BACTERIOPHAGE FELIX O1: GENETIC CHARACTERIZATION AND BIOREMEDIAL APPLICATION

by

Jean M. Whichard

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Committee Members:

Dr. F. William Pierson, Co-chairman

Dr. Nammalwar Sriranganathan, Co-chairman

Dr. James Claus

Dr. William Hueston

Dr. Timothy Larson

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Abstract

Bacteriophage Felix O1 was studied for applicability as a *Salmonella* intervention. Felix O1 s potential as a *Salmonella* therapeutic was explored, as was its utility as a food application. Felix O1 is specific for and infects most serovars within the genus *Salmonella*. The entire 86.155-kb sequence of the phage's linear, double-stranded chromosome was determined. 213 open reading frames (ORFs) were found, including 23 homologues of phage genes ($e<0.008$). Homology searches do not indicate genes that would be expected to increase virulence of *Salmonella*. Thirteen T4 homologues were found, including *rII*A and *rII*B, rapid lysis genes of T-even phages. Site-directed mutagenesis of the *rII*B region was attempted by homologous recombination with plasmids containing *lux*AB of *Vibrio harveyi*. No Δ*rII*B *lux*AB+ recombinants resulted from the methods tried.

Serial i*n vivo* passage was used to select for a longer-circulating Felix O1 mutant using the modified methods of Merril *et al*., (1996). No difference was found in the clearance of wild-type (WT) and Felix O1 following nine serial passages. Injection of 10^9 pfus yielded 24-hour concentrations of 6.5 and 4.9 $\log_{10}$ pfus/ml plasma for WT and 9^{th} passage, respectively. Both isolates were undetectable in plasma by 72 hours, but remained in spleens at 96 hours.

A large-plaque Felix O1 variant (LP) isolated during *in vivo* serial passage was compared with WT for *Salmonella* growth suppression. Spectrophotometric measurement of BHI cultures indicated greater suppression of *S. typhi* by LP than by WT, a difference not seen with *S. typhimurium* DT104. Both isolates

suppressed 24-hour *S. typhimurium* DT104 growth on experimentally-contaminated chicken frankfurters at 22°C. Untreated frankfurters yielded 6.81 $\log_{10}$ *Salmonella* cfus/g, whereas WT and LP-treated samples yielded 5.01 and 4.70 $\log_{10}$ cfus/g, respectively. Both phages suppressed the *Salmonella typhimurium* DT104 growth ($p<0.0001$), but the isolates did not perform differently ($p=0.5088$). Presence of *Salmonella* caused a higher yield of WT phage than from the uninoculated group ($p=0.0011$), but did not affect LP yield ($p=0.4416$). With *Salmonella* present, the 24-hour LP concentration was lower than WT concentration. This supports the surmised LP rapid-lysis phenotype since T4 rapid-lysis mutants typically exhibit lower burst sizes than wild-type phage.

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I. Scope of the problem

Salmonella infection with non-host-adapted serovars represents a significant threat to the health of humans in the United States and worldwide. Recent comprehensive estimates of human non-typhoid salmonellosis indicate that 1.4 million cases occur per year in the United States, 95% of which are from contaminated foods (Mead *et al*., 1999). American consumers recognize and are concerned about the risk of foodborne salmonellosis from meat and poultry products (White *et al*., 1997), and with good reason (Olsen *et al*., 2000). Depending on the testing method used, prevalence of *Salmonella* on raw poultry products was found to range from 2 to 100%, with a median of 30% (Bryan & Doyle, 1995). Prevalence may be high, but actual numbers of *Salmonella* found in processed poultry products may be quite low. As few as 10 colony forming units (cfus) of *Salmonella* may be expected in a whole carcass rinse from a contaminated chicken carcass (Whittemore, 1993). Although the exact infective dose of *Salmonella* is difficult to pinpoint, 10 cfus may be insufficient to cause disease in humans (Blaser et al., 1982, Glynn et al., 1992). One might wonder why human foodborne *Salmonella* is such a problem, or why poultry products are so often blamed for human disease. Even though few *Salmonella* cells may be present in a processed poultry product, many of the risk factors associated with human disease are related to amplification of a typically low initial pathogenic load. These factors include improper cooling, storage, and cooking as well as handling raw contaminated poultry products (Bryan & Doyle, 1995).

Distribution and consumer factors are important in foodborne salmonellosis, but *Salmonella*-contaminated food products remain the ultimate determinant of human disease (Mead *et al*., 1999, Olsen *et al*., 2000). When one considers the different ways food animal species are slaughtered and processed, it is no surprise

that *Salmonella* isolations from poultry products have been and still are more frequent than isolations from swine and ground beef products (FSIS, 2000). Poultry slaughter and processing is unique in that many animal carcasses are exposed to common surfaces and water sources. Poultry carcasses go through a common scald tank, defeatherer and chill tank. Countercurrent flow in scalding tanks, chlorination of chill tanks, and high water turnover rates in each are used to reduce pathogenic bacteria (James *et al.*, 1992b). However, none of these strategies totally eliminate the possibility of cross-contamination among carcasses (James *et al*., 1992b).

Contamination of raw processed poultry products is attributed to many factors, both on the farm and at processing facilities. However, cross-contamination at slaughter and processing is generally viewed as the most important reason for the high prevalence of *Salmonella* in the final product (James *et al*., 1992b, Lillard, 1990, Clouser *et al*., 1995). One study found the most significant increase in prevalence to occur with chilling in a common chill tank (James *et al.*, 1992b). *Salmonella* prevalence at various steps during processing at a plant in Puerto Rico was determined for 250 samples over a seven-day period. When countercurrent scalding was used, there was only a 4% increase in *Salmonella* positives from pre-evisceration to pre-chill steps of processing. However, *Salmonella*-positive samples rose from 28% before to 49% after chilling (James *et al.*, 1992b). Lillard (1990) found as much as a tenfold increase in prevalence of *Salmonella* on carcasses from the beginning to the end of the production line. A significant increase in *Salmonella*-positive carcasses was found following scalding and defeathering steps for three turkey flocks in a conventional processing plant samples (Clouser *et al.*, 1995). *Salmonella*-positive carcasses increased significantly from 3/14 before defeathering to 10/14 positive after defeathering, as measured in serial dilutions of stomached breast skin. Clouser *et al.* (1995) further defined that conventional scalding and defeathering resulted in a much

higher post-defeathering incidence of *Salmonella* than did kosher or steam-spray non-immersion scalding systems. The nature of poultry slaughter and processing illustrates the fact that even a few *Salmonella*-positive birds can cause a significant problem in the processed product.

The federal government has used two approaches to address the public concern and the *Salmonella* risk associated with processed meat and poultry: implementation of updated disease surveillance systems and enforcement of risk-based pathogen control in food animal processing. The National Food Safety Initiative has contributed to improved surveillance of foodborne disease in this country (Mead *et al.*, 1999). This initiative along with other federal monitoring programs has resulted in enhanced foodborne salmonellosis data, and improved monitoring of emergence of antimicrobial resistance (Mead *et al.*, 1999; NARMS, 2000). Recent comprehensive estimates of human non-typhoid salmonellosis indicate that 1.4 million cases occur per year in the United States, 95% of which are from contaminated foods (Mead *et al*., 1999). *Salmonella* is estimated to be responsible for roughly a fourth of foodborne disease hospitalizations, and almost a third of all deaths attributable to known bacterial, parasitic and viral foodborne causes (Mead *et al.*, 1999). Specific food sources responsible for salmonellosis were not reported in this study, but foodborne salmonellosis outbreaks often involve a poultry product vehicle for infection (Olsen *et al*, 2000).

The United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) has addressed an important part of the *Salmonella* problem by requiring meat and poultry processors to identify and control sources of *Salmonella* contamination in their own plants (USDA, 1996). This has resulted in the mandatory implementation of Hazard Analysis and Critical Control Point (HACCP) plans by large and, more recently, small meat slaughter and processing facilities (FSIS, 2000). In order to establish controlled baseline *Salmonella* data

and determine the efficacy of HACCP implementation, USDA FSIS is testing meat samples for *Salmonella*. The official FSIS method uses classical selective culture followed by biochemical and immunological confirmation (USDA, 1996). FSIS reported that 20% of broiler carcasses and 49.9% of ground turkey samples tested were positive before the implementation of Hazard Analysis and Critical Control Point (HACCP) regulations (FSIS, 2000).

Conditions seem to have improved somewhat since HACCP plans have been required of poultry slaughter and processing facilities (FSIS, 2000). The federal government placed the burden of proof on food processors for the microbiological quality of raw meat and poultry, a decision that has resulted in lower *Salmonella* prevalence in products of meat industries subject to HACCP requirements (FSIS, 2000). FSIS may ultimately broaden its scope to include other aspects of animal production in its regulations USDA, 1996), but the current microbiological requirements rest solely on meat and poultry processors (USDA, 1996; FSIS, 2000). FSIS set initial performance criteria for *Salmonella* and *E. coli* in raw products, in addition to requiring establishment of critical control points (CCP's) and adoption of sanitation standard operating procedures (SOP's). Since implementation of the 1996 final rule, prevalence of *Salmonella* in meat products regulated has decreased, including in slaughtered broilers and ground turkey. *Salmonella* prevalence in broilers slaughtered in large plants went from 20% to 10.3%, and ground turkey prevalence decreased to 34.6% (FSIS, 2000). At the beginning of 2000, at least 90% of large poultry plants were in compliance with *Salmonella* performance criteria (FSIS, 2000). Although the results are preliminary and error associated with prevalence determinations was not reported for the FSIS data, lower prevalence in terms of percent isolations does seem promising.

The FSIS recognizes that *Salmonella* performance criteria will likely become stricter now that all plants (large and small) have implemented HACCP and

preliminary *Salmonella* prevalence data has been collected. This means that processors will probably be held to a more strict performance standard as time goes by. Contamination prevention of the final product was almost addressed in the HACCP regulation by requiring some antibacterial treatment of meat during processing. However, this provision was excluded from the final rule for many reasons, including a lack of consensus for the type of method to be required (USDA, 1996). Some of the methods available to processors for reduction of pathogenic bacteria during processing are presented in the following section.

II. *Salmonella* intervention: chemical, physical and biological methods

Many methods for *Salmonella* decontamination of poultry involve chemical treatment of carcasses or processing waters. Trisodium phosphate (TSP) is one example of a pre-chill chemical application currently used by the poultry industry to reduce bacterial pathogens. Ten percent TSP applied in a pressurized spray can achieve statistically significant reduction of *Salmonella* on experimentally-contaminated chicken skin when compared to tap water-treated controls (Wang *et al.*, 1997). However, others have questioned the efficacy of this application because of pH changes that result from the treatment. It is possible that low colony counts from treated carcasses are falsely low due to pH effects on otherwise culturable *Salmonella*. Bianchi *et al.* (1994) reported a significant decrease in experimental contamination with both *S. typhimurium* and *S. arizonae* using a peroxidase-catalyzed chemical dip. The dip also contained citrate buffer, sodium iodide and sodium carbonate. Thirty minutes were required for a statistically significant reduction over a water placebo rinse as determined by a most probable number technique (MPN), and the reduction was less than or equal to 1.2 $\log_{10}$ for both high (1×10^6 cfus/g tissue) and low (3×10^3 cfus/g tissue) starting levels of contamination (Bianchi *et al.*, 1994). Mullerat *et al.* (1994) found dose-dependent *Salmonella*-reducing efficacy with Salmide®, a sodium chlorite disinfectant, alone and in combination with other agents. The authors

observed a significant 0.7 $\log_{10}$ reduction with 54 mM Salmide® on experimentally-contaminated broiler drumstick skin. A reduction of 2.52 $\log_{10}$ was achieved with this dose in combination with ethylenediaminetetraacetic acid (EDTA), and a 1.74 $\log_{10}$ reduction resulted when Salmide® was combined with sodium lauryl sulfate (Mullerat *et al.*, 1994).

