

Biology of *Salmonella*

Salmonella enterica is a species of Gram-negative, motile, facultatively anaerobic rods belonging to the *Enterobacteriaceae* family. First discovered in 1885 by American veterinary surgeon, Daniel E. Salmon, more than 2500 *Salmonella* serotypes have been identified to date. *Salmonella* isolates have been generally classified according to the Kauffmann-White scheme based on the presence of specific antigens contained in the envelope, cell wall and flagella referred to as Vi, O and H antigens respectively. O antigens are comprised of lipopolysaccharide (LPS) complexes that constitute the endotoxins present in *Salmonella*. Although serological analyses such as slide agglutination tests are most often performed to identify isolates, bacteriophages may be utilized to gain valuable information during outbreaks of infection. *Salmonella* also contains extrachromosomal DNA, termed plasmids, which are important due to the presence of genes that confer antibiotic resistances and pathogenecity. More recently, a change of nomenclature was proposed in order to minimize improper medical treatments due to confusion between serotypes (Le Minor and Popoff, 1987). The new nomenclature recognizes all *Salmonella* isolates as a single species.

Analysis of recent incidence patterns in the United States has shown *S.* Typhimurium to be the most frequently isolated serotype in human salmonellosis cases followed closely by *S.* Enteritidis (CDC, 2002). Reports of salmonellosis increase during the summer months, when conditions most favourable to bacterial growth and spread between larger numbers of people exist. Children and infants account for an inordinately high number of salmonellosis cases compared to all other segments of the American population including the elderly. FoodNet estimated that during 2001, 134.1 per 100,000 infants less than one year of age contracted salmonellosis (CDC, 2002). This frequency is almost nine times higher than the average rate of incidence over all age groups during the same time period. In the past, children between one and ten years of age have also shown a much higher incidence rate than average (CDC, 2001). This may be due to a relatively weak immune system with which to combat *Salmonella* (i.e. first exposure). More severe symptoms result thus requiring medical attention more often than adults with salmonellosis. This, coupled with a child's potentially higher incidence of contact with the bacteria (e.g. from soil; Haddock, 1986), may account for higher rates of salmonellosis among

individuals less than ten years of age. Following recovery, individuals are protected against subsequent infections to some extent. Incidence rates among the elderly are marginally higher than in younger adults (CDC, 2001); however, salmonellosis in the elderly tends to be more serious than in other adults. It has also been observed that large outbreaks tend to occur in establishments serving large numbers of people including hospitals, institutions, nursing homes and restaurants.

Reservoirs of *Salmonella* include patients and carriers as well as domestic and wild animals. Black et al. (1960) have stated that salmonellosis can be divided into four different clinical syndromes including gastroenteritis, bacteremia with or without extra-intestinal localization, enteric fever, and the carrier state. A study over a six-year period showed that the syndrome with gastroenteritis was prevalent in 70% of cases involving *Salmonella*. *S.* Typhi always caused an enteric fever type of disease and the carrier condition, both temporary and permanent, almost exclusively occurred with this serotype. Salmonellae causing salmonellosis have an incubation period of 6-72 hours, most often between 12-36 hours following ingestion of contaminated foods. Patients diagnosed with salmonellosis may suffer from acute onset of fever, abdominal pain, diarrhea, nausea and sometimes vomiting. Dehydration can become severe and in some cases life threatening in the very young and elderly. Antibiotic treatment is necessary in fewer than 2% of clinical cases as the disease is usually self –limiting, requiring no hospitalization (CDC, 2001).

Typhoid fever causes serious disease more frequently than salmonellosis. Following ingestion of an infective dose, the bacteria attach to and penetrate the intestinal wall then travel into the blood circulation via the thoracic duct. The organisms then move into the lymphatic system and are engulfed by monocytes, which support further multiplication. The bacteria then traverse throughout the blood stream. Manifestation of symptoms usually occurs 1-3 weeks following contact with the bacterium and include fever, headache, malaise, anorexia, enlarged spleen and rose coloured spots on the trunk. Constipation precedes more severe abdominal symptoms such as ulceration of Peyer's patches in the ileum, which can result in hemorrhage or perforation. Necrosis of lymphoid tissue, hepatitis, liver necrosis, ulcerated larynx, infection of bone and joints, and inflammation of lungs, gall bladder, peritoneum and other organs are further

complications associated with typhoid fever. A fatality rate of 10% is reduced to 1% with antibiotic therapy. Bacteria may remain in organs such as the spleen, liver and gall bladder and are shed intermittently thereafter. This is referred to as the carrier state. Ten percent of patients discharge bacilli for three months after onset, while 2-5% become permanent carriers.

Blood, urine and fecal samples may be tested for the presence of *Salmonella*. Typhoid fever may be diagnosed by culturing the organism from one or more of these sources, depending on the progression of the infection. During the first week blood cultures will commonly test positive. Stool and sometimes urine cultures become positive by the beginning of the second week of infection. Antibody titers demonstrate previous contact with the organism but are not useful for diagnosis unless rising antibody titers are observed. Blood cultures are frequently negative in patients with salmonellosis. Fecal cultures from infected individuals will begin to test positive around one week following infection. Fecal cultures may remain positive for several weeks, even following complete clinical recovery.

Multi-Drug Resistance in *Salmonella*

The use of antibiotics for treating salmonellosis is minimal and mostly reserved for typhoid fever; however, due to rising occurrences of multi-drug resistant strains, antibiotic therapy in general is becoming less efficacious when utilized. In England and Wales, non-typhoidal salmonellae isolated from humans were tested for antibiotic resistance over a ten-year period between 1981 and 1990 (Threlfall *et al.*, 1993). The three most common serovars comprising almost 80% of the total number of 186,244 isolates were *S.* Typhimurium, *S.* Enteriditis and *S.* Virchow. Dramatic increases in isolates resistant to four or more drugs were observed for both *S.* Typhimurium (6-18%) and *S.* Virchow (1-11%). The emergence of *S.* Typhimurium resistant to gentamicin is believed to be a direct result of using apramycin to treat earlier outbreaks of infection in cattle. A similar trend of increasing resistance (15-66%) in isolates from cattle was indeed seen over the same ten-year span. A similar study in the United States from 1979 to 1980 and 1984 to 1985 showed increases of drug resistance in human isolates from 16 to 24% (MacDonald *et al.*, 1987).

Another practice commonly thought to increase drug resistance is the sub-therapeutic administration of antibiotics to serve as growth promotants in animal husbandry. Many countries including the United States now ban the use of growth promoting antibiotics that belong to the same class of antibiotics used in human medicine. In Denmark, the use of avoparcin as a growth promoter was banned due to the concerns of transferring vancomycin resistant *Enterococcus faecium* to humans via the food supply. A study done three years following the initiation of the ban showed a dramatic decrease in glycopeptide resistant *E. faecium* isolated from poultry from 82 to 12%; however, no such decline was seen in swine (Bager *et al.*, 1999). The authors speculate that these conflicting observations may be due to the different production practices used for poultry and swine. Strict regulations of growth promotants coupled with more responsible use of antibiotics by physicians in medical practices are hoped to reduce incidences of human infection with drug resistant strains.

Methods to Control *Salmonella* Contamination in the Food Industry

The food industry, including farmers, processing plants, and food service workers, as well as the consumer must be vigilant in order to minimize consumers' risks of becoming infected with *Salmonella* and other foodborne pathogens. Possible sources of *Salmonella* on the farm include feed and water contaminated by infected birds, rodents and reptiles. Early diagnosis of infections in flocks or herds allows for the proper isolation and treatment of a fewer number of animals before widespread outbreaks within a single farm can occur. The utilization of feed that has been pelleted and subjected to steam has been shown to reduce bacterial counts below detectable levels (Blankenship *et al.*, 1984). Proper construction of buildings to minimize contact between commercial and wild animals is also important.

The concept of competitive exclusion, allowing colonization of flocks with bacterial cultures containing normal flora to prevent subsequent colonization with *Salmonella*, has been investigated for application in the poultry industry (Blankenship *et al.*, 1991). Specific-pathogen-free chicks were challenged with *S.* Typhimurium two days after treatment with competitive exclusion cultures. Samples taken from hatcheries (eggshell fragments and paper

pads), grow-out house environments (feed, water and litter), external and internal poultry samples (breast skin with feathers and excised ceca) and carcasses were analyzed for *Salmonella* contamination. Although competitive exclusion did not completely prevent colonization with *Salmonella*, fully processed carcass contamination dropped from 41% in the control flocks to 10% in the treated flocks. Optimization and implementation of competitive exclusion could be an important tool of a multi-faceted approach to increasing food safety.

Another strategy to prevent *Salmonella* colonization is the utilization of sugars to block potential binding sites. Enterobacteria have both Type 1 and 2 fimbriae that are used for adherence to the intestinal epithelium. Type 1 fimbriae agglutinate red blood cells of most mammalian species; however, agglutination is blocked in the presence of mannose. Meanwhile, Type 2 fimbriae are insensitive to mannose. Although Type 1 fimbriae are found on all *Salmonella* serotypes, Type 2 fimbriae are present on only a few serotypes. Oyofo et al. (1988) have shown that alpha-methyl-D-mannoside, D-mannose, arabinose, and galactose inhibit Type 1 fimbriae positive *S.* Typhimurium adherence to chicken small intestine *in vitro* by 94%, 90%, 82%, and 62% respectively. The work done by Mintz et al. (1983) showed that adherence of a strain of *S.* Typhimurium without fimbriae to Henle intestinal cells was inhibited by alpha-methyl-D-mannoside only. LPS, D-mannose and D-galactose had no effect on adherence. These results suggest that one or more additional factors other than fimbriae exist that are associated with adherence and that these factors may be blocked in order to reduce *Salmonella* colonization.

Processing of thousands of birds per day from transport, hanging, slaughter, defeathering, cutting, washing, and chilling to storage increases the risks of one infected bird cross-contaminating every carcass subsequently processed. The implementation of process controls in slaughter plants such as the use of counter-flow scalder water, post-scald rinse prior to picking and chlorination of chiller water (Drewniak *et al.*, 1954) has also been shown to decrease bacterial contamination.

The irradiation of raw meat and poultry has also shown promise in reducing the presence of pathogenic organisms. Although irradiation has been utilized for years to sterilize food for those with weakened immune systems, the general public's fear of radiation has precluded

widespread acceptance of irradiated products. A recent survey conducted in the U.S. indicated that only 49.8% of the respondents were willing to buy irradiated meat and poultry (Frenzen *et al.*, 2001). Participants willing to consume such products were found to have higher education and household income as well as a greater knowledge of food irradiation. Notable was the finding that acceptance among those having higher foodborne illness risk factors was found to be no greater than those individuals having lower risk factors. Irradiation has been shown to oxidize lipids, causing the release of volatile compounds (e.g. propanal and hexanal), some of which are associated with an unpleasant odor. Irradiation can also turning poultry meat pink (Nam *et al.*, 2002; Du *et al.*, 2001). Vacuum packaging and short-term storage served to minimize lipid oxidation and the presence of volatiles. As is the case with the use of antibiotics, the use of irradiation can result in the accumulation of irradiation resistant pathogens. Since irradiation of meat at levels approved by the FDA only reduces the number of pathogens, those naturally resistant will survive and may reproduce to high levels with subsequent processing. Higher levels of radiation would then be needed to effectively reduce the pathogen load. This would, in turn, increase volatile production, lipid oxidation and colour change, thus potentially making the product less appealing to the consumer.