Hwang and Beuchat (1995) experimentally contaminated wing drumettes with S. *enterica* serovars Dublin, Enteritidis and Typhimurium to demonstrate the efficacy of a 0.5% lactic acid/0.05% sodium benzoate wash in reducing the bacterial numbers. Wings were exposed to the wash for 30 minutes at 4°C, and viable *Salmonella* remaining were counted from serial dilutions of a shaking rinse from each wing. The authors observed significant reductions in *Salmonella* over water-wash controls at all timed samplings, immediately following treatment and each day (eight days total) following treatment when stored at 4°C. Morrison and Fleet (1985) reported a 2 $\log_{10}$ reduction of nalidixic acid-resistant *S. typhimurium* and *S. sofia* on chicken carcasses using immersion in 60°C water for 10 minutes. They effected another tenfold reduction by adding 200 ppm chlorine or 2.5% potassium sorbate to the immersion, both with minimal affects on consumer acceptance (Morrison & Fleet, 1985).

Modified atmosphere packaging could be viewed as a form of chemical intervention for microbial growth as well. Unlike other brief chemical processing treatments, it has the advantage of residual activity during distribution and storage, when temperature abuse may result in a significant increase in pathogenic load. Baker *et al.* (1986) suppressed *S. typhimurium* growth on experimentally-contaminated ground chicken using 80% CO_2 with storage at 2°C for 5 days. The air-stored sample growth rose to over 10^5 cfus by the fifth day, whereas 80% CO_2-stored samples at 2°C were held to below 10^4 cfus. Air versus CO_2 storage differences were less dramatic at higher temperatures (Baker *et al.*, 1986).

Nychas and Tassou observed S. *enteritidis* suppression using 100% CO_2 storage in experimentally-contaminated chicken fillets, although the specifics of their contamination methods are not clearly delineated (Nychas & Tassou, 1996).

Many physical treatments have also been attempted for *Salmonella* control in processed poultry (Davidson *et al*., 1985). Steam decontamination has also been studied for use in poultry. A 52% reduction in *Salmonella*-positive samples was reported using steam for 20 seconds on retail portions of poultry meat naturally contaminated with *Salmonella*. However, statistical significance of these data was not provided, and drawbacks to this method include variable success depending on portion of meat treated and exposure time limitations due to organoleptic effects (Davidson *et al.*, 1985).

Atmospheric compression technology has also been used to decrease *Salmonella* contamination, a procedure that is costly and has detrimental effects on the final product (Meyer *et al.*, 1995; Corry *et al.*, 1995). High voltage electricity and oscillating magnetic fields are both very effective in reducing bacterial viability due to membrane and DNA effects, respectively. However, cost of implementing these methods would be very high (Corry *et al.*, 1995). Application of hydrostatic pressure to meat foods has been explored more recently for reduction of pathogenic bacteria (Ananth et el., 1998). Alpas et al. (1999) treated suspensions of *Salmonella* with 345 MPa of pressure for 5 minutes at 25°C using a hydrostatic unit. Reductions of 5.45 to 8.34 log10 were obtained, depending on the serotype of *Salmonella* treated. Gamma irradiation, an effective non-specific means of reducing both pathogenic and spoilage organisms in meat, has gained consumer acceptance over the past few years (Bruhn, 1995; Frenzen et al., 2000). Penetrating ability is also very good, with minimal effects on the final product. However, installation of irradiation equipment and containment facilities is expensive and likely cost-prohibitive for small processors. This would likely

necessitate central irradiation facilities. Use of radioactive byproducts from the nuclear power industry (^{60}cobalt) and nuclear weapons manufacture (^{137}cesium) continue to hinder efforts to improve consumer acceptance. Additional concerns include fear of toxic byproducts in the meat, personnel safety, and environmental implications of irradiation facilities (Corry *et al.*, 1995). Szczawinska *et al.* (1991) also found that irradiation (1.25 or 2.50 kGy) and storage over time under temperature-abuse conditions contributed to higher *S. dublin* growth in mechanically-deboned chicken meat than in non-irradiated samples. Similar trends were seen with *S. typhimurium* and *S. enteritidis* test strains, but these experiments lacked the same statistical significance. The authors suggest that fewer competing indigenous microflora may allow for better growth of *Salmonella* in an irradiated mechanically-deboned product contaminated after irradiation (Szczawinska *et al.*, 1991). This alludes to the possibility of irradiation providing more of a niche for post-irradiation *Salmonella* growth, which would be cause for great concern. Suffice it to say, many chemical and physical avenues are available for *Salmonella* decontamination. However, most methods have drawbacks such as limited efficacy, detrimental effects on appearance of the meat, lack of consumer acceptance and/or high cost.

Biological control measures are also available for pathogen control in foods, but there are no published examples of live organism applications used at processing for specific control of *Salmonella* in processed poultry. There are numerous examples of beneficial microbes used to manufacture foods (Pelczar et al., 1986). In some cases, organisms or their products control spoilage and pathogenic bacteria (Nuckles et al., 1987). For example, lactic acid bacteria have been used as a biological acidulant in fermented sausage products to lower the pH of the final product (Nuckles *et al.*, 1987).

Bacteriocins are bacterial proteins that are toxic to other bacterial genera and species (James *et al.*, 1992a). Nisin is a heat-stable peptide produced by *Lactobacillus lactis*. This product not only prevents growth of other gram positive organisms, but also retards growth of some gram-negative pathogens, including *Salmonella* (Mulder, 1995). Researchers have reported a 3-$\log_{10}$ reduction of *Salmonella typhimurium* on experimentally-contaminated chicken skin when nisin was added to packaging film (Natrajan & Sheldon, 1995).

Competitive exclusion in the live bird is an example of a pre-harvest *Salmonella* intervention using a biological entity (Reid & Barnum, 1982; Seuna *et al.*, 1984). This approach employs beneficial gut flora organisms that are administered orally early in the bird's life. Appropriate use results in prevention of colonization by pathogens. Administering beneficial bacteria to young chickens in order to prevent *Salmonella* colonization and shedding (competitive exclusion or the Nurmi concept) has been used successfully (Reid & Barnum, 1982; Seuna *et al.*, 1984). There is currently one product approved by the Food and Drug Administration (FDA), Preempt®, which consists of a mixture of competitive exclusion bacteria designed for spraying on chicks and poults at the hatchery. Spraying ultimately results in consumption of these beneficial bacteria.

III. Bacteriophages

A. Biology

Bacteriophages are ubiquitous viral particles which infect and sometimes kill their bacterial host. Some phages have a relatively broad intragenus host range, whereas others are very narrow in host range, infecting only specific species or strains of a bacterial genus. Excluding the filamentous phages that can produce progeny without killing the host cell, phages exhibit one of two life cycles. Phages are either lysogenic (temperate) or lytic (virulent), depending on their ability to exist in the bacterial host

without killing it. Virulent phages obligately kill the host in the process of making and releasing progeny, whereas temperate phages can either lyse the cell or exist quiescently in their host in a lysogenic state. Phages in the lysogenic state do not produce progeny, but they may at some point end this coexistence with the host by producing progeny and lysing the cell (Pelczar *et al.*, 1986). Temperate phages such as λ have the capability of incorporating their DNA into the host bacterial chromosome or, such as phage P1, existing in a plasmid form in the host's cytoplasm (Yarmolinsky & Sternberg, 1988). Phages in the lysogenic state are also called prophages. They express the few genes necessary to continue in their lysogenic state, and do not express other genes necessary for host lysis and progeny formation. If conditions are right, a prophage can end this coexistence with the host chromosome and produce mature phage progeny by a process called induction. Various agents that damage DNA, such as chemicals and ultraviolet light, can be used to induce prophages to produce virions (Roberts & Devoret, 1983). Lytic (virulent) phages, on the other hand, obligately lyse the host cells they infect. Virulent phages are incapable of incorporating into the host genome, but instead kill their host in the process of completing their life cycle. However, some lytic phages can control the timing of host-cell lysis, depending on conditions surrounding its host (Mathews, 1994) .

Lysis inhibition (LIN) is an elegant mechanism that T-even phages possess for delaying lysis of the *Escherichia coli* host cell (Paddison *et al.*, 1998). Although obligately lytic, T4, one of the most well-characterized T-even phages, delays lysing the host it has infected if superinfection with other T-even phages occurs. Superinfection occurs when extracellular concentration of phage is high or if the concentration of infected cells is high because progeny emerging from nearby cells can superinfect

(Abedon, 1994). The competitive advantage of this mechanism lies in the greater number of progeny that result when lysis is delayed. This prevents phage from wasting resources by releasing progeny when there are few uninfected host cells. Additionally, this mechanism may ultimately improve survival since more progeny are produced (Paddison *et al.*, 1998).

Lysis inhibition and the genes involved are central to the study of T4 and the very paradigms of molecular biology. The "r factors" were identified as the source of mutation resulting in a rapid-lysis phenotype (Hershey, 1946), and the "r" designation came to be used for genes whose mutation was associated with rapid lysis. Genes *rII*A and *rII*B are among the most well-characterized genes of the T-even phages (Mathews, 1994). Mutational study of *rII* was convenient because of the means available for screening. Mutants were readily identified by their larger, clearer plaque phenotype, and they were unable to plaque on strains of *E. coli* harboring phage λ in the lysogenic state. The consequent focus on *rII* substantially contributed to characterization of classical middle promoters and specific *rII* mutations that conferred a rapid-lysis phenotype (Stitt & Hinton, 1994; Snyder & Kaufmann, 1994; Paddison, 1998). The rapid lysis effected by *rII* mutants appeared to be due to an escape from the mechanism of lysis inhibition, but the exact nature of the involvement of *rII* remains a mystery (Paddison, 1998).

Historically, it was thought that *r* genes, including *rII*A and *rII*B, were necessary for lysis inhibition to occur. Phages with functional *rII* genes exhibited lysis inhibition given the appropriate conditions. Conversely, phages mutant in *rII*A or *rII*B produced a rapid lysis phenotype that was thought to be due to freedom from lysis inhibition. These *rII* mutants produced larger, clearer plaques than wild-type phage on solid media, and

a rapid-lysis phenotype in liquid culture. Rapid lysis seen in infections with *rII* mutants occurs regardless of superinfecting phage, so it was assumed that mutation conferred a freedom from lysis inhibition. One model purported that *r* genes may in some way be involved in preventing egress of lysozyme (product of gene *e*) into the periplasmic space. Lysis inhibition would occur by somehow blocking passage of lysozyme though a membrane holin (product of gene *t*). Preventing lysozyme from accessing the peptidoglycan is a successful means of delaying host-cell lysis. This theory is partly true, but more recent data has further defined the specific roles of genes *rI*, *rII*A, *rII*B and *rIII* in lysis inhibition and rapid lysis (Mathews, 1994; Paddison et al, 1998).

It is more likely that *rI* is responsible for true lysis inhibition rather than *rII*. A few key pieces of evidence contradict the previous theories of *rII* involvement in lysis inhibition. For instance, presence of *rI* is absolutely required for the mechanism to be active (in all strains tested), whereas *rII*B does not always have to be present for lysis inhibition to occur (required on *E. coli* B, but not required for lysis inhibition in strain K). Also, *t* mutants, that lack a functional holin that is thought to be necessary for egress of lysozyme, are constitutively lysis-inhibited. The possibility of *rII* interaction with gene *t* holin seems less likely since mutants of *rII* can partially suppress the lysis inhibition phenotype of *t* mutants. That is, *rII* mutation confers rapid lysis by some mechanism. If this were solely due to an absence of interaction with *t* holin, then an *r* mutant would not negate the constitutive lysis inhibition seen in the *t* mutant. This latter evidence instead supports *rII* mutant rapid lysis by a mechanism other than freedom from lysis inhibition. Mutation of *rI* (and *rIII*) are not able to confer lysis in the lysis-inhibited *t* mutants, which supports their involvement with the *t* holin in the classical model. The rapid lysis seen in

rII mutant infections may have appeared similar to freedom from lysis inhibition, but disruption of cellular energetics and consequent rapid cell death likely occurs by a separate mechanism than classical lysis inhibition (Paddison *et al.*, 1998).

B. Bacteriophage-based *Salmonella* Detection

Bacteriophages have been exploited as a detection method for bacteria (Sarkis *et al.*, 1995; Pearson *et al.*, 1996; Ulitzur & Kuhn, 1987). The fact that bacteriophages only infect live bacteria offers an advantage over immunological and molecular biological detection methods, which are independent of viability. In terms of foodborne disease, viable organisms that will replicate given the opportunity are of utmost concern. Techniques are available for construction of recombinant phages expressing bioluminescence by inserting luciferase (*lux*) genes (Ulitzur & Kuhn, 1987). For example, luciferase-coding mycobacteriophages have been developed to detect viable mycobacterial cells (Sarkis *et al.*, 1995; Pearson *et al.*, 1996). Since live cells are required for phage infection and subsequent expression of *lux* genes contained in the phage DNA, viability of bacterial cells can be assessed, a useful adjunct in antimicrobial susceptibility testing (Sarkis *et al.*, 1995; Pearson *et al.*, 1996). Ulitzur & Kuhn (1987) described the cloning of bacterial luciferase (*lux*) genes into the genome of lambda phage. Infection of *E. coli* by the recombinant phage L28 resulted in *lux* expression and bioluminescence which could be measured within a short period of infection. As low as 10 cfus/ml were easily detected. Loessner *et al.* (1996) developed a luciferase-coding recombinant A511 phage for detection of viable *Listeria* in foods and environmental samples.

In meat processing settings, Kodikara *et al.* (1991) were able to detect bacterial contamination of work surfaces using a luciferase-producing recombinant bacteriophage specific for the Enterobacteriaceae. This testing procedure required about one hour, versus at least 18-24 hours required for more traditional culture techniques. The application was useful not only for inanimate surfaces but also for carcasses when contamination levels exceeded 10^4 cfus per gram of tissue or square centimeter of surface sampled. Four-hour enrichment increased the sensitivity of the test, and the relationship between traditional log number of colony forming units and bioluminescence remained linear (Kodikara *et al.*, 1991).

Researchers have used luminescent phages to detect low numbers of *Salmonella* in artificially contaminated sewage, soil and water (Turpin *et al.*, 1993; Stewart *et al.*, 1989). This *lux*-MPN method employed a recombinant P22 phage and provided identical results compared to a traditional most probable numbers determination (Turpin *et al.*, 1993; Stewart *et al.*, 1989). Bennett *et al.* (1997) developed a Felix O1 biosorbent capture system for subsequent detection of *Salmonella* by PCR, and IDEXX (Westbrook, Maine) produced a phage-based detection system for *Salmonella* in foods. The phages in the IDEXX test express ice nucleation gene when *Salmonella* is present. The IDEXX *Salmonella* BIND® assay and technology has since been purchased by Biocontrol, Incorporated (Indiana, Pennsylvania), and the assay is used by meat processors and agencies including ConAgra, Keebler and the Iowa Department of Agriculture. There is a fixed cost associated with equipment necessary to run the assay ($3450 for the BIND® SuperCooler), and the cost for each test is about $5.50 (Goans, 2000).

One of the problems in developing phage-based detection methods is finding a phage or phages with the appropriate host range. Although relatively few *Salmonella* serovars are responsible for the majority of human disease (Centers for Disease Control and Prevention, 1999), a desirable detection method would detect the majority of the over 2,000 serovars, such that emerging serovars responsible for infection could be identified. Chen and Griffiths have addressed this problem by using a mixture of many phages for the development of their assays (Griffiths, 1998). Marker gene expression (luciferase) appears to show a linear relationship with increasingnumbers of *Salmonella* numbers (Chen & Griffiths, 1996). Hirsh and Martin (1983) addressed the wide variety of *Salmonella* serovars by developing a detection method that employed phage Felix O1, which has broad host-range within the genus *Salmonella*. Their assay, designed for detection in milk, employed a high-pressure liquid chromatographic (HPLC) measurement of Felix O1 progeny following incubation with the suspect product. Phage-treated samples that contained *Salmonella* produced a positive detector response not seen in the uncontaminated controls. Although as few as 5 organisms were detected using this method, some strains could not be detected (including one of the *S. agona* and three of the S. *enteritidis* strains). The authors suspected that the potential for false negatives would make this assay less attractive for industry-wide use (Hirsh & Martin, 1983). Ulitzur and Kuhn (2000) also succeeded in producing a Felix O1 recombinant for *Salmonella* detection by inserting bacterial luciferase genes into an essential region of the Felix O1 chromosome. Their recombinant maintained the broad intragenous host range and relative specificity for the Salmonellae. However, no data was provided regarding its applied performance (Ulitzur and Kuhn, 2000).

C. Bacteriophage-based *Salmonella* intervention

Bacteriophage data as it pertains to foods consists mainly of prevalence studies and exploitation of phages as a detection method for bacterial contamination (Loessner *et al.*, 1996; Kodikara *et al.*, 1991; Kennedy *et al.*, 1986). Nevertheless, there are a few examples of attempted phage-based bacterial intervention in food animals and meat products. In fact, phages are found in many food products, including meats and even some further-processed foods (Kennedy *et al.*, 1986). Greer and Dilts (1990) tried to use phage to reduce spoilage of ribeye steak by *Pseudomonas* species. The researchers combined seven *Pseudomonas* phages in order to maximize efficacy against a wide variety of naturally occurring isolates. *Pseudomonas* in stomached samples was counted daily, and shelf life was defined based on appearance daily by an experienced sensory panel. On some experimental days, significantly lower numbers of *Pseudomonas* were isolated from phage treated steaks. However, sensory panel results indicated that no appreciable shelf-life extension was achieved by phage treatment. The authors attribute the shortcomings of phage in prolonging shelf-life to the high percentage of naturally-occurring isolates that were not susceptible to the "cocktail" of 7 phages. Greer and Dilts (1990) also lamented the dearth of *Pseudomonas* phages with broad host range.

Research addressing the potential of phages for pre-harvest pathogen control in food animals is limited. One published attempt at phage-mediated reduction of *Salmonella* in poultry was a feeding trial performed in chickens (Berchieri *et al*., 1991). When one-day-old chicks were dosed concurrently with 10^6 cfus of a nalidixic acid-resistant *S. typhimurium* and 10^{11} pfus of F2.2 phage, a significant reduction in mortality resulted over a 24-day period compared to birds dosed with *Salmonella* only. In a similar experiment, transient decreases in *Salmonella* were noted at

various points in the gastrointestinal tract, but the effect disappeared after 3 hours post-inoculation (Berchieri *et al*., 1991). A dose-response experiment showed that 10^{11} pfus was necessary to significantly decrease mortality over untreated controls when 10^{8} cfus of *S. typhimurium* strain F98 were given concurrently, whereas 10^{9} pfus did not significantly decrease mortality (Berchieri *et al.*, 1991). Berchieri and colleagues went on to address mortality in chickens and colostrum-deprived calves suffering from K1+ *E. coli* septicemia using a K1-specific phage parenteral treatment. Phage administration dramatically reduced morbidity and mortality in chickens, and may have controlled bacterial proliferation to a certain degree in colostrum-deprived calves (Barrow *et al.*, 1998).

Though there is little data addressing use of phages for *in vivo* control of *Salmonella*, there are published attempts to use phage to control combat *E. coli* infections. Research in phage therapy for bacteremia in the early 1900's was frought with inconsistent results and failures which are now attributed to a lack of understanding of phage physiology and host range (Merril *et al.*, 1996; Levin & Bull, 1996). Potential therapeutic uses for bacteriophages were ultimately eclipsed by the advent of antibiotics in the 1940's. However, interest in parenteral use of these viruses was recently renewed due to emergence of multiple drug-resistant pathogens (Merril *et al.*, 1996).

Studies performed by Smith and Huggins in the 1980's showed promise for the use of coliphages in the treatment of *E. coli* septicemia in mice and perinatal diarrhea in livestock, sometimes achieving an even greater reduction in mortality using phages versus antibiotics (Levin *et al*., 1996). However, dosage amounts and schedules did not appear to be optimized

for the antimicrobials used. These researchers were able to improve survival rates in mice suffering from *E. coli* septicemia by administering a K1-specific phage (Levin *et al*., 1996). Ninety-nine percent of phage-treated mice survived versus 3% of controls when a multiplicity of 10 pfus per cfu of *E. coli* was given, both given intramuscularly in separate legs. Splenic and circulating bacterial concentrations were decreased and held at or below the minimum detectable level (10^2 cfu/g tissue) in phage-treated mice, versus a progressive rise from 10^4 to at least 10^6 cfus/g tissue in untreated controls (Levin *et al*., 1996).

Conversely, Reynaud *et al.* (1992) did not succeed in their attempted "phagotherapy" of enteropathogenic *E. coli* enteritis in rabbits. Although their lytic phage CF0103 was effective against *E. coli* 0103 in an *in vitro* setting, reductions in mortality were not achieved at an MOI of 100. Both phage and bacteria were administered at the same time to experimental animals by gastric gavage. Phage treatment apparently delayed onset of mortality, but overall mortality was 90% in experimentally-infected animals whether they were given phage or not. The authors allude to emergence of a resistant subpopulation of organisms as the culprit for mortality in phage-treated groups, but they also suggest that nuances associated with conditions in the gastrointestinal tract may be the reason for low efficacy (Reynaud *et al.*, 1992).

Merril *et al.* (1996) successfully diminished clinical signs of *E. coli* septicemia in one-week-old BALB/c female mice using a long-circulating mutant of a virulent λ phage, bacteria and phage given intraperitoneally. Symptom scoring results on a progressive scale of 1 to 5 improved with phage treatment. Symptom scores for treated mice averaged 1, versus an average of 3 for the untreated group. Phage was administered at a

multiplicity of 100 plaque forming units (pfus) per colony forming unit (cfus) of bacteria. Neither statistical significance nor counts of circulating bacteria were provided (Merril *et al.*, 1996).

The scope of phage-based intervention is increasing. Alavidze *et al.* (2000) studied mixtures of lytic phages for their efficacy in decreasing *Salmonella* on contaminated surfaces. The phages they studied include members of the Myoviridae and Siphoviradae. The researchers targeted a range of serovars including those isolated from poultry species. The researchers claim that a "cocktail" of three phages at an MOI of 0.01 reduced surface contamination from 1×10^7 cfus to undetectable levels in less than 48 hours. Intralytix, the company conducting this research, is pursuing poultry applications for this technology (Travis, 2000).

D. Bacteriophage Felix O1

Bacteriophage O1 (also called phage Felix O1, 01 or 0-1) is a member of the A1 group of the Myoviridae (Ackermann, 1973). It was first referenced by Felix and Callow and is the anti-O phage used in the original scheme for the identification and typing of *Salmonella typhi*, (Felix & Callow, 1943). Phage O1 is fairly unique among *Salmonella* bacteriophages by virtue of its broad host range within and its specificity for the genus. Of 15 serogroups tested in one study, all but 2 included members that could be productively infected by phage O1 (Kallings, 1967). Furthermore, Kallings found that in general, other gram negative enteric bacteria are resistant to lysis by phage O1. This specificity and broad intragenus host range can be contrasted with many other *Salmonella* phages, including epsilon 15, P22 and many of the P22-like phages used in *Salmonella* phage typing (McConnell *et al.*, 1992; Merril, 1996; and Callow, 1959).