HACCP and the HACCP-Based Inspections Model Project

The Food Safety and Inspection Service has taken great strides in attempting to improve the quality of the final meat product. In 1996, the Pathogen Reduction and Hazard Analysis and Critical Control Point Systems (HACCP) were introduced to scientifically monitor and meet safety performance standards by shifting the onus from FSIS personnel to the processing establishments. Under HACCP, each step in processing is examined and controls set to eliminate possible contamination. The following year, the HACCP-Based Inspection Models Project (HIMP) was implemented to more effectively use inspectors. By placing carcass inspectors at critical points on the processing lines, a higher proportion of unacceptable carcasses are identified and removed. FSIS personnel ensure that plant workers are properly executing the process control procedures including the appropriate handling of carcass defects.

To study the efficacy of the new project, FSIS solicited voluntary participation of plants that slaughter market hogs and young poultry. Performance standards were established by FSIS for both food safety and non-food safety defects. In order to protect the consumer acceptable food safety defects, which include birds with infections or fecal contamination, were set to zero. Non-food safety standards, comprising a wide assortment of defects such as bruising, disease and dressing defects, were set at the 75^{th} percentile of then current levels achieved under traditional methods of inspection. Therefore improvement in 25% of plants was needed to satisfy the new non-food safety standards.

In order to evaluate HIMP, FSIS gathered baseline data in plants under the traditional system. Research Triangle Institute, an independent consulting firm, collected microbial and organoleptic data. Following a period of transition to implement new practices, data were again collected and compared to baseline data. Both baseline and HIMP implemented data of fourteen young chicken plants are shown in Table 1.1 (USDA, 2002). More than 600,000 birds in total were inspected.

Marked reductions in defects in both food and non-food safety categories have been observed. Food safety defects have been reduced by 99.9% but still do not meet the zero tolerance goals. Defects in four of the five non-food safety categories have decreased in excess of 50%. The USDA plans to increase participation in the project and seeks further improvement to maximize the safety of the American food supply.

Table 1.1. Percentage of young chickens positive for defects in plants with traditional inspection practices and plants implementing HIMP (USDA 2002).

Defect Category	Baseline Data (% of Carcasses)	HIMP Data (% of Carcasses)
Food Safety		
1. Infectious	0.1	0.0003
2. Contamination (e.g. fecal material)	1.5	0.2
Non-Food Safety		
1. Animal Disease	1.7	0.9
2. Miscellaneous (e.g. bruises, sores, etc.)	52.5	24.9
3. Contamination (e.g. digestive content)	18.6	9.4
4. Dressing Defects (e.g. feathers, etc.)	80.0	54.2
5. Dressing Defects (e.g. digestive tract tissue)	20.8	6.8

Bacteriophages: An Alternative Approach to *Salmonella* Control

Bacteriophages, also known as phages, comprise a dozen families including thousands of species. They are ubiquitous, being found anywhere the bacterial host resides. Co-discovered in 1915 by Frederick W. Twort and in 1917 by Felix d'Herelle, bacteriophages have been studied primarily in the last three decades to gain insight into molecular mechanisms such as DNA replication and recombination. Morphology between phages varies greatly. Phages may be icosahedral, filamentous, tailed, isometric or pleiomorphic. Tailed phages consist of a head, where genetic information is stored as double stranded DNA, single stranded DNA, positive sense RNA or negative sense RNA, and the tail. The tail serves as a means of attaching to the host preceding injection of the contents of the phage head into the bacterium for initiation of replication. The genomes of phages such as M13 are small, comprised of fewer than 6,500 base pairs while the genome of the often-studied T4 consists of approximately 168,800 base pairs. Some phages have two distinct lifestyles available to maximize chances of survival, known as the lytic and lysogenic cycles.

During the lytic cycle, hundreds of phage progeny may be produced. The newly formed phage escape the host by the actions of a lytic enzyme system which breaks bonds in the bacterial cell wall, rupturing the bacterium and releasing the contents of the cell into the environment. In contrast, the goal of the lysogenic cycle, otherwise known as lysogeny, is not to produce a viable phage particle (virion), but to integrate the genetic material of the phage into the bacterial chromosome. The host survives and reproduces, each time replicating the integrated phage DNA. Under conditions stressful to the host including irradiation by ultraviolet light (Lwoff *et al.*, 1950) integrated phage genes are transcribed and the lytic cycle initiated. Lysogeny has been identified as a factor in the transference of virulence factors into bacteria. This process, termed transduction, has been shown to confer virulence in species such as *Corynebacterium diphtheriae* (Freeman, 1951).

Due to the utter dependency of phages on their hosts, phages must be extremely specialized and efficient. Many phages have a narrow host range as a result. This characteristic of phages was only fully understood in the latter part of the 20th century. Prior to the discovery

of the antimicrobial properties of penicillin by Fleming in 1928 and its subsequent wide spread use, d'Herelle investigated the therapeutic applications of phage for patients with bacterial infections such as the protection of the Soviet army against dysentery and gangrene. Although experiments during this time were not well-controlled and met with criticism in the West, phage became a daily part of life in the USSR, where d'Herelle established the Elivia Institute of Bacteriophage. The emergence of wide spread multi-drug resistant strains of bacteria today has necessitated a revival of interest in the study of phage as therapeutic agents.

In Europe, phages are now being used topically to treat burn victims, preventing bacterial infections with pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *P. aeruginosa* develops new resistances readily and can break down pig skin which is commonly used for skin grafts of burn victims. Soothill et al. (1988) have shown the ability of bacteriophage to control the *in vitro* growth of *P. aeruginosa*. Such efforts may prove to reduce both nosocomial infections and recovery times.

Bacteriophage Felix O1

In the West, phages have been classically used as identification and diagnostic tools. Bacteriophage Felix O1, also known as phage O1, 01 or O-1, has been utilized to type *Salmonella* Typhi. Bacteriophage Felix O1 has the ability to lyse as high as 99.5% of *Salmonella* isolates and only 0.3% of non-*Salmonella* species (Thal and Kallings, 1955). Bacterial strains belonging to OE groups 1-4 show considerably less sensitivity to Felix O1 with only 87.5% of strains able to be lysed (Fey *et al.*, 1978). Felix O1 attaches to the terminal N-acetylglucosamine branch of the bacterial LPS core, as shown by Lindberg et al. (1967, 1969, 1970) and is more accessible in rough strains than in smooth strains (Westphal, 1975). Felix O1 is also thought to be a virulent phage, unable to undergo the lysogenic cycle.

The genome of bacteriophage Felix O1, containing 86,155 base pairs of double stranded DNA, has been recently sequenced (Whichard, 2000). Genomic analysis has revealed the presence of 96 open reading frames (ORFs) of which only 20 show significant homology to

genes of other phages and bacteria. The most frequently occurring homologies (11) are with those of phage T4, a related phage that also belongs to the A1 group of the Myoviridae family (Ackermann, 1973).

These characteristics make Felix O1 an excellent tool not only for diagnosis and identification, but also for applications associated with therapeutic or bioremedial uses. The bioremedial utilization of phages on farms (on the litter) and in poultry processing plants (in chiller water, on equipment and the final meat product) may serve to decrease the numbers of *Salmonella* found in these areas (Whichard, 2000). Assays have incorporated the use of Felix O-1 with high-pressure liquid chromatography (HPLC) in order to detect the presence of *Salmonella* in milk within 24 hours of sample collection (Hirsh and Martin, 1983). The lower detection limit of this assay was 10^6 organisms per ml of solution thus requiring enrichment for practical application.

Felix O1 has also been engineered to contain luciferase genes (Ulitzur and Kuhn, 2000). When placed into a sample containing *Salmonella*, the phage genes are expressed and light is emitted which can be detected by the use of luminometer. Since classical microbiological techniques require one to two days to positively identify the presence of *Salmonella*, the construction of rapid assays such as this have the potential to reduce losses in processing plants and would allow for quicker treatments in hospitals and on farms.

Lee and Harris (2001) have studied Felix O1 to determine its efficacy in treating *Salmonella* Typhimurium infection in swine. Three hours following intranasal administration of $5x10^8$ colony-forming units (CFU) of *S.* Typhimurium in three-week-old pigs, $2x10^{10}$ plaque forming units (PFU) per ml was delivered both orally (6 ml) and intramuscularly (4 ml). The animals were killed after 6 hours and tonsil, liver, lung, blood, spleen, ileocecal lymphnode, and cecum contents samples were harvested and analyzed for presence of bacteria. Statistically significant decreases ($P<0.001$) in the number of bacteria were seen in tonsil and cecum contents samples. Bacteria in the contents of the cecum remained high ($3.6x10^5$ CFU). The authors remain hopeful that although administration of Felix O1 alone may not be sufficient in fighting

infection, its refinement and use as a primary agent in a phage cocktail would be an effective short-term strategy to combat *Salmonella* infection in swine.

An area of concern associated with phage therapy is phage clearance by the reticuloendothelial system (e.g. spleen cells) of the infected host. Merril et al. (1996) have isolated naturally long-circulating bacteriophages using the serial-passage technique. Phage were propagated on mutator *Escherichia coli* in order to increase its rate of mutation. Infected mice were injected intraperitoneally with phage and blood collected after seven hours. The phage present in these samples were propagated and injected again *in vivo*. This cycle was repeated for a total of ten passages. Two isolates, Argo1 and Argo2, were found to remain in the circulatory system of naïve mice statistically significantly longer (P=0.003) than the original phage stock. Symptoms were observed to be less severe in mice that had been infected with *E. coli* (i.p.) 30 min prior to injection with long-circulating phage (i.p.; MOI of 500) than in those mice treated with wild-type phage. It should be noted, however, that these results were not found to be statistically significant.

Barrow et al. (1998) were able to demonstrate statistically significant decreases in mortality of chickens infected with *E. coli* when phage were administered as a therapeutic agent. Bacteriophage R, administered intramuscularly, was able to combat *E. coli* inoculated both intramuscularly and intracranially. The simultaneous administration of 10^4 PFU phage was sufficient to protect three-week old chickens challenged with 10^6 CFU of *E. coli* intramuscularly, while 10^8 PFU phage were needed to protect against the same number of bacteria inoculated intracranially. Phage were found in the brains of the birds that had been inoculated intracranially thus demonstrating the capacity of phage to cross the blood-brain barrier. Dubos et al. (1943) also reported this phenomenon.

More recent investigations have attempted to circumvent problems associated with the clearance of phage by the immune system by utilizing lytic enzymes produced by bacteriophage. Lytic enzymes share the specificity found in the bacteriophage in which it was produced. Nelson et al. (2001) have shown the *in vitro* killing of group A streptococci by lysin isolated from bacteriophage C_1. One thousand units of lysin was sufficient to completely kill 10^7 CFU bacteria

while 100 units decreased the viability of Streptococci by 3 log in 5 seconds, 4 log in 1 minute and 5 log in 10 minutes. The ability of this lysin to prevent colonization of the upper respiratory tract by group A streptococci was also studied. Upon treatment with 250 units of lysin, the incidence of colonization after 24 hours decreased from 70.5% in control mice to 28.5% ($P<0.03$). These results reveal further avenues that may be pursued to more effectively use phage in the treatment of disease.

Study of Bacteriophage Felix O1 Gene Expression

The purpose of this project was to identify and classify functional bacteriophage Felix O1 genes according to observed times of expression during the lytic cycle. The genes hypothesized to be functional were the 96 previously identified by Whichard et al. (2000) using GeneMark®. A sensitive assay, reverse transcriptase-polymerase chain reaction (RT-PCR), was utilized to amplify RNA extracted from samples at times 0, 5, 10, and 25 min following the addition of phage. These time points were found to yield the greatest amount of RNA for early, middle and late expressed genes in bacteriophage T4 (Karam *et al.*, 1994), a phage which shares the greatest number of homologous genes with Felix O1. Primers designed for each of the 96 putative ORFs using sequencing information enabled the investigation of each ORF separately.

RNA is converted to cDNA during RT-PCR by the action of reverse transcriptase. This enzyme is then inactivated by high temperature, followed by many cycles of strand denaturation, primer annealing and strand extension by *Taq* polymerase. In this fashion, the original mRNA template for the gene in question can be amplified millions of times and detected by ethidium bromide staining on an agarose gel. If equal amounts of RNA are used for the time points of each gene and RT-PCR is not allowed to run to saturation, crude relative quantification can be performed by comparing band intensities.

Analysis of the Felix O1 proteome would give further insight into the life cycle of the phage; however, such a study would require a very sound infrastructure including equipment, expertise and funds. Due to the immense scope of a proteomic project and the lack of proper

resources it was decided to investigate only the transcripts formed during the lytic cycle of the phage. These investigations may serve as a foundation for the engineering of Felix O1 to aid in its use as a bioremedial or therapeutic agent in the future.

Engineering of the Felix O1 genome would be facilitated with this knowledge. Functional genes may be replaced by constructs while integral components such as the promoter sequences remain intact for the successful expression of the construct. Classifying functional genes according to time of transcription would also give clues as to the function of the proteins synthesized. For instance, most proteins in the phage head and tail are produced late in the lytic cycle. Also, functional genes that have been found to be homologous to genes in other systems might be exploited to combat *Salmonella*, such as the gene encoding lysozyme.

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CHAPTER 2: APPROACH AND METHODS REFINEMENT

Over the past decade the scientific world has seen an explosion of knowledge associated with mapping entire genomic sequences of organisms, including our own species. Vast amounts of data have resulted from such projects with the onus quickly turning to extraction of relevant data for elucidation of the problem in question. This usually requires the study of both transcripts and proteins produced by the organism.

The sequencing of bacteriophage Felix O1 was no different in this respect. Following sequencing, only a small number of homologs were found to exist. Also, conflicting results were given by GeneMark® and MIPS PEDANT® regarding the number of open reading frames (ORFs). It was therefore planned to investigate the proteins produced by bacteriophage Felix O1 during the lytic cycle. A project such as this would provide insight not only into which ORFs were functional and the time during which the ORFs were expressed, but it would also confirm the nucleotide limits of each ORF and the corresponding molecular weight of the protein produced. Preliminary experiments were performed with the bacteriophage Felix O1 virion.

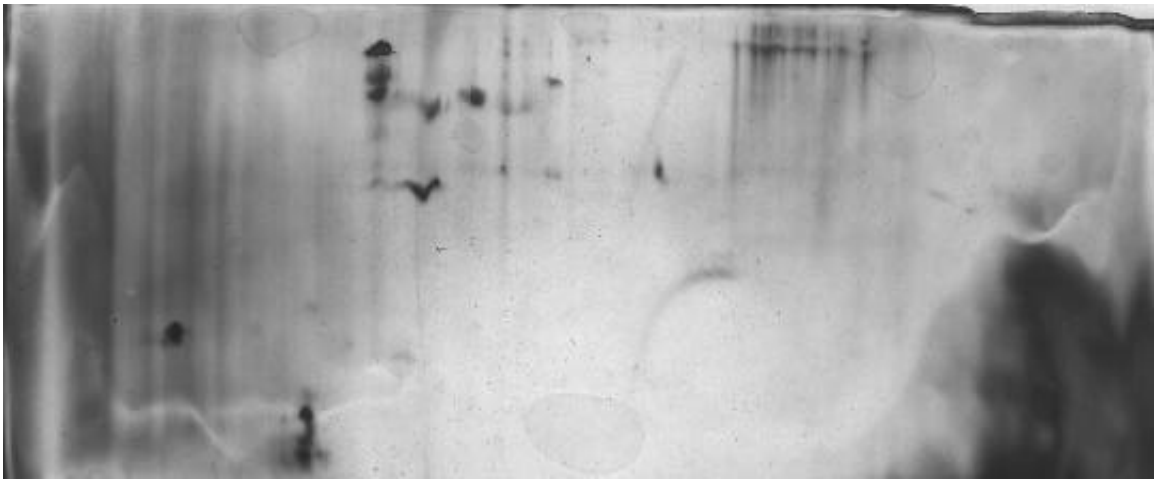
Shown below (Figure 2.1) are structural proteins of bacteriophage Felix O1 that have been isolated using two-dimensional gel electrophoresis (2-DE). Attempts were made to identify each protein by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS; see chapter 4 for more details). Several technical problems were encountered during the course of these experiments. The 2-DE gels showed strong background staining. Also, the mass spectrometer that was used had marginal resolving capabilities that made protein identification difficult. Although we were able to narrow identification to a half dozen proteins in some circumstances, we were unable to positively identify any one protein. The extent to which the protocol used to digest the proteins and recover the peptides may have hampered identification is unknown. Facilities, external to Virginia Tech, were then contacted due to the lack of equipment and expertise on campus. The high cost of protein identification, which in some circumstances exceeded $250 per protein, precluded us from finishing the proteomics based work.

In order to elucidate which ORFs were functional coding sequences it was then necessary to investigate the transcripts produced by bacteriophage Felix O1 during the lytic cycle. To further characterize these ORFs into early, middle and late expression, gene expression at three distinct time points during the lytic cycle was studied. Preliminary results of these expression experiments revealed many problems that were encountered (Figure 2.2). It was immediately apparent that the samples had been contaminated in some fashion as suggested by the presence of bands at time 0 (Fig. 2.2, lanes 2, 6, 10, etc). It was concluded that the bacterial stock used in these experiments had become contaminated with phage. Also, PCR results showed the presence of DNA at all time points.

Decontamination of all equipment, including micropipettors and laminar flow hoods, successfully prevented contamination of a fresh, phage-free stock of *S*. Typhi. Samples were frozen in ethanol and lyophilized to ensure arrest of transcription at the desired time points. The volume of DNase used was increased as was the incubation time for DNA digestion; however, these refinements did not eliminate all residual DNA. It was necessary to separate the RNA from DNA using TRIzol® following initial nucleic acid extraction. Any residual DNA was then eliminated by using 100 units of DNase and incubated for 120 min at 37°C. It was decided to add this additional step rather than replace the original extraction with TRIzol® due to the large volumes of TRIzol® (1 L/sample) that would be required due to the large number of bacterial and phage cells.

Figure 2.1. Bacteriophage Felix O1 structural proteins isolated by two-dimensional gel electrophoresis. 30 ug of CsCl-purified bacteriophage Felix O1 proteins separated by isoelectric charge for 20,000 VH and by molecular weight using SDS-PAGE.

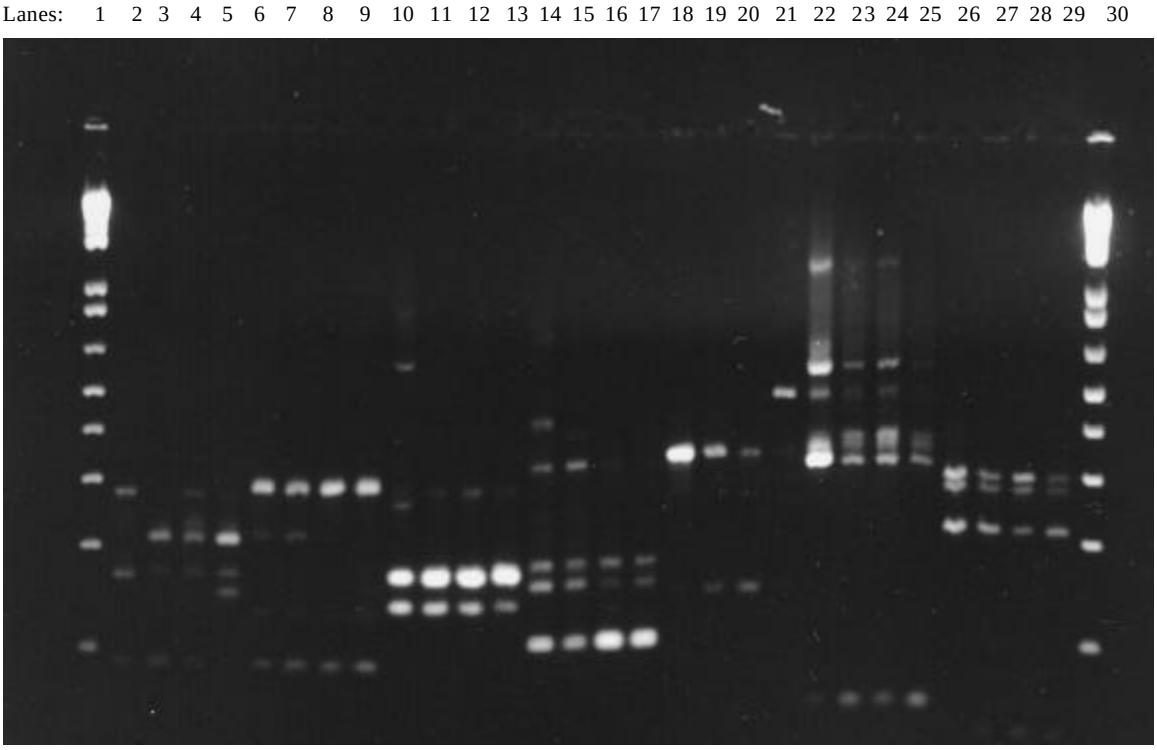
pH 3 pH 10



Further refinements were also needed to reduce the number of bands seen in the gels. In some lanes (Fig. 2.2, lanes 22-24) as many as five bands were present which was evidence that the conditions during amplification were not sufficiently stringent. The general rule of thumb, which suggests that primer annealing temperatures should be approximately 5°C lower than the melting points of the primers, was followed initially. To increase stringency, ORFs with similar optimal annealing temperatures, as suggested by PrimerSelect®, were grouped together in 2.5°C intervals for the RT-PCR reactions. For example, all ORFs with optimal primer annealing temperatures between 50.1°C and 52.5°C were studied using a primer annealing temperature of 52.5°C. This strategy also maintained the ability to run many RT-PCR reactions simultaneously. The amount of each primer used in each reaction was reduced from 30 pmoles to 20 pmoles to help reduce the number of bands.