The somatic receptor for phage Felix O1 was deduced by observing different strain susceptibility to adsorption and lysis by phage O1, and was definitively determined by mutational analysis of the lipopolysaccharide core (Hudson *et al.*, 1978; MacPhee *et al.*, 1975). Phage O1 adsorbs to the bacterial cell surface by virtue of a terminal N-acetylglucosamine in the lipopolysaccharide core, and the adsorption rate constant is determined by presence of this structure in the core and accessibility depending on O-side chain length. Since the core structure is a common feature of many *Salmonella*, it is logical that phage O1 adsorbs to and infects most members of the genus. Felix O1 phage resistance occurs when: i. the LPS core N-acetylglucosamine receptor is sterically blocked, ii. when presence of a prophage prevents infection (Kallings & Lindberg, 1967), or iii. When a mutation affects structure of the LPS core Hudson *et al*., 1978). Mutations that affect core LPS structure include *gal*E, *gal*U and those in some *waa* genes (Hudson *et al*., 1978).

Felix O1 can be very sensitive for routinely identifying *Salmonella* isolates. Kallings (1967) used a spot test to determine susceptibility of various *Salmonella* strains to a standard dilution of Felix O1 phage. He applied a loopful of phage suspension (1.9×10^{11} pfus/ml) to bacteria distributed over a small area of an agar plate using a loopful of liquid suspension (1.0×10^{8} cfus/ml). Following incubation at 37°C for 5 hours, cultures were screened for confluent lysis. Using this method, Kallings obtained positive reactions for 99.9% of one group of 2,260 *Salmonella* isolates. Another panel of 211 isolates yielded 99.5% positives. The percentage of these 211 *Salmonella* detected by this method decreased dramatically when the titer of the Felix O1 suspension used for spotting was lower, and only 47.4% of strains gave positive results when the

concentration of the Felix O1 suspension was 2.0 x 10^7 pfus/ml. Although use of a high-titer Felix O1 suspension resulted in a highly-sensitive assay, this came at the expense of more false-positive non-*Salmonella* isolates. In addition, group E *Salmonella*e are generally resistant to phage O1 (Kallings, 1967). Tsang et al. (1997) criticized Felix O1 phage's reliability as a screening method because of the phage's inability to detect some members of the genus. These authors succeeded in isolating the M105 mouse monoclonal antibody that detects both Felix O1-sensitive and resistant strains of *Salmonella*.

Although Felix O1 is capable of lysing most *Salmonella* serovars, applicability of Felix O1-based detection methods and/or interventions depends on the serovars likely to be encountered and the impact of false-negatives. Hirsh and Martin (1983) recognized the shortcomings of their Felix O1-based detection assay because key strains were not detected. Conversely, Stefanov *et al.* (1987) promoted use of Felix O1 as a supplemental detection method for poultry because many of the major poultry isolates were successfully detected.

IV. Scope of the work performed

The niche that Felix O1 occupies as a natural parasite of most *Salmonella* serovars made this phage a logical candidate for phage-based *Salmonella* intervention. Emergence of multiple drug-resistance coupled with the intensive production and processing of food animals that may harbor *Salmonella* have inspired the research described here. Though many modalities exist for the control of *Salmonella* infections and contamination of food products, this zoonosis is still an important problem. It is possible that phages such as Felix O1 represent the future of fighting bacterial disease, and they may be applicable both in preventing transmission as well as fighting infection once it occurs. The work

done in this project was designed to test two specific hypotheses important to the future of applied bacteriophage research.

A. Hypotheses

Based on published work and progress in the area of bacteriophage research, I hypothesized that:

1. *Salmonella* contamination in poultry production or processing can be reduced through the application of bacteriophage Felix O1.
2. Phage Felix O1 can be mutagenized to increase its virulence and improve its applicability as a salmonellacidal agent.

B. Specific aims

The following specific aims were addressed in order to test the above hypotheses. The specific goals of this work were to:

1. Determine Salmonellacidal activity of wild-type phage Felix O1 as an application for the poultry industry.
2. Determine the nucleotide sequence of phage Felix O1 necessary for the purpose of generating recombinants.
3. Mutagenize genetic loci of Felix O1, and select one or more recombinants based on stability and virulence against *Salmonella*.
4. Determine the potential of a recombinant Felix O1 as a biological *Salmonella* intervention for the poultry industry.

The following dissertation includes chapters describing work pertaining to each of the above specific aims. Chapter 2 summarizes our sequencing of the Felix O1 chromosome. This includes the entire 86.155-kb genome sequence as well as putative functions of open reading frames found in the genome. Chapter 3 describes the attempts to mutagenize the *rII*B region of Felix O1, an approach that was unsuccessful. Chapter 4 includes our attempts to isolate a longer-circulating

variant of Felix O1 through *in vivo* serial passage in mice. It was from these *in vivo* studies that several large-plaque (LP) variants were isolated. Of five isolates tested, the large-plaque phenotype on solid media was stable for two of the variants. Of these two, one was consistent in producing slightly better suppression of *S. typhi* growth in liquid culture, as determined turbidometrically. Although no specific mutants were obtained using the methods in Chapter 3, the stable LP variant proved useful in applied experiments. Chapter 5 describes the different behavior of LP and WT, and culminates in efficacy results for each isolate in an experimental contamination model. The efficacy of WT and comparative efficacy of WT and LP is expressed in terms of ability to suppress growth of *Salmonella typhimurium* DT104 in temperature-abused poultry frankfurter samples. Conclusions and future directions for this line of research are presented in Chapter 6.

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Chapter 2: Bacteriophage Felix O1: Genetic Characterization

I. Abstract:

Bacteriophage O1 (also called phage Felix O1, 01 or 0-1) is a member of the A1 group of the Myoviridae (Ackermann, 1973). It is the anti-O phage used in the original scheme for the identification and typing of *Salmonella typhi* (Felix *et al*., 1943). The researchers have pursued this phage as a bioremedial application for the intervention of *Salmonella* contamination and the treatment of salmonellosis. In order to generate recombinants and address safety issues, the genome was sequenced. Felix O1 contains a linear, double-stranded DNA chromosome that is 86.155 kilobase pairs in length. Open reading frame analysis revealed homology with several other phages, including many putative T4 homologues. However the overall organization of the chromosome is quite different from that of T4. Chromosomal features include homologues of the T-even phage *rII* genes and a putative tail fiber gene. However, no T4-homologous introns were found. Since the phage lacks significant homologies with genes associated with integration, a lytic life cycle is surmised.

II. Introduction

The emergence of multiple drug-resistance among bacteria such as *Salmonella* reminds us of the vigilance necessary to successfully combat bacterial disease. The process of finding and marketing safe & efficacious antimicrobials from the thousands of promising candidates is long and tedious. In some cases, the success of a new antimicrobial is quickly supplanted by emergence of pathogens made resistant by the very drug developed to treat them (Heisig, 1996; Reyna *et al.*, 1995).

Antimicrobial resistance and the intricacies of developing new antimicrobials for treating resistant infections are causing researchers to look for alternatives to antibiotics. Some scientists are revisiting bacteriophage-based interventions for bacterial diseases (Merril *et al*., 1996). Before the advent of antibiotics, Felix

D'Herelle among others pursued this avenue (Merril, 1996; Levin & Bull, 1994). Historically, the lack of understanding of phage host-range and infection dynamics contributed to phage therapy's demise while new, highly effective antibiotics were being developed. However, now that bacterial infections have become more and more difficult to treat as resistance to antimicrobials increases, researchers are once again attempting to exploit the host-parasite relationship between bacteria and their phages for therapeutic purposes (Merril *et al*., 1996; Levin & Bull, 1994).

Bacteriophage O1 (also called phage Felix O1, 01 or 0-1) is a member of the A1 group of the Myoviridae (Ackermann, 1973). It was first used by Felix and Callow in the original scheme for the identification and typing of *Salmonella typhi*, (Felix & Callow, 1943). Phage O1 is fairly unique among *Salmonella* bacteriophages because of its broad host range within and its specificity for the genus. Of 15 serogroups tested in one study, all but 2 included isolates that could be productively infected by phage O1 (Kallings, 1967). Furthermore, Kallings found that other gram-negative enteric bacteria are generally resistant to lysis by phage O1. This specificity and broad intragenus host range can be contrasted with many other *Salmonella* phages, including epsilon 15, P22 and many of the P22-like phages used in the Anderson *Salmonella* phage typing scheme (McConnell *et al.*, 1992; Merril *et al*., 1996; and Callow, 1959). Ability to lyse the majority of *Salmonella* serotypes and relative specificity for the genus are desirable qualities for a biological *Salmonella* intervention. The reason for these properties can be explained by Felix O1-host receptor interaction.

Phage O1 adsorbs to the bacterial cell surface by attaching to a terminal N-acetylglucosamine in the lipopolysaccharide core, and the adsorption rate constant is affected by presence of this structure in the core and accessibility depending on O-side chain length. Since the core structure is a common feature of many *Salmonella*, it is logical that phage O1 adsorbs to and infects most members of the genus (Hudson *et al*., 1978). The somatic receptor for phage Felix O1 was

deduced by observing different strain susceptibility to adsorption and lysis by phage O1, and was definitively determined by mutational analysis of the lipopolysaccharide core (Hudson *et al.*, 1978; MacPhee *et al.*, 1975). Felix O1 phage resistance occurs when the LPS core N-acetylglucosamine receptor is sterically blocked, when presence of a prophage prevents infection (Kallings & Lindberg, 1967), or when a mutation affects structure of the LPS core (Hudson *et al.*, 1978). Mutations that affect core LPS structure include *gal*E, *gal*U and those in some *waa* genes (Hudson *et al*., 1978).

The ultimate goal of this research was to develop phage-based *Salmonella* interventions applicable at various points during animal production and processing, critical points that contribute to the transmission of this zoonosis: *Salmonella* infections in animals, horizontal transmission among food animals, and contamination of foods of animal origin. To accomplish that goal, this research involved determination of the entire genomic sequence of phage O1, an aim undertaken for two reasons. First, determining the genes present and, to some degree, the organization of the genome was desirable. This provides the basic information necessary to produce specific mutants of Felix O1, mutants that may be more applicable for specific purposes. The second reason for sequencing this viral genome was to address safety of the phage. Some phages carry virulence genes. One example is phage CTX of *Vibrio cholerae* that can render avirulent *V. cholerae* virulent by transfering cholera toxin genes into the infected bacterial host (Waldor & Mekalanos, 1996). Sequencing the genome allowed us to screen the sequence for virulence genes by doing homology searches, thus enabling us to gauge its safety as a parenteral treatment, environmental or food application. For these reasons, the entire 86.155 kb sequence of phage O1 and its basic genomic organization are presented here.

III. Materials and Methods:

A. Phage and Bacterial Strains

The stock of phage Felix O1 used for all genetic studies was kindly provided by Dr. Hans Ackermann (Felix D'Herrelle Bacteriophage Stock Center, Universite Laval, Quebec, Canada). Dr. Ackermann also provided the host strain of *Salmonella typhi* (ViA, phage type Tananarrive) used to propagate the phage.

B. Isolation of Felix O1 DNA

Phage Felix O1 was propagated on the bacterial host using solid media. Specifically, a suspension of Felix O1 was mixed with an equal volume of a 0.5 McFarland suspension of *S. typhi* (approximate multiplicity of infection between 0.25 and 1.0). The mixture was immediately spread onto the surface of trypticase soy agar plates containing 0.5 mM $CaCl_2$ and incubated overnight at 37°C. Phage was collected the following day by adding 9 ml of suspension media to the 150-mm plates, scraping the surface with a glass spreader, and pooling the resulting liquid on ice. The pooled suspension was removed from the ice and was treated for 8-10 minutes with chloroform. The residual bacterial debris was removed from the aqueous phase by centrifugation (10,000 rpm for 10 minutes in an IEC refrigerated centrifuge using rotor 872), and the result was a crude phage lysate. DNA was extracted directly from the crude lysate using a Bio-Rad Prep-a-Gene® kit (*Hin*dIII library construction) or a Qiagen® Lambda Midi or Maxi kit (for randomly-sheared library construction and direct genomic and PCR primer walking). Kits were used according to manufacturer's instructions with one exception. Twice the recommended volume of DNase-containing Qiagen buffer L1 was used in phage DNA isolation for random library construction. This was done in order to ensure degradation of *Salmonella* genomic DNA before phage DNA was extracted from the capsids.