Hurdles such as these are routinely encountered and overcome during the process of experimentation but are rarely included in manuscripts. The refinements made during my studies have been included in the hopes of facilitating future research of bacteriophage Felix O1.

Figure 2.2. Preliminary RT-PCR results of bacteriophage Felix O1 gene expression. 1 ul of total RNA extracted from samples taken at 0, 5, 10, and 25 min post-infection was used in each reaction with Ready-To-Go RT-PCR Beads®. 5 ul of each product for ORFs 17 (lanes 2-5), 38 (lanes 6-9), 75 (lanes 10-13), 79 (lanes 14-17), 82 (lanes 18-21), 86 (lanes 22-25), and 118 (lanes 26-29) were run on an ethidium bromide stained, 1.5% Agarose-1000® gel for 75 minutes and visualized with ultraviolet light.



CHAPTER 3: TEMPORAL ANALYSIS OF BACTERIOPHAGE FELIX O1 GENE EXPRESSION

Abstract

Bacteriophage Felix O1, also known as enterobacteria phage O1, has been used to type *Salmonella* Typhi and is an excellent candidate for use in bioremedial and therapeutic applications. It has extremely high intra-species specificity and is strictly virulent in nature, unable to undergo lysogeny. To facilitate the development of the bacteriophage for use in these areas, the full sequence of the genome had been elucidated previously. In this work, identification and classification of functional coding sequences via reverse transcriptase-polymerase chain reaction was performed.

All of the 115 putative open reading frames (ORFs) studied were found to be functional. 53.0%, 9.6%, and 18.3% of the ORFs studied were found to initiate expression early, middle or late in the lytic cycle, respectively. Expression of the remaining 19.1% ORFs was evident when the amount of total RNA was increased and when samples were taken at a later time point. Comparisons between bacteriophage Felix O1 and the phage with the most shared homologs, phage T4, revealed many similarities in times of gene expression.

Introduction

Felix O1 (also known as enterobacteria phage O1; International Committee on Taxonomy of Viruses #02.043.0.00.032) is a bacteriophage of the *Myoviridae* family. It has been utilized to type *Salmonella* Typhi and has the ability to lyse as high as 99.5% of *Salmonella* isolates and only 0.3% of non-*S. enterica* species (Thal and Kallings, 1955). Bacteriophage Felix O1 is believed to be a virulent phage, unable to undergo lysogeny. These characteristics have encouraged investigation of bacteriophage Felix O1 as a rapid diagnostic tool in the food industry (Hirsh and Martin, 1983). The addition of luciferase genes (Ulitzur and Kuhn, 2000) provides a quick and easy alternative to classical microbiological techniques.

The Centers for Disease Control (CDC) have estimated that more than 1.4 million cases of salmonellosis occur annually in the United States with more than 500 casualties as a result (Mead *et al.*, 1999). Salmonellosis was the most frequent laboratory diagnosed foodborne infection by the Foodborne Disease Active Surveillance Network (FoodNet) in 2001 (CDC, 2002). Annual economic losses in the US between 0.5-2.3 billion dollars (Frenzen *et al.*, 1999) provides an additional impetus to develop strategies to minimize the staggering health and economic effects of these infections.

As global incidences of drug resistant *Salmonellae* escalate at alarming rates (Threlfall *et al.*, 1993; MacDonald *et al.*, 1987) the scientific community has had to redouble its efforts to combat salmonellosis. Many countries, including the United States, now ban the use of certain antibiotics as growth promotants in hopes of curbing the incidence of resistant strains. It is also widely believed that the over-prescription of antibiotics by medical doctors and their improper use by patients have also contributed to this predicament.

The uncertain future of antibiotics has rejuvenated study of bacteriophage therapy, a field all but abandoned in the West since the introduction of antibiotics in the 1940's. Soothill et al. (1988) have demonstrated the efficacy of bacteriophage in controlling growth of *Pseudomonas aeruginosa* on pig skin, which is routinely used for skin grafts. Additionally, studies regarding the efficacy of bacteriophage Felix O1 as a therapeutic agent in swine have shown statistically significant decreases in the number of *Salmonella* present in tonsil and cecum contents samples following treatment (Lee and Harris, 2001). The work described in this paper was performed in order to facilitate the development of bacteriophage Felix O1 as an agent for *Salmonella* control.

The genome of bacteriophage Felix O1 has been fully sequenced (Whichard *et al.*, 2000) and open reading frames (ORFs) greater than one hundred nucleotides in length predicted by both GeneMark® and the Munich Information Center for Protein Sequences (MIPS) PEDANT® software. GeneMark® predicted the presence of 96 ORFs while MIPS PEDANT® predicted 220. Of the homologs found, bacteriophage Felix O1 shares gene homology most frequently with phage T4.

In order to determine functional coding sequences experimentally, reverse transcriptase-polymerase chain reaction (RT-PCR) was used. All 96 ORFs predicted by GeneMark® and 19 of the genes predicted by MIPS PEDANT® were investigated not only for presence or absence of transcripts but also for time of transcription.

Materials and Methods

The stocks of both *Salmonella* Typhi (ViA, phage type Tananarrive) and bacteriophage Felix O1 were provided by Dr. Hans Ackermann (Felix D'Herrelle Bacteriophage Stock Centre, Universite Laval, Quebec, Canada). Bacteriophage Felix O1 was stored in low salt buffer (0.05M Tris HCl, pH 7.5, 0.05M NaCl, 0.01M $MgSO_4$) while the stock of *Salmonella* was stored in 20% glycerol. Both stocks were kept at –80°C until used.

Bacteriophage Felix O1 was propagated as previously described (Whichard *et al*., 2000) and adjusted to a concentration of $1x10^{10}$ plaque forming units per ml (PFU). Ten ml of Bacto™ tryptic soy broth (Invitrogen; Carlsbad, CA) was inoculated with a single colony of *Salmonella* Typhi and incubated overnight without shaking at 37°C. The following day, 1.6 ml of the starter culture was added to 80 ml of fresh broth and incubated for four hours in a shaking water-bath at 37°C to an optical density (OD) of one. This culture was then subjected to a centrifugation of 4000 x g and re-suspended in 8 ml of broth to a concentration of approximately $1x10^9$ colony forming units per ml (CFU).

To three of the four 2 ml aliquots of *Salmonella*, 2 ml of bacteriophage Felix O1 was added, resulting in a multiplicity of infection (MOI) of 10. Low salt buffer was added to the remaining sample to serve as a control (i.e. time zero). Transcription was arrested in each sample at designated time points (0, 5, 10, and 25 min) by freezing samples in ethanol and dry ice. These times were shown to yield the greatest amounts of early, middle and late mRNA in phage T4 (Karam *et al*., 1994) and were utilized in these experiments due to the frequency of gene homology between the two species of phages. The four samples were then lyophilized overnight (Freezer Dryer Model 75018-18; Labconco; Kansas City, MO) and nucleic acids

isolated using the Micro to Midi Total RNA Purification System® (Invitrogen; Carlsbad, CA) the following day. Due to the high content of DNA in the samples, TRIzol® (Invitrogen; Carlsbad, CA) was then used to separate DNA from RNA. Residual genomic DNA was removed by the addition of 100 units of DNase I (Sigma; St. Louis, MO) and incubation at 37°C for 120 minutes. Next, the RNA was purified using the Micro to Midi Total RNA Purification System® and eluted in a final volume of 225ul RNase-free dH_2O. Additionally, a sample ($1x10^{10}$ PFU) of bacteriophage Felix O1 alone was processed in order to confirm the absence of mRNA in the phage stock.

Primers for the 96 ORFs predicted by GeneMark® and 19 of the ORFs predicted by MIPS PEDANT® were designed using the program PrimerSelect from the LaserGene® software suite (DNASTAR, Inc.; Madison, WI) and are listed in Appendix A. RT-PCR was performed for each gene at each of the four time points for a total of 464 reactions using Ready-To-Go™ RT-PCR Beads (Amersham Pharmacia Biotech Inc.; Piscataway, NJ) in a Mastercycler® gradient thermocycler (Brinkmann Instruments, Inc.; Westbury, NY). Twenty pmol of each primer (One Trick Pony; Ramona, CA) and 1ul of total RNA were used in each reaction. Reverse transcription was allowed to occur for 30 min at 42°C followed by inactivation of the reverse transcriptase by incubation at 95°C for 5 min. Thirty-two cycles of denaturation, primer annealing and strand extension were performed at 95°C (30s), 40-55°C (30s) and 72°C (60s) respectively. Primer annealing temperatures varied and were dependent upon primer attributes. Primers were grouped into 2.5°C intervals based on ideal primer annealing temperatures as predicted by Primer Select. For example, all primers with ideal primer annealing temperatures between 50.0°C and 52.4°C were subjected to primer annealing temperatures of 50.0°C. Ten ul of each of the RT-PCR products were separated via gel electrophoresis using 1.5% Gibco Agarose-1000® (Invitrogen; Carlsbad, CA) at 100V for 75 minutes. The ethidium bromide-stained bands were visualized by ultraviolet light.

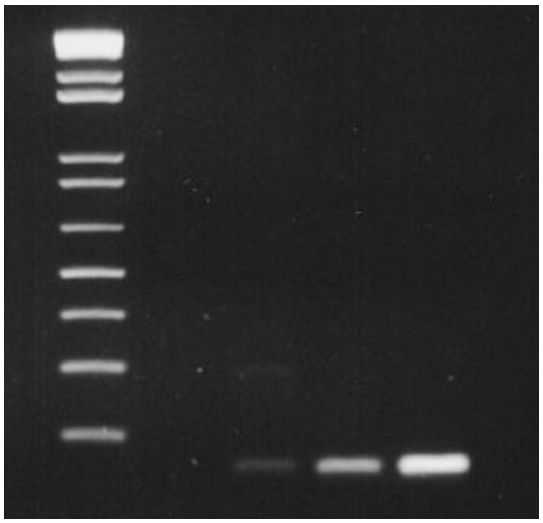
Results

The results of the RT-PCR experiments are shown in Table 3.1. Increasing levels of expression were observed for many genes. Those ORFs that did not show expression for time points 0 through 25 were re-run at time 60 min with 3 ul of template and scored for presence or absence of expression. Representative gels showing early, middle and late gene expression are shown in Figure 3.1.

Figure 3.1. Bacteriophage Felix O1 transcription analysis. The representative RT-PCR products at the indicated time points are shown as ethidium bromide-stained agarose electrophoresis bands. A. Early, increasing transcription of ORF 24. B. Middle, increasing transcription of ORF 291. C. Late, low level of transcription of ORF 159. D. Transcription of ORF 133 seen only at time 60 minutes.