C. Sequencing Strategy

A *Hin*dIII library of Felix O1 DNA was constructed in pBluescript SK +/- using DH5α competent cells. DNA inserts were partially sequenced from the extracted plasmids using T3 and T7 sequencing primers on an ABI 377 automated sequencer with BigDye Terminator chemistry (Advanced Center for Genetic Analysis, University of Minnesota). A randomly-sheared genomic library was made according to the methods of Roe et al. (1998). Briefly, the library was constructed by nebulizing phage DNA, blunt-end ligating fragments (0.5-1.5 kb) into *Sma*I-linearized bovine alkaline phosphatase-treated pUC18, and transforming *E. coli* (Stratagene Epicurian Gold® ultracompetent) with the ligation product. Phage insert DNA was sequenced from the extracted plasmids as described for the *Hin*dIII restriction library. A primer walking technique was used to obtain the remainder of the unknown sequence data and to enhance coverage over some of the known areas of the genome. Primer walking was performed at the Virginia Tech Sequencing Facility on phage genomic DNA preparations (Qiagen Lambda Kit; Valencia, California) and PCR products of the phage genomic preparations. Approximately 284 primers were used to achieve closure and to optimize sequence coverage. Direct genomic sequencing reactions entailed 99 cycles of amplification with addition of Thermofidelase® reagent (Fidelity Systems, Gaithersburg, Maryland). PCR amplifications were performed using Amersham-Pharmacia Ready-to-Go PCR beads® and were prepared for sequencing by purifying with EDGE® columns. Because different strategies were employed to complete the sequence, all segments were size-confirmed by performing PCR with various combinations of sequencing primers over the entire length of the chromosome. A direct genomic preparation of Felix O1 DNA was used as the template, and the size of PCR products was confirmed by agarose gel electrophoresis (data not shown).

D. DNA Sequence Analysis

Perkin-Elmer ABI Editview® software (version 1.0.1; Foster City, California) and Sequencher® (Genecodes version 3.1.1; Ann Arbor, Michigan) were used to align raw sequence data and to produce the Felix O1 chromosome consensus. Open reading frame analysis was performed by the Munich Information Center for Protein Sequences using PEDANT software (http://pedant.mips.biochem.mpg.de/), and with tBlastX software from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The MIPS ORF analysis was compared with the SwissProt peptide database, and ORFs were searched with tBlastX against the nr peptide sequence database. The entire genomic sequence and a list of open reading frames is available from the National Center for Biotechnology Information (Accession number AF320576; http://www.ncbi.nlm.nih.gov).

IV. Results:

A. Sequence analysis of the Felix O1 genome

Each nucleotide of the 86.155-kb double-stranded DNA genome of Felix O1 was sequenced at least once from each strand, with an average coverage over the entire genome of 7.32. The genome is double-stranded and linear, but the most likely mode of replication (rolling circle mechanism) precludes definition of precise ends. The chromosome has been numbered starting at the adenine of the start codon of the largest T4-homologous open reading frame, the putative *rII*A gene. A linear map is presented in Figure 1 and includes homologies with other phages, GC content, and *Hin*dIII restriction sites.

Figure 1. Linear map of bacteriophage Felix O1. The top line represents the scale in 1000-bp increments. The next line below gives open reading frames (ORFs) that show significant homology (e≤0.008) with other phage products. ORFs above the line in blue are read from the positive direction, and ORFs read from the negative strand are given below the line in fuchsia. *Hin*dIII restriction sites are shown as vertical red marks on the next line. The green line represents GC content calculated over 18-bp segnments, shown as deviation from the 39% average genomic GC content (standard deviation=5%).

B. Open Reading Frame Analysis

Two hundred and thirteen open reading frames (ORFs) were identified in the Felix O1 chromosome. Criteria for definition as an open reading frame include presence of a start and a stop codon and a nucleotide length of greater than 100. Nested ORFs (ORFs contained within a larger ORF) were not included. Putative function of ORFs was defined using the SwissProt database and those with significant homology ($e \leq 0.008$) were screened for homology with other phages using the tblastX search engine (National Center for Biotechnology Information). Both methods compare deduced phage O1 amino acid sequences with a peptide database. The 23 ORFs that showed significant homology ($e \leq 0.008$) with actual and hypothetical bacteriophage products are listed in Table 1. The peptide lengths and calculated molecular weights are also given for these 23 ORFs. The DNA sequence 15 bp upstream of the start codon is presented for each ORF for identification of possible ribosomal binding site features. Nucleotides in boldface print are homologous with the 3' end of 16S *S. typhi* ribosomal RNA sequence, a sequence which is complementary to the Shine-Dalgarno sequence that is employed in the translation initiation process (Miller *et al.*, 1994).

Table 1. Felix O1 open reading frames homologous with other phage genes. (Continued on page 53).

Open Reading Frame Number (orientation, + or -)	Potential ribosomal binding site sequence (15 nucleotides upstream of italicized start codon, ATG, TTG or GTG)[a]	Nucleotide coordinates	Putative Protein Size (a.a.)[b]	Putative Protein Molecular Weight (kDa)[b]	Homologous gene & function[b]	Phage source of homologous gene[b]
1 (+)	TAAG**AGGA**AAGT**AT**T*ATG*	1-2364	788	89.2	*rII*A: rapid lysis protein	T4
4 (+)	AG**A**GA**GAG**TAATAAG*ATG*	2447-3553	369	41.7	*rII*B: rapid lysis protein	T4
10 (+)	TAAAC**AGGAG**AACCA*ATG*	3657-4202	182	20.8	Hypothetical 20.7-kDa vs-regB intergenic protein	T4
29 (+)	TGGGA**GAG**A**TG**T**T**AA*ATG*	7205-7987	261	29.0	Hypothetical 17.5-kDa tk-vs intergenic protein	T4
75 (-)	AGCAG**AGGAGG**CATA*ATG*	19230-18769	154	17.2	*p*13: Putative lysis protein	APSE-1
79 (-)	AGAGGC**TAA**AA**AG**AA*ATG*	20114-19233	294	31.5	12: Major tail subunit	HK97
111 (+)	ATATGGT**TAA**A**G**C**G**C*GTG*	29283-30077	265	28.2	Complements λ red function	Felix O1
160 (+)	AGC**AGGA**AAAATGTA*ATG*	41956-44181	742	80.4	Unknown	SK1
180 (+)	TGT**AGGAG**C**T**T**A**ATA*ATG*	49975-51138	388	40.8	*gp*37: tail fiber	TuIa (T4)
184 (+)	TAG**AGGA**CAAA**A**ATA*ATG*	51192-53537	782	84.1	*gp*37: tail fiber	TuIa (T4)
191 (-)	**AAGGA**CACTT**T**TGAT*ATG*	55122-54226	299	34.2	*td*: thymidylate synthetase	T4
195 (-)	GA**AAGGA**TT**T**ACAAA*ATG*	55669-55127	181	20.5	*frd*: dihydrofolate reductase	T4
218 (-)	**TAAGG**TA**G**GT**A**AGAC*ATG*	60865-60365	167	19.4	Hypothetical gene 3.8 protein	T7
220 (-)	TT**T**TT**G**C**AG**TAATGT*ATG*	63576-60850	909	104.4	DNA-directed DNA polymerase	T7
235 (+)	AT**TAA**T**G**G**GG**GT**A**AA*ATG*	65090-65830	247	28.9	*g*1: deoxynucleotide monophosphate kinase	T4
239 (+)	AACTAC**AA**C**GA**A**G**A**G**G*TG*	66029-68017	663	75.0	4A: DNA primase/helicase	T3 & T7
254 (+)	A**TA**T**GGAG**AGCCATA*ATG*	69415-70458	348	39.9	Gene 6: exodeoxy-ribonuclease	T3

Table 1 (continued).

Open Reading Frame Number (orientation, + or -)	Potential ribosomal binding site sequence (15 nucleotides upstream of italicized start codon, ATG, TTG or GTG)[a]	Nucleotide coordinates	Putative Protein Size (a.a.)[b]	Putative Protein Molecu-lar Weight (kDa)[b]	Homologous gene & function[b]	Phage source of homologous gene[b]
257 (+)	**AAG**AC**G**A**T**TTCACTT*TTG*	70415-70936	174	20.4	Hypothetical protein ORF 7903	Phi-YeO3-12
269 (+)	CTGT**AGGA**CTCA**A**AAA*ATG*	73119-75350	744	85.1	*nrd*A: ribonucleoside diphosphate reductase, α chain	T4
277 (+)	GTT**TAA**CA**A**A**G**AA**A**A*TTG*	75603-76733	377	43.7	*nrd*B: ribonucleoside diphosphate reductase, β chain	T4
285 (+)	TG**TAA**A**G**G**GG**CA**AT**A*ATG*	77226-79367	714	80.7	*nrd*D (*sun*Y): anaerobic nucleoside diphosphate reductase	T4
292 (+)	**T**GGT**GAGGT**A**A**CTAG*ATG*	80177-80659	161	18.7	*nrd*G: anaerobic ribonucleotide reductase activase	T4
305 (+)	GG**A**TT**AGGT**ATTAAA*ATG*	83289-83777	163	18.8	Hypothetical gene 3.8 protein	T7

[a] Nucleotides in boldface are homologous with the 3' end of the 16S ribosomal sequence of *S. typhi*. The reverse complement sequence used for comparison is: TAAGGAGGTGATC, and includes the putative Shine-Dalgarno feature (Miller *et al.*, 1994).

[b] Putative functions of open reading frames was determined by the Munich Information Center for Protein Sequences (MIPS) and by tblastX sequence search engine (National Center for Biotechnology Information, 2000). Putative protein lengths and molecular weights were determined by MIPS PEDANT analysis software

V. Discussion:

Several genes associated with structure, assembly and nucleic acid metabolism have been identified based on homology with other phage products. Some of the putative genes identified are homologous with the T-odd phages T3 & T7 as well as phages that infect non-Enterobacteraciae. Thirteen out of 23 ORFs are homologous with coliphage T4 (Table 1).

Although many of the Felix ORFs are homologous with T4, the apparent organization of the Felix chromosome based on the ORF homologies does not resemble that of T4. For example, Felix rapid lysis gene *rII*A resides upstream from *rII*B as it does in T4. However, in T4, *gp*37 is less than 10 kb downstream from *rII*B and is read in the opposite direction, whereas the two putative *gp*37 homologues in Felix O1 are over 40 kb away from *rII*B and are read in the same direction as rIIB. Felix tail fiber gene (*gp*37). Also, Felix O1 dihydrofolate reductase gene (*frd*) is found upstream of the putative thymidylate synthase gene, *td* (as it is in T4). However, the nucleoside triphosphate reductase genes *nrd*A and *nrd*B are just downstream of *td* in T4, and these putative genes in Felix O1 are 17 kb away from *td*, and are read from the opposite strand as their homologues in T4. Another interesting finding is the Felix homology with *vs*-*reg*B (20.7 kD) and *tk*-*vs* (17.5 kD) intergenic hypothetical proteins of T4. These intergenic region homologues exist in Felix O1 without the expected homology with the genes that define the intergenic regions (valate synthase, thymidine kinase and *vs*, *tk*, and *reg*B),. Again, this example illustrates the difference in overall genomic organization, In phage O1, these hypothetical proteins are just downstream of *rII*A and *rII*B, whereas in T4 they are over 50 kb upstream.