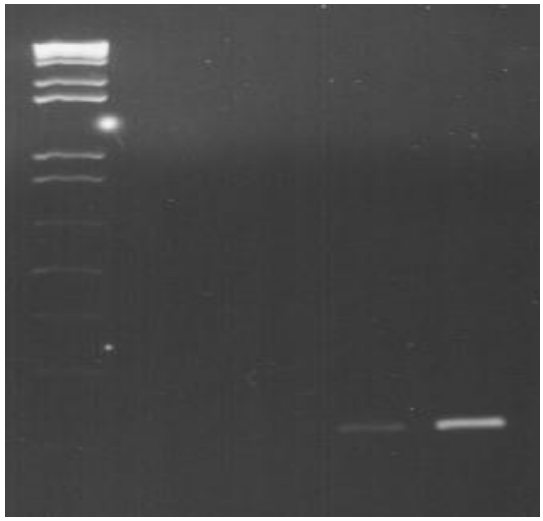
A. ORF 24

Time (min) 0 5 10 25



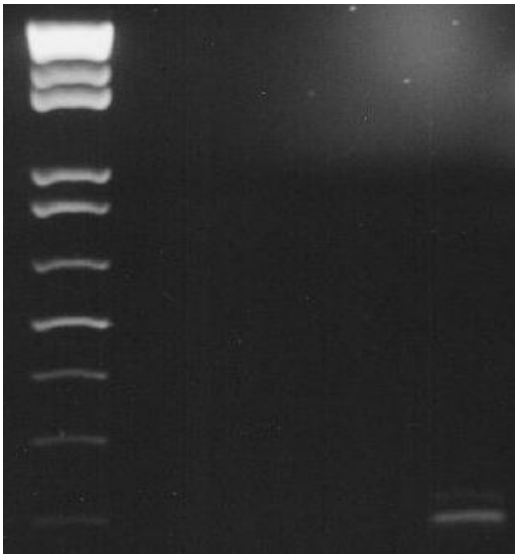
B. ORF 291

Time (min) 0 5 10 25



C. ORF 159

Time (min) 0 5 10 25



D. ORF 133

Time (min) 0 5 10 25 60

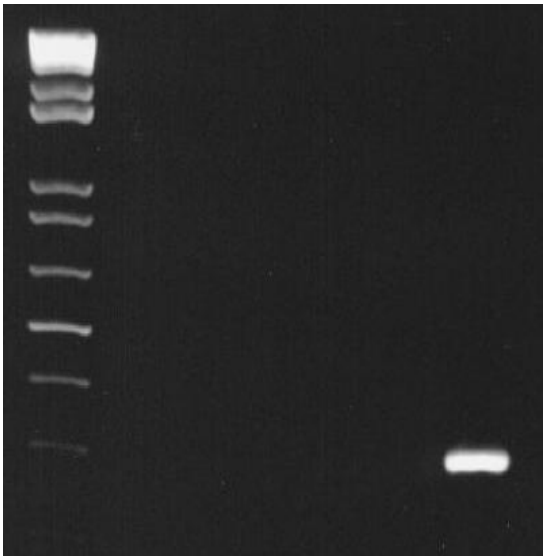


Table 3.1. Bacteriophage Felix O1 gene expression patterns. Levels of transcription at 0, 5, 10, 25 and 60 min (if needed) are listed. None, low, medium and saturated expression are indicated by -, +, ++, and +++ respectively. The methodology allows for only a rough comparison of transcription levels within genes and is not quantitative.

	Time (min)			
ORF		Early	Middle	Late
	0	5	10	25 (60)
1	-	-	-	+
4	-	++	+++	+++
10	-	+	+	++
17	-	+	+++	+++
20	-	-	+	++
23	-	-	+	+
24	-	+	++	+++
28	-	+	++	++
29	-	+++	+++	+++
38	-	+++	+++	+++
43	-	+	+	++
45	-	++	++	++
48	-	+++	+++	+++
50	-	++	++	++
51	-	+	+	++
54	-	+	+	+++
55	-	+	+	++
57	-	+	++	+++
65	-	-	-	- (+)
67	-	+	+	+
69	-	+	+	++
71	-	-	-	++
73	-	-	-	+
75	-	+	+	++
79	-	-	-	- (+)
81	-	-	-	- (+)

Table 3.1 (continued).

	Time (min)			
ORF		Early	Middle	Late
	0	5	10	25 (60)
82	-	+	+	++
85	-	+	+	++
86	-	-	-	+
100	-	+	+	+
109	-	++	++	++
111	-	+	+	+
115	-	-	-	- (+)
118	-	-	-	+
123	-	-	-	- (+)
129	-	+	+	+
131	-	+	+	++
133	-	-	-	- (+)
135	-	+	+	++
138	-	+	+	+
143	-	-	-	+
146	-	+	+	+
148	-	++	++	+++
149	-	+	+	+
150	-	-	-	- (+)
155	-	-	-	- (+)
158	-	-	-	- (+)
159	-	-	-	+
160	-	+	+	++
165	-	+	+	++
168	-	+	+	+
169	-	-	-	+
171	-	+	+	+
173	-	-	-	- (+)

Table 3.1 (continued).

	Time (min)			
ORF		Early	Middle	Late
	0	5	10	25 (60)
174	-	-	-	- (+)
177	-	+	+	+
179	-	+	+	+
180	-	-	-	- (+)
184	-	-	-	- (+)
189	-	+	+	++
190	-	+	+	+
191	-	+	+	++
195	-	+	+++	+++
198	-	-	-	+
199	-	-	+	++
203	-	-	+	++
204	-	+	+	++
207	-	+	++	+++
210	-	-	+	++
211	-	-	+	++
215	-	-	-	+
220	-	+	+	+++
229	-	-	+	++
231	-	-	-	+
235	-	-	-	+
239	-	+	+	++
248	-	-	-	+
254	-	+	+	+
259	-	-	-	- (+)
261	-	-	-	+
262	-	-	-	- (+)
269	-	+	+	++

Table 3.1 (continued).

	Time (min)			
ORF		Early	Middle	Late
	0	5	10	25 (60)
277	-	-	-	+
282	-	-	-	+
285	-	-	-	- (+)
291	-	-	+	++
292	-	-	-	- (+)
293	-	-	-	+
294	-	-	-	- (+)
295	-	+	+	++
296	-	-	+	++
299	-	+	+	++
301	-	+	++	+++
305	-	-	+	++
308	-	+	++	+++
313	-	+	+	++
P12	-	+	+	++
P33	-	+	+	++
P40	-	+	+	++
P49	-	++	++	++
P61	-	+	++	+++
P62	-	-	-	+
P97	-	-	-	+
P102	-	+	+	+
P113	-	-	-	- (+)
P167	-	+	+	+
P170	-	+	+	+
P200	-	-	-	++
P218	-	+++	+++	+++
P250	-	-	+	++
P255	-	-	-	- (+)

Table 3.1 (continued).

	Time (min)			
ORF		Early	Middle	Late
	0	5	10	25 (60)
P257	-	-	-	- (+)
P268	-	-	-	+
P276	-	-	-	- (+)
P298	-	+	++	+++

Discussion

It is immediately apparent that all of the ORFs investigated, whether identified by GeneMark® or MIPS PEDANT®, are functional coding sequences expressed at some point during the Felix O1 life cycle. The majority of these ORFs are expressed early in the lytic cycle (61 of 115, 53%). Almost one in five ORFs (22 of 115, 19%) studied failed to show expression during times 0-25 min post-infection; however, all of these ORFs were visibly expressed at 60 min. Although the expression of these genes may in fact initiate between 25 and 60 min, the amount of total RNA used for the 60 min experiments was three times that for the other time points. Therefore it is also possible that these genes were expressed at levels too low to be detected by this RT-PCR method between times 0 and 25 minutes post-infection.

Due to the presence of functional coding sequences of all 19 ORFs identified by MIPS PEDANT® it is important to establish which of the remaining 105 ORFs may be functional even though only three of these ORFs have shown any significant homology to peptides in the National Center for Biotechnology Information (NCBI) database. ORFs P218 and P305 are both homologous to the hypothetical gene 3.8 protein of bacteriophage T7 (e=2e-07 and e= 5e-12 respectively) while ORF P257 shows homology to a hypothetical protein from bacteriophage phi-Ye03-12 (e=7e-06).

The expression patterns of phage T4 genes have been previously studied (Karam *et al.*, 1994) and those with significant homologies to genes in bacteriophage Felix O1 were compared to our findings. A full list of bacteriophage Felix O1 homologs are shown in Table 3.2. Genes *td*, *frd*, and *nrdA* are expressed early in the lytic cycle in both phage T4 and Felix O1. The *gp37* gene, which encodes for a tail fiber protein, is expressed late in both bacteriophages. Genes *nrdD* and *nrdG* initiated expression late in the Felix O1 lytic cycle but were found to be transcribed early in the phage T4 lytic cycle. This may be due to low but undetectable levels of transcription of the Felix O1 genes. Expression of the remaining homologs was detected at similar times. Additional investigations would be required to more accurately compare expression patterns between phages.

The primary goal of this research was to identify functional coding sequences in Bacteriophage Felix O1. Expression of all 115 ORFs was observed at or before 60 minutes post-infection with the majority of ORFs expressed early in the lytic cycle. Crude quantification of expression within genes revealed increased expression over time for most ORFs. Investigation of an additional 105 putative ORFs is necessary in order to determine the remaining functional coding sequences.

Table 3.2. Bacteriophage Felix O1 homologs (Whichard *et al.*, 2001).

ORF	Homologous Gene and Function	Source of Homolog
1	*rIIA*: affects cell membrane ATPases of host to delay lysis	T4
4	*rIIB*: affects cell membrane ATPases of host to delay lysis	T4
10	Hypothetical 20.7 kDa vs-regB intergenic protein	T4
29	Hypothetical 17.5 KDa tk-vs intergenic protein	T4
75	*p13*: putative lysis protein	APSE-1
79	12: major tail subunit	HK97
111	Complements λ red function	Felix O1
160	Unknown	Sk1
180	*gp37*: tail fiber	TuIa (T4)
184	*gp37*: tail fiber	TuIa (T4)
191	*td*: thymidylate synthetase	T4
195	*frd*: dihydrofolate reductase	T4
218	Hypothetical gene 3.8 protein	T7
220	DNA-directed DNA polymerase	T7
235	*g1*: deoxynucleotide monophosphate kinase	T4
239	4A: DNA primase/helicase	Phi-Ye03-12
254	Gene 6: exodeoxyribonuclease	T3
257	Hypothetical ORF 7903 protein	Phi-Ye03-12
269	*nrd*A: ribonucleoside diphosphate reductase, α subunit	T4
277	*nrd*B: ribonucleoside diphosphate reductase, β subunit	*S*. Typhimurium
285	*nrd*D: anaerobic nucleoside diphosphate reductase	*S*. Typhimurium
292	*nrd*G: anaerobic nucleoside diphosphate reductase	T4
305	Hypothetical gene 3.8 protein	T7

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CHAPTER 4: APPLICATIONS OF BACTERIOPHAGE FELIX O1, POSSIBILITIES AND HURDLES

Future Experiments

The genome of bacteriophage Felix O1 has been fully sequenced (Whichard, 2000) and now many of the functional coding sequences have been elucidated as described in Chapter 3. Although 115 ORFs were investigated using RT-PCR it became readily apparent that functional coding sequences exist beyond those identified by GeneMark®. It is therefore imperative that the remaining 105 ORFs predicted by MIPS PEDANT® be studied. Primers that may be used in these subsequent investigations are listed in Appendix B. Of these ORFs eight are completely contained within larger genes, necessitating the use of a different assay such as the Northern blot. If both overlapping genes were functional, two bands of different sizes would result when using a probe that would bind to the common sequence.

Microarray technology may also be employed in order to confirm the RT-PCR results and to more accurately determine relative mRNA levels throughout the lytic cycle. Microarrays are commonly used to determine relative expression levels of hundreds or thousands of genes simultaneously. Probes specific for each gene studied are hybridized to different areas on a glass slide. Two pools of mRNA are extracted at each of the desired time points. The first pool is then labeled with one of two fluorochromes, Cy-3 or Cy-5. The other fluorochrome would then be incorporated into the second pool of mRNA. Equal volumes of Cy-3 and Cy-5 labeled mRNA from different time points are then allowed to bind to the probes on the glass slide. Following a set of washes to reduce nonspecific binding, a laser is used to fluoresce the samples. The two fluorochromes used emit light at different wavelengths, allowing for quantitative comparison of gene expression.

Since microarrays can only assay expression levels between two samples, multiple experiments would be required to investigate Felix O1 gene expression over four different time points. Each time point would be compared to the three other samples twice for a total of 24 experiments. For example, the first assay would require sample A to be Cy-3 labeled and sample

B Cy-5 labeled. The second assay would then require the labeling of sample A with Cy-5 and sample B labeled with Cy-3. This is needed in order to reduce artifacts inherent to microarrays. The natural progression of these investigations would then necessitate study of Felix O1 proteins.

For many organisms including both eukaryotes and prokaryotes the vast majority of ORFs are found to be strongly homologous to at least one gene in another species. Light may be shed on the characteristics of an organism if the functions of homologous genes are assumed to be similar; however, bacteriophages tend to have very few homologous genes. Felix O1 is a typical bacteriophage in this respect. Only 24 of 220 ORFs (10.9%) show strong homology with genes of other organisms and further study will be required to elucidate important characteristics of the phage. This information could then be exploited in applications focused to combat *Salmonella*.

The identity and relative amounts of proteins produced by Felix O1 can be investigated using two-dimensional gel electrophoresis (2-DE) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS). 2-DE is a method of separating proteins first by isoelectric point, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins according to molecular weight (O'Farrell, 1975). After staining with either Coomassie brilliant blue or silver stain, the individual proteins are excised from the gel and digested with trypsin. The digestion products are then analyzed by MALDI-ToF MS, a highly sensitive tool that can analyze molecules with masses ranging from 100 to one million Daltons (Karas *et al.*, 1988).

A pulsed laser beam ionizes the protein fragments with the assistance of a matrix, alpha cyano-4-hydroxycinnamic acid. A voltage is applied causing the ions to travel through the vacuum and to strike the detector. The detector records the intensities and flight times of each group of ions. These data are represented by a mass spectrum. Lower molecular weight species will travel faster through the vacuum and thus strike the detector before higher molecular weight species. The spectra of fragment masses produced would then be compared to putative spectra of Felix O1 ORFs in order to identify the proteins. The sensitivity of the spectrometer is the key in distinguishing the exact masses of protein fragments. This strategy allows not only for the

identification of proteins but also for confirmation of putative sequence nucleotide limits of the ORFs. In addition, it is possible to identify unique proteins not previously predicted to exist.

To investigate the function of these proteins several methods may be employed. The utilization of 2-DE and MALDI-ToF MS to identify structural proteins as well as other proteins present in the Felix O1 virion could prove advantageous to separate proteins into pre and post replicative protein categories.

Mutation analysis studies, while laborious, have been used successfully in the past to determine functions of specific proteins in phages such as phage T4 (Drake and Ripley, 1994). A single gene is rendered nonfunctional and the effects of the mutation on the phenotype are observed. The gene in question is then assumed to play a role, either directly or indirectly, in producing the wild type phenotype. For example, if a mutant phage did not produce tail fibers the mutagenized gene would be assumed to encode for either the tail fibers themselves, or for other proteins necessary for proper tail fiber production. Examples of agents that may be used to cause missense or nonsense mutations include but are not limited to UV irradiation, hydroxylamine, nitrosoguanidine, and 5-bromouracil.

Bioremediation and Therapeutics

Bacteriophage Felix O1 is a strong candidate for application to many facets of the *Salmonella* problem for both human and veterinary medicine. Classically used to type *Salmonella* Typhi, Felix O1 may also be used as a rapid diagnostic tool (Fey *et al.*, 1978; Ulitzur and Kuhn, 2000), bioremedial agent (Whichard, 2000) or therapeutic agent (Lee and Harris, 2001).

There are several avenues that could be explored in the development of the Felix O1 as a bioremedial tool. Food crops that may be susceptible to *Salmonella* contamination could be sprayed with a mixture containing Felix O1 prior to or during processing. Phage cocktails could also be utilized on the feedlots and in the housing of swine and poultry production facilities.

Application of phages to equipment, chiller water and the final meat product itself may also prove beneficial.

The use of Felix O1 alone as a therapeutic agent has not yet proven to be highly efficacious (Lee and Harris, 2001). Since *Salmonella* is an intracellular pathogen, Felix O1 will most likely have little effect on the bacterium after migration into macrophages. Complications such as phage clearance by the reticuloendothelial system have encouraged investigation and utilization of the phage lytic enzyme system in lieu of a complete phage for therapeutic applications (Nelson *et al.*, 2001). Relatively large phages such as Felix O1, which contain double stranded DNA, require at least one holin along with a lysozyme in order to cause bacterial lysis (Nelson *et al.*, 2001). A coding sequence homologous to the phage T4 lysozyme has been identified in Felix O1 (ORF 75); however, no holins have been located. Holins will most likely be transcribed during the middle and/or late stages of the lytic cycle (Karam *et al.*, 1994). Using the transcription data from chapter two, the number of potential holin genes may be reduced to a more manageable set. The lytic action of proteins from these genes along with the putative lysozyme could then be investigated.

Possible applications of Felix O1 holin and lysozyme products would mirror those of the intact phage. One additional application that may prove beneficial would be the incorporation of holin and lysozyme genes into bacteria normally found in the intestinal flora of the organism for which protection is needed. Constitutive expression of the genes and release of their products may prove useful to combat acute *Salmonella* infections and prevent *Salmonella* colonization. This concept could be extended and applied to protect organisms from many other species of bacteria. A single construct could be engineered to contain lytic enzymes from many phages as well as genes to direct transport of the enzymes to the extracellular environment. It would be important to include genes from several phages that have the ability to infect the same bacteria in order to reduce the appearance of resistant bacteria. Investigations concerning *Salmonella* resistance to Felix O1 have shown that many resistant colonies become rough (MacPhee *et al.*, 1975). Rough colonies are generally less virulent than smooth colonies. Furthermore, those mutants that retained smooth characteristics were found to be more sensitive to antibiotics (MacPhee *et al.*, 1975). . These phenomena are observed due to the ability of Felix O1 to bind to

the LPS core (Lindberg, 1967, 1969, 1970), which is integral to the bacteria. Therapeutic applications of phage may therefore enhance the efficacy of antibiotics versus drug resistant strains of *Salmonella.*

Regardless of how Felix O1 is employed to fight salmonellosis the public may be apprehensive of consuming a virus. Due to possible transfer of bacterial resistance or virulence factors, any phages used must be strictly virulent. Felix O1 is one example of such a phage. Public concerns facing genetically modified organisms (GMOs) and irradiation of meat are widespread; however, education has proven to increase acceptance of irradiation practices (Frenzen *et al.*, 2001). Similar strategies may be needed to inform the public regarding the safety of bacteriophages to humans and their benefits over traditional antimicrobials. The ability of phages to evolve to once again infect previously resistant bacteria is an example of one such advantage.

Overall, the development of phage and their proteins as effective antimicrobial agents has proven a difficult task. Due to the relatively unique genetic makeup of each phage, antimicrobial candidates will require a great deal of investigation. This work represents a continuation in such an endeavor. Only through persistency and ingenuity will strides be made to further exploit one of nature's oldest biotic relationships.

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Appendix A. Primers used in the RT-PCR experiments to investigate bacteriophage Felix O1 gene expression. The prefix P- denotes those ORFs predicted by MIPS PEDANT®. The remaining ORFs were predicted by GeneMark®.

ORF	Upper Primer	Lower Primer
1	AAGGCAGTAGGTGACACAGTTG	TTACCGAAAATTGCCTCTACAGC
4	AAAGAGGGCAAGCTGACTAAAA	GAACGCATCTCAACAGCACCAT
10	TGGTGAGAATAACATGGGTAAA	TTCGCAGCATAATCAAAGTCTCT
17	GTTAAAGAAGGTGCTCCGTATC	ACCGTTGCAGCATCAGTAAAGA
20	AGGTGTCTGGGGAGGAT	CTTGGGAGTTTTTGAATGTA
23	GTACCTAGATAATGGCTGGAAA	TGGAGGTACGGCAACAACT
24	ATCGCAGTAGTAGGAACAAGAA	ATAAATCAACATGCTCAATACT
28	ATGGCTGACTTCTGTAAA	CGTTGTCCTTCGTGGTCTA
29	TGCAGGTATCGGTGGACTTGA	GCTCTTCGACAGGTGGGTAACT
38	CGGCGTGACTGTTAAAAATCCT	TTCCTCTAAATCATCACCGTCAC
43	GAGGATTTACGGGCTGAAC	AACTTGATGCGCTATTACTACAC
45	ATGGCTATTAATAACCGTGAA	GCCGTCTAGCGTGATACC
48	GAATAAAGGCGAAGATGTAAGC	GAGGATAACCGGATTCACGA
50	ATCAGAAAACGGGGCTTATT	AACAGTATTTACAGGGGCTTTAT
51	GCACTGATAACGCCTTTGA	AGTATAGCCATCCTTGAGAAC
54	GAGTGAATCCGTTGTTGTTATC	AGCAGCTTGTCTTCTCAGTA
55	AGCGGCTATCATACTCAAAT	GTTCTCACTTACTAGGGGATGT
57	CCTTCTTATCGGATTTCTTACTA	AATGAGCAGCCTGTTACTTTG
65	AATGCACTGCGCTTGTTAC	AATATCAGTGTTTGCCTTGTTG
67	GATTCACCACGCATACCTG	AGACAACTACAACCATCCTAAA
69	GTTGACTTTGTTATTGCCTACCA	GTTGTTTTCCCTATTTGTGACTTC
71	AATACCGTTAAGTTCATCAAA	GTTAGCCGTTACAGTGGAG
73	TGCCTTTCCAGAACAGATTT	TCAAGAGGCGACAAGGTG
75	TTCCACCACTGCATAGCCTCTG	CTGCCGGAATCCCAACAATC
79	CAGTTGCGCCTTCTTTGGTGTA	AATGGAAGAAGGGTGGTGAGGA
81	TAACCTTTGTGATGCGGAGAT	TAAGGTTAAAGGTATTGTTGTT

Appendix A (continued).