Irrespective of secondary structure, strength of expression has been found to depend heavily on sequence and spacing of the Shine-Delgarno sequence from the initiation codon (Chen et al., 1994, Ringquist et al., 1992). The upstream sequences of the ORFs presented in Table 1 were analyzed for putative ribosomal binding sites in order to gauge the likelihood that they code for proteins. The

small size of the Shine-Delgarno sequence precluded searching the genome systematically for putative ribosomal binding sites. Instead, the nucleotides upstream of the proposed start site for each of the ORFs in Table 1 were aligned with a sequence complementary to the 3' end of the *S. typhi* rRNA in order to identify potential ribosomal binding sites. The sequence used for alignment includes the portion identified by Ringquist et al. (1992) to maximize translational efficiency.

The historically reported optimal spacing between the Shine-Delgarno sequence and the translation initiation codon varied from anywhere from 5 to 13 nucleotides, depending on the reference nucleotides used. Chen et al. (1994) employed a more systematic method to define spacing, and thus were able to determine a much smaller range of optimal spacing. Aligned spacing refers to the number of nucleotides between the nucleotide corresponding to the 3'U of the sequence 5'UAAGGAGGU3' and the adenosine of the start codon AUG. Based on these reference points, the optimal aligned spacing is 5 nucleotides, determined both experimentally and by recalculating spacing reported previously (Chen et al., 1994). Using these criteria and the putative Shine-Delgarno sequences reported in Table 1, several of the ORFs exhibit the optimal spacing, including the *rII*B, *tk-vs* intergenic, *gp*37, *frd* and gene *6* homologues.

Included in the list of T4-homologous ORFs are the three genes of T4 found to contain introns (I-TevI, I-TevII and I-TevIII in genes *td*, *nrd*D and *nrd*B genes, respectively). No such introns were found in the Felix O1 sequences homologous with these genes. This does not rule out the possibility of introns in the Felix O1 chromosome, but if introns are present, they apparently do not reside in the same genes that contain them in T4 or are not homologous with those found in T4.

Phage O1 is most likely a virulent phage, committed to lysing cells it infects and lacking the ability to incorporate into the host chromosome as do temperate (lysogenic) phages such as λ and P22. We base this conclusion on a few classical

experiments as well as on the organization and contents of the phage genome. We approached the search for a Felix lysogen in *Salmonella typhi* using the modified methods of Douglas (1975). First, a few potential lysogens were collected from Felix propagation spread plates (colonies that grew on Felix propagation plates, colonies apparently unaffected by the phage). These colonies were subcultured 5 times to remove any extracellular phage from the potential lysogens. Saline suspensions of each suspect were exposed to ultraviolet light for 10, 20, 30, 40, 50, 60, 90 & 120 seconds, and then spread on agar plates. After incubation, none of the lawns of bacteria exhibited any plaque formation, although viability was affected at 90 and 120 seconds for all strains. Although these data are limited and the studies are certainly not exhaustive, the isolates tested are either not lysogens or are not inducible by ultraviolet light. The colonies that grow on propagation plates are more likely the result of selection for and growth of resistant isolates, resistant because of the nature or accessibility of lipopolysaccharide components important for attachment of Felix O1.

Sequence analysis points to a virulent versus a temperate life cycle for Felix O1 as well. Most of the significant homologies found are with T4, a lytic coliphage. In addition, the presence and organization of putative *rII*A and *rII*B genes further emphasizes the similarity between phage O1 and T4. In fact, *rII* genes in T4 are partly responsible for delaying host cell lysis when the multiplicity of infection is high or when superinfection by other viruses occurs (e.g. when there are relatively few host cells available for continued infection). This mechanism allows for some modulation of the life cycle of virulent phages under certain conditions since lysogeny is not an option, an alternate process that maximizes the number of progeny resulting from the infected host cells.

The only homology that might point to a temperate life cycle was that of a short segment of Felix O1 DNA homologous with a portion of the repressor gene *c*I of lactococcal phage Tuc2009. Not only is the homology low (e=0.64), but the homologous portion only spans a short segment of the N-terminal DNA-binding

portion of the Tuc2009 repressor protein. Three of the highly conserved amino acids (a.a.) present in most repressor proteins are present in this Felix sequence (glutamine, a.a. 25; alanine, a.a. 29; and glycine: a.a. 33). However, the Felix sequence lacks the helix-turn-helix motif of a DNA-binding protein and lacks the hydrophobic brace of Tuc2009's repressor as well (Van de Guchte *et al.*, 1994). This segment is also homogous with the helix-turn-helix N-terminal portion of *cpg* and *cng* genes of *Lactobacillus* phage phi-g1e. However, homology with *cpg* ends after amino acid 70 of the 132-amino acid product (Kodaira *et al.*, 1997). Although nucleotides 56820-57146 of Felix O1 probably do not code for a functional repressor protein, presence of the helix-turn-helix feature may indicate functionality of the segment as a DNA-binding protein of some kind.

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Chapter 3: Mutagenesis of Phage Felix 01

I. Abstract

Mutagenesis of the Felix O1 chromosome was undertaken in order to produce a recombinant with predictable timing of lysis that could also be used to detect viable *Salmonella* by virtue of a reporter gene. Homologous recombination was used to delete the *rII*B region of the Felix O1 chromosome and simultaneously insert promoterless *lux*AB genes of *Vibrio harveyi*. The necessary steps for producing recombinants were based on the modified methods of Loessner *et al*. (1996). This included designing the recombinant plasmid, cloning it into *E. coli*, transforming *Salmonella* with the plasmid, infecting plasmid-containing *Salmonella* with Felix O1 WT, and enriching and screening for *lux*-expressing recombinant phages. Two approaches were used to produce a plasmid for recombination with phage DNA. First, hybrid PCR primers containing 17 and 20 base pairs of Felix O1-homologous DNA surrounding the *rII*B region to be deleted were used to amplify *lux*AB genes from plasmid pQF110. This product was successfully ligated with pUC18 to form plasmid pUCflux12. This plasmid was used to transform competent *E. coli*, and was subsequently electroporated into *S. typhimurium*. No recombinant phages resulted from infection of the plasmid-containing *Salmonella* with Felix O1 WT, enrichment and screening for *lux* expressing phages. The second approach employed separate amplification of *lux*AB and much longer flanking sequences of Felix O1 DNA upstream and downstream of *rII*B (1.0 and 0.3 kb, respectively), and necessitated ligation of all three fragments with a plasmid vector. Simultaneous ligation of the *rII*B upstream, *lux*AB, and *rII*B downstream fragments was unsuccessful, but opportunities still exist for stepwise ligation and cloning of fragments since all three fragments are available on PCR2.1® plasmids in separate TOP10® *E. coli* clones (Invitrogen). Although the site-directed mutagenesis of the *rII*B region

described proved unsuccessful, avenues for mutation of this region and others remain and are presented herein.

II. Introduction

As described previously (Chapter 2), homology was found between Felix O1 DNA and *rII*A and *rII*B genes of phage T4, and the arrangement of *rII*A and *rII*B in Felix O1 is similar to that found in T4. The putative coding regions for *rII*A and *rII*B are located in the Felix genome between nucleotides 1 and 3552. The *rII*B locus was targeted because it was one of the few putative genes identified through sequencing the Felix O1 genome that would probably affect dynamics of infection/lysis. Products of *r* (rapid lysis) genes in T4 are involved in the phenomenon known as lysis inhibition. T4 is a virulent (obligately lytic) phage. However, under certain conditions timing of lysis is delayed by this mechanism. Specifically, lysis inhibition is a delay of lysis of a bacterial cell productively-infected with phage T4. Lysis inhibition occurs when the multiplicity of infection (MOI) is high. It also occurs when host cell concentration is high because phage progeny emerging from neighboring cells can superinfect nearby cells. The cumulative effect of lysis inhibition is seen in liquid culture as persistent turbidity that can last for hours. T4 *rII* mutants possess a rapid lysis phenotype which was thought to be due to freedom from lysis inhibition. The rapid lysis effected by rII mutants occurred even when the MOI or host cell concentration is high. Phage mutant in *rII* genes also produce a larger, clearer plaque phenotype on solid media. Although the exact function of *rII*B is unknown, it has been historically hypothesized that the protein may interfere with the lysis inhibition mechanism itself (Abedon, 1994). More recent evidence shows, however, that *rII* products may effect rapid lysis via a pathway independent of the lysis inhibition mechanism (Paddison *et al.*, 1998). Regardless of the underlying mechanism, the net effect of *r* mutation and rapid lysis is the same and is potentially desireable for a phage-based *Salmonella* intervention.

In theory, inactivation of the *rII*B locus in Felix O1 should produce an *rII*B mutant that might perform better than the wild-type phage as a *Salmonella* intervention. Assuming this locus is functional in Felix O1, such a mutant would have lysis characteristics that were predictable and independent of MOI or host cell concentration. Since bacterial concentration (and consequently MOI) in a processing setting may be quite variable, addressing this locus through mutagenesis was a logical choice. The *rII*B locus was chosen over *rII*A because of its closer sequence similarity to the T4 genes and because there was more complete data for this gene than for *rII*A during the time mutagenesis experiments were being designed.

III. Materials and Methods

A. Overview

In practice, the goal was to achieve simultaneous deletion of *rII*B and insertion a screenable marker that would enable the recombinant to be used as a *Salmonella* detection assay. The *lux*AB genes of *Vibrio harveyi* were chosen as the marker. The product of *lux*AB is a bacterial luciferase that cleaves a long-chain aldehyde (such as nonanaldehyde) producing light as a byproduct. Nonanaldehyde (also called pelargonaldehyde) is a diffusible substrate that can easily be added to cells infected with a *lux*-expressing phage (Loessner *et al.*, 1996). Luminescence output following addition of the substrate indicates the presence of cells susceptible to phage since infection of a susceptible host is necessary for phage gene expression. Luminescence output (relative luminescence units, or RLU's) can be measured with a luminometer or scintillation counter upon addition of the diffusible substrate (Ulitzur and Kuhn, 2000).

Two approaches were used to remove the *rII*B genes while inserting *lux*AB into the Felix O1 genome, and both approaches employed homologous recombination of phage DNA with a plasmid. The *lux*AB genes were subcloned into a plasmid with Felix homologous DNA on either side of *lux*AB. The Felix-homologous DNA sequences placed before and after *lux*AB on the plasmid were sequences from the upstream and downstream ends of *rII*B, respectively. The goal was to clone this plasmid into a strain of *Salmonella* that could then be infected with Felix O1 wild-type. The Felix-homologous DNA flanking the *lux*AB genes was included in order to allow for a double crossover recombination event between the plasmid and Felix genomic DNA. Upon lysis of the infected plasmid-containing cells, a certain percentage of progeny containing *lux*AB in place of *rII*B would be expected. These recombinants could be isolated using a selective enrichment procedure for *lux*-expressing recombinant phage. Figure 1 outlines the general steps necessary to obtain a *lux*+ Δ*rII*B mutant.

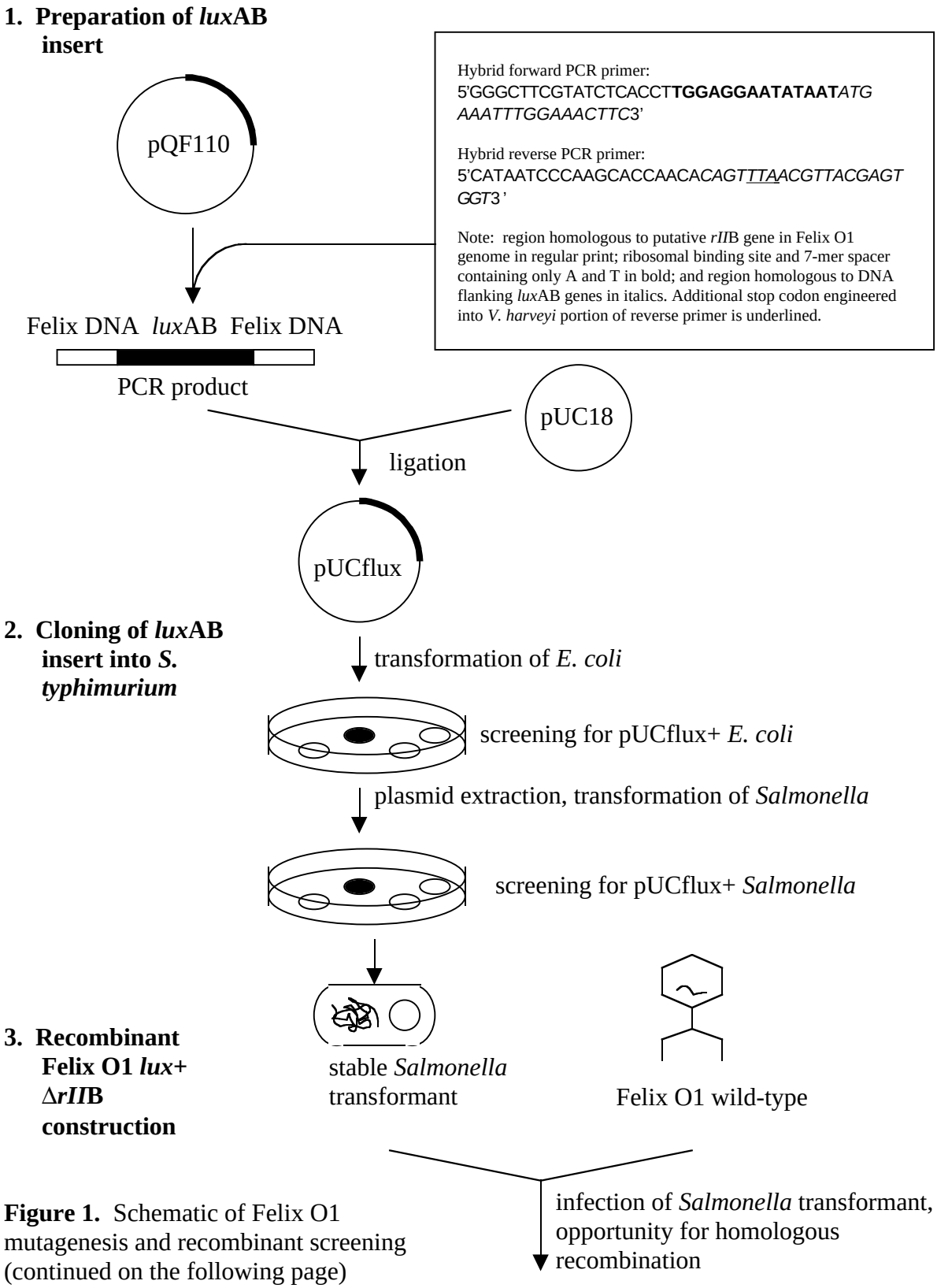

Figure 1. Schematic of Felix O1 mutagenesis and recombinant screening (continued on the following page)

4. Recombinant Felix O1 enrichment and screening

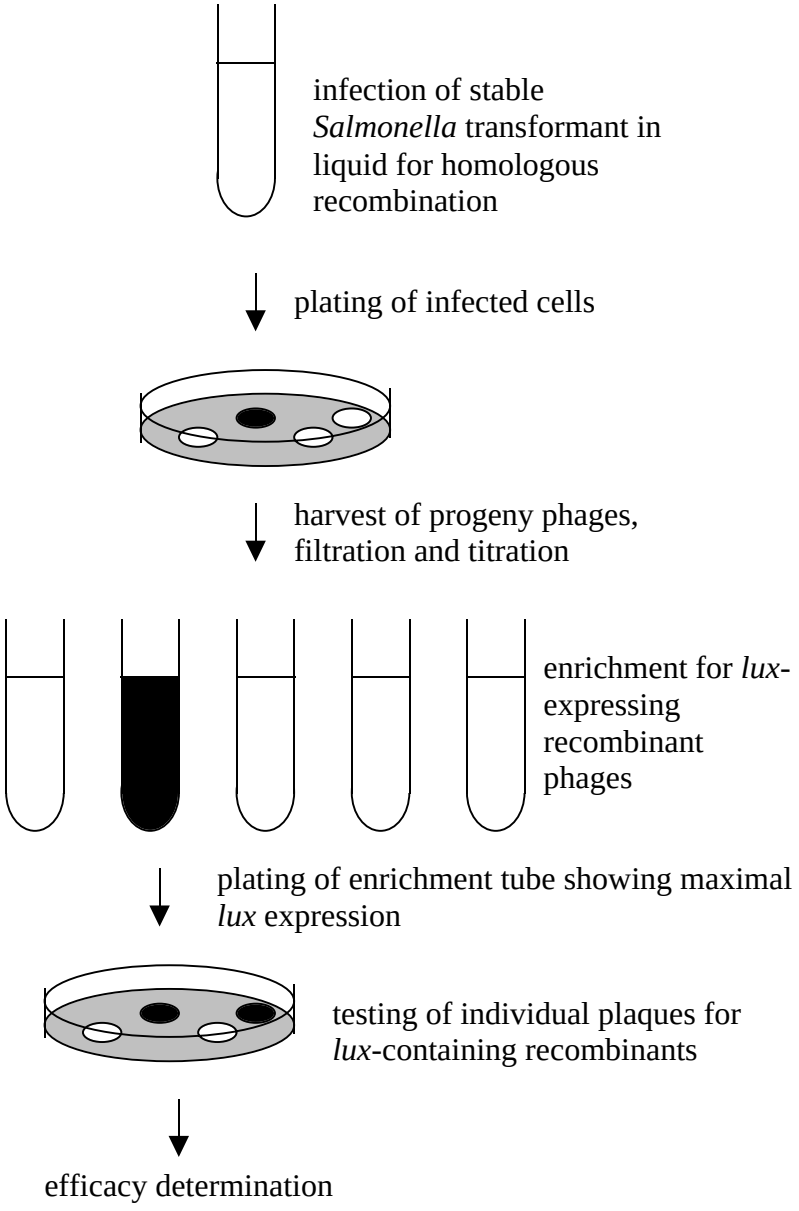

Figure 1 (continued)

The first approach involved construction of a *lux*AB plasmid with relatively short flanking sequences of Felix-homologous DNA. This approach employed a pair of hybrid PCR primers to amplify the *lux*AB genes of *V. harveyi* from plasmid pQF110. The primers were hybrid in that the 50-mer and 41-mer used to amplify the 2.1-kb *lux*AB genes contained 17 and 20 base pairs of Felix DNA on their 5' ends respectively.

The second approach to mutagenesis included much larger Felix-homologous flanking regions on the *lux* plasmid in order to enhance chances for a double-crossover event between the plasmid and Felix genomic DNA. This was achieved by separate amplification of the Felix sequence upstream of *rII*B (1.0 kb), the *lux*AB genes (2.1 kb) and a 0.3-kb Felix-homologous sequence downstream of *rII*B. These three fragments were to be ligated simultaneously into pUC18 for use in subsequent transformations.

B. Plasmid design and construction

The following plasmids were used for cloning and mutagenesis: pBluescript II SK +/-® (Stratagene), pQF110 (ATCC), pUC18 (*Sma*I-linearized, BAP-treated®, Pharmacia) and PCR2.1® (Invitrogen). The following enzymes were used for cloning, restriction and modification. Type II restriction endonucleases included *BamH*I, *BsrG*I, *Csp*45I, *Nhe*I, *Pst*I, *Sac*I, *Sal*I, *Xba*I, *Xho*I (New England Biolabs and Promega). Other enzymes used included T4 ligase (New England Biolabs, 202S), Klenow DNA polymerase (New England Biolabs, 210S), Shrimp alkaline phosphatase (Amersham Life Science, USB US70092) and T4 polynucleotide kinase (Amersham, E70031Z).

Polymerase chain reactions for amplification of *lux*AB genes of *Vibrio harveyi* were performed using Perkin-Elmer GeneAmp® reagents (kit number N808-0009) and Perkin-Elmer Amplitaq® polymerase (N801-0060). *lux*AB genes were amplified from Qiagen plasmid preparations of pQF110 in a Hybaid Omnigene® thermocycler. Amplification of large Felix-homologous flanking sequences used in the second mutagenesis approach was performed using Qiagen Lambda kit preparations of Felix O1 genomic DNA.

C. Transformation of *Escherichia coli*

The following strains of *E. coli* were used for cloning: DH5α (for general cloning, Gibco), Epicurian coli Gold Ultracompetent® (for general cloning, Stratagene), JM109 (strain in which pQF110 was provided, ATCC) and TOP10® ultracompetent (strain provided with plasmid PCR2.1®, Invitrogen).

All *E. coli* transformations were carried out with the commercially-available competent strains listed above and were performed according to manufacturer's instructions. Briefly, 1-5 ng of plasmid DNA was added to 50- or 100-ul aliquots of cells. Mixtures of competent cells and plasmid DNA were incubated on ice (one hour), followed by a mild heat shock step and another ice incubation (five minutes). Transformed cells were grown with shaking at 37C for one hour in the designated media, and appropriate dilutions were plated onto LB agar containing 100 ug/ml ampicillin, 400 ug IPTG and 800 ug X-gal. Presumptive transformants were selected based on growth on ampicillin-containing media and white colony phenotype (β-galactosidase, α-complementation screening). Transformants were further characterized by restriction analysis of plasmid extracts and PCR to confirm the appropriate *lux*AB insert.

Successful transformants are stored in LB broth containing 10% glycerol at –80C.

D. Preparation of competent *Salmonella*

Unlike for *E. coli*, there are no commercially-available *Salmonella* competent cells. Competent cells were prepared for strains of *Salmonella* according to the methods of Ausubel *et al.* (1987). Briefly, an overnight LB broth culture was diluted 200-fold in prewarmed LB (2.5 ml of culture in 500 ml broth). The culture was grown with shaking at 37°C to an optical density (600 nm) of 0.5-0.6. The cells were chilled rapidly and were pelleted by centrifuging at 4200 rpm in an IEC T-20A refrigerated centrifuge (rotor 872) at 2°C. The pelleted cells were washed twice with ice-cold sterile water (same centrifugation protocol as for pelleting). After washing, the pellet was resuspended in 40 ml of ice-cold water containing 10% glycerol, and 100-ul aliquots were frozen on dry ice and stored at -80°C until use. Competent cells for the following strains were prepared and frozen at -80°C:

1. *S. typhi* phage type Tananarive (Hans Ackermann, Universite Laval, Quebec, Canada)
2. *S. typhimurium* LT2 SA 3046 (*Salmonella* Genetic Stock Centre (SGSC), Calgary, Canada). Genotype: metA22 metE551 trpC2 fla-66 rpsL120 H1-b H2-e,n.x “cured of Fels 2” xyl-404 hsdL6 hsdSA29 hsdSB ilv-452 leu-3121 galE(+) rfa-3077 (DOC(S), Ffm(S), C21® at 42C; DOC®, FO(S) at 30C.)
3. *S. typhimurium* LT2 SA 3047 (SGSC). Genotype: metA22 metE551 trpC2 fla-66 rpsL120 H1-b H2-e,n,x “cured of Fels 2” xyl-404 hsdL6 hsdSA29 hsdSB ilv-452 leu-3121

galE(+) rfa-3078 (DOC(S), Ffm(S), C21® at 42C; DOC®, P22(S), FO(S) at 30C.)

4. *S. typhimurium* LT2 SA 3394 (SGSC). Genotype: metA22 metE551 ilv-452 leu-3121 trpC2 xyl-404 galE856 hsdL6 hsdSA29 hsdSB121 rpsL120 H1-b H2-e,n,x fla-66 nml(-) Fel-2(-) recA1 pro lys.
5. *S. typhimurium* LT2 SA 3552 (SGSC). Genotype: metA22 metE551 ilv-452 leu-3121 trpC2 xyl-404 galE856 hsdL6 hsdSA29 hsdSB121 rpsL120 H1-b H2-e,n,x fla-66 nml(-) Fel-2(1) recA1 pro lys / F'lacI(q)Z(M15) proAB Tn10.