ORF	Upper Primer	Lower Primer
82	GTTCTTACTTGTTTCTGCGTCTG	GAGGGGTCTTCACTATGTTCAAA
85	GACCAAAAGGCTCTCGTAACA	GTCTGCTGCAATGGCTTTCA
86	TCGAGCCGTAGCAATGTAGAAA	AACCACTTAGCTGCCAAACCTG
100	ATGAAAGATGGTAAGAGAACT	TGGCAATTTGAACATAGC
109	GATGGCTCTGGAGAAACAAAG	GGATGAATAAAGAATGCCAACA
111	ATTCAGTGGTATTATGGGTGTT	AGGGCAGCTTCTTCTTCA
115	TCTATGGATGCACTTCTTGAT	TGTAGCAGTCTCTTCTTTCTTA
118	TTATGGCCTGCTGCAAAGAAAC	CTCCCAATCAGAAACCATCACA
123	GATGTTCTTGCTATGGGTCAGT	GCGGCTTTGGCTATTTG
129	GACAGTTCATGCCTTTTG	CATATACTTTGCCTTTGAC
131	ACCCCAACGACTATTATTTTACA	CGCATTTATCGTTAGCATCC
133	TCAAGAGGCCAAAAGAAAAT	CTGTAAAGGTTGCGTAGGTATC
135	GGTACAAGCCGGAGAAGAAGC	AGGCCAGTTGGTACGAAATCA
138	TGGCTTGGCAACAGATTACT	TTAGCCACCTTTAGCGTCAG
143	GACCCAGCCAATAACCCTCTT	TCGTATCTGGCAGCATCACC
146	AGTTATTGGGGTTCCTTCAGC	AACCTCTTTTAATTGCCTTTTG
148	GTAGTAAAGCGTAAAGTCTCTG	ATGGGATAATTGCTGTGA
149	TGAAGAAACAGGGGCTACAGT	TTACCCCTTTGGAGTCTACATCT
150	TTGCTTGGGGTAATGCTCA	GCCCCTGCTAAGATACCTG
155	TAATCTACGACCCATCAGG	GTCTAACTTCTCACAACCAAAA
158	TGGGGCAAGAGATGGTAT	AACAGTTGCCCCTTGAAAT
159	CACTGACTGATGCTGCTGTA	CATTTTTCCTGCTCTTCTAA
160	CGCAGGCTTTAGGTCTTGTGATT	CATTGGCCTTTTTGGGTTCGTAA
165	TACTGGAAAGACCGCTACTACA	CCCCTTCTTATTCGGATTCACT
168	ATGGTTCTTGACGCTATCT	AATGCCCTTTCTCTTCC
169	ACCTCCGACGACACCACTT	CAACACTTCCCCAGACACTAAA
171	CATTTCGGTGACTCTGTGTTA	TGGTGCGTGTGCTCATTA
173	TTTTAAGGGCTGGTGAAG	TGTCTGTTAAAATATCTGTTGTA
174	ACGGCTTTAAACGCTGTA	GTTCTGGCAATAATGTCACC
177	ACAGCGTACACATTAGCAAAAA	TACTTCAGCCATAACACCATAAT
179	GGTAACCAGCTAAAAGTATT	GCTCAGTGGCAAAAGAC

Appendix A (continued).

ORF	Upper Primer	Lower Primer
180	CTAACTGGTGGTGGACATACTC	AGCGGTTCTTTGCCATACATA
184	ATTTCACGGGCACTAACACG	ACCTTCGCCATCACTAACAACT
189	TTGCGGGTAAGACAGACA	TACGAACTCATCATCAATAGG
190	ATTTTTAGTGTTATTGGGATGTA	AAGGGTTTTGGTAAGTTTTG
191	CTTCCCTGCGTGTTTGTAGTT	TGGCTTTAGCACCTTGTCAT
195	TTGACTGAATCTTAGCGAAAAT	TGCCGTGGCCTCAACATA
198	TTTACCTCCCTCAGAAGATTACT	TCACATGTGGCGACGAA
199	AAACAGCCTGAACATCTACACC	AAGATTAAGCATACTGAGAAGA
203	TCCGTTTGCGTGAAGTG	ATCCCAGAAGCGGTTATG
204	GCAAGAACGCGATACTGAT	GATTGTTAAAGGTGTTGCTCTA
207	CTCGAATAACTAGCCCAACAGG	TGACGAAGCCCATGATAAGTG
210	ATTTTGTTTGTATAGGATTGTC	ATTCTTCGTGTTGATGGTA
211	CAAACTTATGAATGATGCTCTC	TCTACCGTTATGATGTTGTTC
215	ACTCCAGTACGGTCTCTTG	AGTCTGCGCACATTCTTAT
220	CTGGGCTGAGTTTTGGTCTACG	GAGCGGTGTGGTTTGAGTTATGT
229	AAAAGAGGTTGCAAAGTGGTA	TTCAGAACGTGGATAATAGTGC
231	AGTCTGATAACGGTGCTTTTG	TTCGTGGGCTTAATTCTTCTAC
235	CTATTGACACGTTTGGGGTTTA	TGTCACGCTCATATTTTGTAGAA
239	AAGGGGCAATTGACCAAGATAA	AATGTCAACTCCGCAACCAGAT
248	ACTCTGCGTTACCTGCTGAA	GCTACACGCTTACCATTTACTT
254	AAGAGCTTGGCCTGACATTTA	GCAGCCTTTTTACCCATCTTTTC
259	CTGGTAAGACTCGGAAGAAGA	ATATTGGCATTGTAGAAGAGTC
261	TGACAGCAAGATTGAAGAACT	CATTAGGTGCTGCATTTGAGAT
262	GTTCTTGACGGCTTGATTTACCT	AACGCATGTCTCAGATACTACC
269	AGACCCGCAGATTGAAGATTTA	TACTGCGTGACCAACTGCCATA
277	GGCAATGGGAAGCAGACAC	TAGCCCTTTCAGAATCACTTTAT
282	ATGGGCAAAACCTAAAACCTGT	CCTCTGCCATAAAATTCTGTAGC
285	GGGGTTACTGCGCTTGATGA	TACTGGATTCTGCCGCCTCTAC
291	TAAGATACTCAACATTCCATACG	GCGCAAGTCATTAGCATA
292	TGCTTTAACAGGGAGTCTTG	AATCACCTTGCCACCCATAC
293	CAGTGGAGAGCCGAGTAAGTC	TCCAGAGTCCCATTTCGTATC

Appendix A (continued).

ORF	Upper Primer	Lower Primer
294	ATTAGGTATGCTCACTGGT	TTATTTAGCCTTCTTATTACG
295	CAGGTTTTATTAGTGATGATGA	CTCTTTTGCTTCTCCAGTAA
296	TCCGTTATTACCCACACTACACA	TATCCCCTTGCCTCTGGTTAT
299	AAGGCGGTAATGGTTGTGAA	GTTACGTTTGTTACTGCCATTG
301	ATGACCCTCTGATTGATTACTTA	GATGTTTTGCTGCTTCTACG
305	GAATACTTTTGCGGAAGATTT	CATGAAGTTCTATTCGTTTTCTA
308	AACATCTTTGGGGGAACTATTA	ACAGGCTGGGTGGTCAA
313	ACTGACGGGTAAGGTTGC	CTGTTTGTTTGGATTGTAGAAG
P12	TGAAGACACCGCAAGAGAAGT	AACGGCAGCATGATTAGCA
P33	CGCTCAGGTCGGGCAAGAAAA	AGGGGGAGTAAGAGGGGGAGA
P40	CGTCGTGTAGTGATTTGCTTAT	CTTTGCCATTTACAGTTATCTCA
P49	GAATAAAGGCGAAGATGTAAGC	GAGGATAACCGGATTCACGA
P61	ACTTTCCCTCTAGGTGTTACG	ATCGCAGAAGCCAGAATA
P62	ACTGGCGTTGTGTTTCTCTT	GTACAGTTTATTTCCGACCAC
P97	ACAAGAAGCACCCAAAGAGAAG	TGCATTGTAGCCAGTGAGTAGT
P102	ATTCGGATGTTGCTTTGGTA	AGGTCTTTGCATAGTCTTGTTTA
P113	AAATGGATATGTGCCTGTCTG	AAAAGCCCTGTCTAAAAATACT
P167	GAACTGGAAAGGCATTACAAAA	CATCAGATAGCGTCAAGAACCA
P170	TTTGCAAGGTTACTACAGAAGA	ATGGATTTTGCAACGAACTC
P200	GAGATACCGCAAATTTTAGCAA	AAACGGAAGAAGAAAGATGAAT
P218	CATGTTATAGCTGCCCTCCAC	ACGCCGTACACTAACACTTCAA
P250	AGAAGACGGTAACAAACTG	GGCTTACAACCAAACTTCTC
P255	AGGTGCTTGTGTAGTTGAG	AGTCTGTCTTTCCCATTC
P257	GCACTATGACCCCGAAACA	GTGCAGCCCATCTACCTCTT
P268	GTTAAGCACGGGGACACAGT	AACAACGTAATTACCTCGCATCT
P276	TTGCGAGGGTTGTGGTGT	TAGCGTGATTTCTCCTTTCTTTAT
P298	CCCCTAAGATGACCTACGA	AACCATTACCGCCTTCACT

Appendix B. Suggested primers for use in additional expression studies. The primers listed below may be used in future RT-PCR studies in order to elucidate transcription patterns of the remaining ORFs predicted by MIPS PEDANT®.

ORF	Upper Primer	Lower Primer
P11	CGCACCTGCAAAGTACCTAATG	CCAGCAGTGCCAACGATGAC
P13	AGAACAGTGCAGGGATTTTGAA	GGATTGCCCAGTCTCTTTGTC
P14	ATGCGTGGTTTTGGGACTA	CACCTTCTGCACATTCTTCTCT
P15	TTACGGCAAACAAATCTGAAG	ATACTCCCGAATGTTACCGTCTG
P18	GTTAAAGAAGGTGCTCCGTATC	ACCGTTGCAGCATCAGTAAAGA
P19	AAGAAGGTGCTCCGTATCG	ACCCCAGCTTTGACAGTG
P21	CCCAAGTAAGTGAGAGCGATAA	TAAAAGCCGTCACGATAGAACT
P22	TTACCACGACCAAAAGAAAC	TGGCACAGCACATTATCC
P25	TATGGAACAATCGCAGTAG	TTTTGAAACGCTCCATC
P26	TGCTTACGTGGCTTCATCTC	TTTTCGTGTCTTTTTATCTCTGG
P27	TTACGTGGCTTCATCTCTG	GTATCACGTCCGAACATTT
P31	CTCAGGTCGGGCAAGAAAAATC	GGGGGAGGGGGAGTAAGAGG
P32	CTGTAGAGAAATCTATAGAGAA	GGGCTGGGCTGGCTCTC
P33	TTTTAGAATTGTGCAGGTAGGT	AATTTTTCTCTTGACTTTTG
P34	AGCCCCTCTCCCCCTCTTACTCC	ATGAACGGGCGCACACGACTAC
P35	GAAGCTGACTATTGCAACTATGT	GTAAATTGCTGTCTGCCTTGTAA
P36	AAGAATTTTACAAGGCAGACA	TACCGCTTCATAAATAATAGTG
P37	CAAAAATGAAAACTGTAGACG	CACGCCGCCTTGATGGT
P39	TTGCCGCGAAAGTTGTC	TTCCTCTAAATCATCACCGTCAC
P41	GGTTAGCAATAGCCTCGTAGA	GCCGCTTTATTTGCTCATCACT
P42	GTGGCTTAAAAGACTACTAC	ATTCCCTCCCCGTTGATG
P44	TGTAAAGTAAGCCCCAGAT	ATAGCCATTTTTAAGTTCTCC
P46	TAGGCAAATCAAAGGTAATG	GTGGCAAAGAAATCAGC

Appendix B (continued).

ORF	Upper Primer	Lower Primer
P47	CGCTGATTTCTTTGCCACTT	CATCACAGACATCACGAAAATC
P52	GCACTGATAACGCCTTTGA	GTATAGCCATCCTTGAGAACTT
P56	GCATTGCTTTTTGAACGACT	AAATCCGATAAGAAGGCTGAAA
P58	AAGAACGCCAGTACCATAACAA	TCCCTAAGAGTAACAAAACCAA
P59	CTTATTCTTTTCGTCAACAACTC	TACCGGTATCATAGCACATTTT
P60	TGGTTTTGTTACTCTTAGG	TGAATGGAACAAACGAT
P63	CGCTCCTTCAGGTCACTACTT	CTTTTAAATTTTTCATGGAGACT
P64	ATAGCTCCCATCATAATCAGTT	TCAGCCCGTGGAAAGGTA
P66	TTCAACAAGGCAAACACT	CCGAAGAGCAACAGGAT
P68	GACTTTGTTATTGCCTACCAT	TAAAAAGCTCAATACTAAACAA
P70	GTTTAAGAGGCCACCATACAG	ACTAGAGCGTGAACTTGACCT
P72	CCAAGCAAAAACAGGACAA	TAGCCGTTACAGTGGAGATG
P74	ACAATGCGTACAAGAGAAC	CTAAGGAGAAAACTATGAAACT
P78	CAGTTGCGCCTTCTTTGGTG	ACAGGTGCGACAGGTTAC
P80	CTGTAAAGCAACTCATCACTA	GGCTATTTTACCGCTTACCTAT
P83	CTGCAATAGAGAAACGAAGTAA	AGGGCAATGGCTCAATCTA
P84	CTAATTGGGAACTACACATA	AAATCAAATAAACCGAAGAA
P91	TAAGAATTGCGGGTATAGAGAA	GGCGGGTGCAGGAGTCG
P92	ATGGTAGCGGTGGGGACTG	TGGCGGCTACGATGAGACTT
P94	TTACAGAAGACCGTTTACAGC	TGGTCGCATACTCACAGGT
P95	GGTGATGCGGCAGACTTTTA	TGAGACCCTTGCATTACCTTATC
P96	TCAGTCGCAGATAAGGTAA	CCGATAAATTGGAGGTTCA
P98	CTACTCACTGGCTACAATG	CCTTCTGCCTTTCGGTTAT
P99	GGATGTGCATTCGGCTGAT	TTGAAATTGGTACTGTGAGAAG
P101	AGCGGGTCTTCCTGTAG	GCCGTTGCTCATGCCTTAGA
P103	TTAGTGCATCCATAGTTTA	AGCTTTCATCTTTTGGTAT
P104	GGTTCGATTCCCTCTGG	TCTTTGCATTGACTTTGAA
P105	CAAGCAAATGATAAAAGGAAAG	AGTTAGCGCACAAGACAATACA
P106	CTGTGCAAGACTTCGTGATG	CTTTTAATAGCCTCTTTCCTT
P107	AGTTTAATGCTGCTTTCGTAT	GTGCCGCTAACTCAACTC
P108	GGTTCGAATCCCTTATCCT	AGAATGGAACTACGAAAGGTG

Appendix B (continued).

ORF	Upper Primer	Lower Primer
P110	TCTACCTTTTGGATTCAG	AGTTATAAGCTTTGTTTCTC
P157	CTAGAGTAATTAATGGGAAGAA	GGACAACAATTTTACCCAACT
P161	TCTAGCGAGGGTATTTCACAGTT	GTTCCCAATCACAAGACCTAAA
P162	TGGTTTGGTTGGTGGTGGTATCT	CTGCTGCGGCTGCTTCTCC
P164	TGGAGAAGCTATCAACATC	AATATCCACCATCTTAGTAGC
P166	GGAATTTGACAGCCCTATGC	GTGACGGTTTGCCCTGATT
P175	TGCCAGAACCAACCCTATG	TGCACCAACTTCCTCAGC
P176	TATTCAGTACACCATTGCTAAG	GTGTTACAGTCCTTTCATCAA
P178	GTATAACGCAACATTTTCAC	GACCCTTGTTAGCATCTGTA
P183	AAGCATTTAGCGTTGAA	ATCTGCCATTATTTTTGTC
P188	TTGCGGGTAAGACAGACACA	TCCCAATAACACTAAAAATACC
P196	GCGGTGTGGGTTGGTATGA	ATGAAAATTGAACACTGCTATG
P197	TCAAAAACTGGCTCAAGAACC	AGAGAACATGGGAGGGACTATC
P201	GAGATACCGCAAATTTTAGCAA	AAACGGAAGAAGAAAGATGAAT
P202	AATCATCTTGTGGGGCATAA	TCTTGATGATTATCTTAACGATG
P205	TGCAGCTTCTGTTTTCTAA	CTGCAAATATTAAAGAGAAGC
P206	ACTGGATACGATGGTTG	AGAGATGACTGATAAGGTAA
P208	GTATCACCAAGCCCAACAATG	GCCTGAGCGTCGTGAAGT
P209	CTTTACGGTCTACTTGTTTCTTGT	CATGCGTTGCTGTAGGTGAC
P212	CAAACTTATGAATGATGCTCTC	TCTACCGTTATGATGTTGTTC
P213	TAACCGTAGTATGTGATTGACC	CTTATAGCCTCCCTCCAGA
P214	TGACCATTTTTGCCGTAGC	CCTCTTATAGCCTCCCTCCAG
P216	GATGTCTCCAATACTCAATGTCA	CTATCAAGAACCGTAAGCAGAA
P217	AGAACCTGAAAGAGATGAAT	ATGCAGCAATGATGAAAG
P219	GAGCATTTCCGTATTTATCAGT	ATTTTATTTACGACCCCGACAA
P232	TTAATCAATGCCGTAGAGA	AGCACCGTTATCAGACTTGT
P233	TTTCCTTATTGACATGCTTATCT	TTCTTCTACGCCCCAATCA
P234	ATTCCAGCTATCTCTACGA	ACACCGCCAGTCATCTC
P236	GAAACTAAAGAGACTCCACAGG	AACTCCATAGCTTCCGTAACTT
P237	GCTGAATGGCTGAGTAGAT	TGTCACGCTCATATTTTGTAG
P238	AAGTTATTGACCACCGTTTAGT	TGTAGTTCTGCCTTCAATCC

Appendix B (continued).

ORF	Upper Primer	Lower Primer
P246	ATGAAATGCGATGGAGAGGTT	GAGGCAGCGCAATGATGA
P247	AATAAATTTCAGGAAGAGTGTT	GAGGCAGCGCAATGATGA
P250	CTACAGCGTATCAATTCTGGCA	AGCCTTCTGGATTTTTCTT
P251	AGAAGGCTGGGATAAAGTCAC	ACCAAGCATAGCCAGTTCATCT
P253	TGGCGGTAACGGTGCTAAT	TCTTCGTCATCTGGGTCTGG
P256	TCAAGGGAACCGTAAAGA	TCAAACCCATCAAGAATCA
P258	CAGGCTGGCATTCTTGTAT	AGTTGTTGTCTGACCTGCTAC
P260	CTCGGAAGAAGATAGTCA	TTGGATAATGTAAAAGTCA
P263	TGGGAATCTTTAAAATCTCAATC	ACAGGTAAATCAAGCCGTCAAG
P264	GAAGCAATCAAATCTCCCTAAA	AACGCATGTCTCAGATACTACC
P265	TATTTCTATGACGGTGTTTATGA	ACACGGTATTTCTCCAAGTTTCT
P266	TTATGAATGACCCGTTACTA	TGTGTTGGCAGATTTCA
P267	GTTAAGCACGGGGACACA	TCATTCTGCCACATATCGTAAAA
P281	AAAAGCTCGTGGTATTGAACA	ATGGCATCTGACGAACTGG
P283	TTTGGGAAGGTTTGGTTGTT	TTTGTTCATTATTGCCCCTTTAC
P284	TTTGGGAAGGTTTGGTT	GAGTTCTCTTAGTTTTTCAGTTA
P289	TAGTATTATTGGGTTGTTCATC	TCATCGTTTTTGGTATTTTA
P290	TTAAAATACCAAAAACGATGAT	AGCTAACCCTAAAAATGG
P297	CCACCAATACGACCCTTCT	GTTCAGTGTTGCAATAGTGTTCT
P300	CTATAAAGCAGCAATGGGTAAA	GACGACGGCTTCAATAACAGT
P312	CTCTGAGCAGTTCGGTTAC	GATTTTCATAGTTTTCTCCAG
P313	ACTGACGGGTAAGGTTGC	CTGTTTGTTTGGATTGTAGAAG
P314	ACTGACGGGTAAGGTTGC	CTGTTTGTTTGGATTGTAGAA
P315	TTGGTAGTGTAGCAGTGTTTGTC	CCTGCTCTTGTGTACCCTTCTC

VITA

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1996-1998	B.S.	Radford University; Radford, Virginia Major: Biology

Professional/Research Experience:

2001-present: Graduate Research Assistant; Department of Biomedical Sciences and Pathobiology, Department of Large Animal Clinical Sciences; VMRCVM; Blacksburg, VA. Responsibilities: analysis of bacteriophage Felix O1gene expression by RT-PCR, study of bacteriophage Felix O1 proteins by 2-DE and MALDI-ToF MS.

1999-2000: Graduate Teaching Assistant; Department of Biology; Virginia Polytechnic Institute and State University; Blacksburg, VA. Responsibilities: Teaching and grading of six biology laboratory courses.

1998-1999: Laboratory Specialist, Department of Neurology; University of Virginia, Charlottesville, VA. Responsibilities: studied efficacy of ketamine treatment of status epilepticus, analysis of GABA receptor expression associated with epilepsy by *in situ* hybridization and Western blots.

Publications:

Borris, D. J., Bertram, E. H., and Kapur, J. 2001. Ketamine controls status epilepticus. Epilepsy Research. 42:117-22.

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