E. Electrotransformation of *Salmonella*

Once the desired plasmid was successfully cloned into *E. coli*, attempts were made to move the plasmid into *Salmonella*. The goal was to obtain a plasmid-containing *Salmonella* that could be infected with Felix to facilitate recombination between Felix genomic and plasmid DNA. The *lux*-containing plasmid with Felix flanking DNA was moved from *E. coli* into electrocompetent *Salmonella* using electroporation. Electrotransformations of competent *Salmonella* strains were performed using either a BTX Electro Cell Manipulator 600® or a BioRad Gene Pulser II®. The methods of O'Callaghan and Charbit (1990), which are specific for *S. typhi* and *S. typhimurium*, were used as a guideline for electroporations.

The plasmid design for the first mutagenesis approach (with 17 & 20 bp of Felix O1 flanking DNA) was successfully obtained in *E. coli* Stratagene XL10 gold Ultracompetent cells. This plasmid was extracted from the *E. coli* transformant using a Qiagen Qiaprep Spin® kit or a Qiagen Midi® plasmid extraction kit. The first electrotransformation efforts were made

with *S. typhi* phage type Tananarive, since Felix O1 shows a high efficiency of plating (EOP) on this strain of *Salmonella*.

The critical parameters for successful electrotransformation include field strength and pulse length (duration of application of electricity to cells). Sanderson *et al.* (1995) and O'Callaghan *et al.* (1990) give the following ideal conditions for electroporation of *Salmonella*: field strength = 12.5 kV/cm, pulse length = 5 ms. Field strength is equal to voltage divided by gap length, and gap length is the distance between electrodes in the electroporation cuvette. 0.2-cm gap length cuvettes were used. In order to achieve the recommended field strength of 12.5 kV/cm using 0.2-cm gap length cuvettes, an electroporation voltage of 2.5 kV was used.

Pulse length was determined by the following equation:

$$(1) \qquad T = R \times C$$

Where T is time (pulse length, in seconds), R is resistance (ohms, Ω), and C is capacitance in farads (F). O'Callaghan *et al.* (1990) further recommend using 200 Ω and 25 uF in order to achieve a pulse length of 5 ms. (Multiplying 200 Ω by 2.5 x 10^{-5} F gives 0.005 seconds). The settings on electroporation units allow for flexibility in choosing voltage, resistance and capacitance, depending on the application. However, the lowest capacitance that could be achieved with the BTX 600 was 50 uF. So, by equation (1), the closest capacitance that could be achieved according to O'Callaghan's recommended parameters was 50 uF and a corresponding resistance of 100 Ω, which gives a pulse length of 5 ms. This equated to a higher capacitance and lower resistance than recommended in order to achieve the appropriate pulse length. Many attempts were made with the BTX unit, and several parameters were modified at different times to try to improve results. Changes that were

tried included amount of plasmid DNA added (25-200 ng), resistance (24-186 ohms) and addition of a heat shock step before electroporation (45°C for 30 seconds).

F. Felix infection of transformants and recombinant phage screening

The methods of Loessner *et al.* (1996) were used with modification for the Felix O1 infection of *lux*-containing transformants and selection of *lux*+ recombinant phage progeny. Briefly, a 0.5 McFarland standard suspension of the *Salmonella* containing pUCf*lux*12 was prepared. One hundred microliters of the bacterial suspension was mixed with Felix O1 wild-type (MOI of about 1) in molten 0.7% agar cooled to 46-50°C. The soft agar mixture was poured onto the LB ampicillin plate and incubated overnight at 30°C. Phage was eluted from the plates the following day (in 10 ml suspension media) for several hours at 4°C with intermittent shaking. The eluate was treated with chloroform, and the aqueous phase was centrifuged (4000 x g, 10 minutes, 4°C) to remove residual bacterial debris. Modification of Loessner's method consisted of these chloroform treatment and centrifugation steps. The supernatant was filtered (0.22 um) and heat treated for 30 minutes at 37°C to inactivate residual luciferase which may have been produced by the pUCf*lux*12-containing host strain. (*V. harveyi* luciferase is preferable to some other bacterial luciferases by virtue of its lability at 37°C). The phage titer of the filtered, heat-treated eluate was determined (by plating serial dilutions on *S. typhi*), and the eluate was diluted to 10^8 pfus/ml.

The next phase was designed to enrich for *lux*-expressing phage. This would be done by selecting progeny from and subculturing aliquots with the highest luminescence output containing the fewest phage. Specifically, the diluted (10^8 pfu/ml) eluate was mixed with an equal

volume of log-phase *S. typhi* culture and allowed to adsorb for 20 minutes at 30°C. The mixture was then diluted with suspension media to yield portions containing 10^5, 10^4 and 10^3 pfus/ml, and each dilution was aliquoted into 20 1-ml samples. After additional incubation at room temperature (60-80 minutes to allow for luciferase expression), tubes were loaded into a Berthold Autolumat LB 953®. 50 ul of 0.25% nonanaldehyde (Fluka) in 70% ethanol was injected into each tube, followed immediately by photon output measurement in relative luminescence units (RLU's). Tubes containing the lowest concentration of phage that exhibited the highest RLU output were repropagated on *S. typhi* by dividing the sample among 3-4 soft agar overlays. The phage from subsequent propagations was eluted, chloroform treated, filtered, heat treated, titrated and diluted as before, and the same luminescence determinations were made.

Additional steps to further enrich samples containing *lux*-expressing phage were not performed because no samples were obtained that exhibited luminescence attributable to anything other than background. Had positive samples been obtained, the enrichment would have been continued by testing for luciferase activity in even less concentrated phage samples (10^2 pfus/ml). Eventually, instead of eluting plaques from plates with suspension media, recombinants would have been enriched from sub-propagations by pooling 10-plaque picks and testing their luciferase activity (RLU output) after incubation with susceptible *Salmonella*. Finally, single plaque picks would have been tested and kept based on their luciferase production.

G. Specific mutagenesis attempts

Two approaches were used for the simultaneous deletion of *rII*B from and insertion of *lux*AB genes into the Felix O1 genome. The first employed amplification of *lux*AB genes of *V. harveyi* using hybrid PCR primers . They are referred to as hybrid because they contained 17 and 20 base pairs of Felix-homologous DNA on the 5' end of the forward and reverse primers respectively. The short Felix-homologous pieces are derived from the genomic sequence upstream and downstream of *rII*B. The second approach employed much longer Felix-homologous flanking regions obtained by separate amplification and ligation of Felix upstream, *lux*AB and Felix downstream segments into a plasmid.

1. Hybrid Primer Approach

The sequences and attributes of the hybrid primer used to amplify *lux*AB from plasmid pQF110 are given in Figure 2.

Forward Hybrid Primer:

5’ – GGGCTTCGTATCTCACCT**TGGAGGAATATAAT***ATGAAATTTGGAAACTTC* – 3’

Reverse Hybrid Primer:

5’ – CATAATCCCAAGCACCAACA*CAGTTTAACGTTACGAGTGGT* – 3’

Figure 2. Forward and reverse hybrid primers. The region homologous to putative *rII*B gene segments in Felix O1 genome is given in regular print; ribosomal binding site and 7-mer spacer containing only A and T in bold; and region homologous to DNA flanking *lux*AB genes in italics. Additional stop codon engineered into *lux*AB flanking portion of reverse primer is underlined.

Primer design was guided by the work of Loessner *et al.* (1996) who successfully produced a luciferase-expressing listeriophage A511 using a similar approach. Seventeen and 20 base pairs of Felix-homologous sequence were present on the 5' ends of the forward and reverse primer. The actual sequence of the Felix O1-homologous portions used in the primers was chosen such that the majority of the coding region of *rII*B would be deleted if a double-crossover event occurred between the Felix O1 genome and the amplicon resulting from use of these two primers. In addition, the forward primer included a ribosomal binding site with perfect complementarity to the 3' end of *S. typhi* rRNA. The forward primer also contained a 7-mer spacer between the ribosomal binding site and the start codon which is designed to maximize translational efficiency. The reverse primer included an extra stop codon to ensure dissociation of RNA polymerase thus minimizing transcriptional read-through by RNA polymerase. The luciferase genes contained on pQF110 were promoterless.

Amplification of *lux*AB genes was performed according to the thermocyclying conditions given in Figure 3.

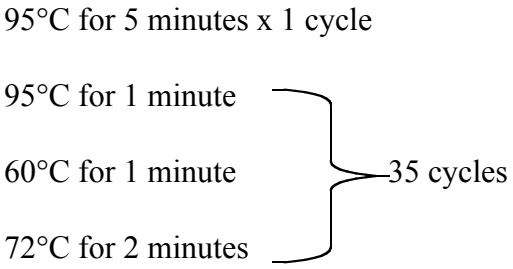

Figure 3. Thermocycling conditions for the hybrid primer approach. These conditions were used for amplification of *lux*AB from pQF110 using hybrid PCR primers.

Although the desired 2.1-kb product resulted from amplification of pQF110 using the hybrid primers in Figure 2, another 1.0-kb band was also produced. Increasing the annealing temperature to 65°C did not remedy the nonspecific amplification problem, and in fact resulted in less of the 2.1-kb product. Reamplifying the 2.1-kb band extracted from an agarose gel also did not alleviate the 1.0-kb band, indicating that there is another annealing site for one of the primers within the 2.1-kb *lux*AB fragment. Gel purification steps were used to prepare DNA for ligation. Excision of the appropriate 2.1-kb fragment from agarose gel prior to ligation helped prevent the possibility of cloning the extraneous 1.0-kb fragment.

The 2.1-kb PCR product was inserted into a *Sma*I-linearized, bovine alkaline phosphatase-treated pUC18 using a blunt-end ligation protocol. A blunt-end ligation protocol was used because cutting the product for compatible end ligation with the plasmid would have done away with the desired Felix O1-flanking sequences in the PCR product. The PCR product was "blunt-ended" prior to ligation according to a protocol available from the University of Oklahoma (Roe, 1998). Briefly, the entire contents of two 25-ul PCR reactions (amplified using the hybrid primers) was pooled and gel-purified using a Qiagen QiaEx II® gel extraction kit. The resulting 27-ul gel extract was combined with 5.0 ul of T4 polynucleotide kinase buffer, 5.0 ul of 10 mM ribosomal ATP, 7.0 ul of dNTP's (0.25 mM each in adenosine, cytosine, guanine and thymine), 3 Units T4 polynucleotide kinase and 10 Units Klenow DNA polymerase. The mixture was

incubated at 37°C for 30 minutes and then gel-extracted again. The gel extracted, blunt-ended, TE buffer-eluted 2.1-kb fragment was ligated with *Sma*I-linearized, BAP-treated pUC18® using 1.0 ul of highly concentrated T4 ligase (400 units/ul, New England Biolabs). Ligations (experimental and control) were incubated overnight at 4°C and the mixtures were used to transform Stratagene XL10 Gold Ultracompetent cells. Presumptive positive transformants were chosen based on ampicillin resistance (100 ug/ml in LB agar) and white colony phenotype on solid media containing 14 μg/ml IPTG and 29 μg/ml X-gal. The plasmid inserts were further characterized using restriction enzyme analysis (*Sac*I/*Xba*I double digests to extract the insert) and PCR of plasmids extracted from transformants using the hybrid primers in Figure 2. Ultimately, transformants 2, 4, 12 and 18 were saved from the second transformation attempt, and transformant 12 was the source of pUCf*lux*12 used in all *Salmonella* electrotransformations.

Many unsuccessful attempts were made to electrotransform *S. typhi* phage type Tananarive with pUCf*lux*12. *S. typhi* was abandoned, and subsequently electrocompetent cells were prepared for strains SA 3394 and SA3552 of *S. typhimurium* LT2 (SGSC, Calgary, Canada). These strains were successfully transformed with pUCf*lux*12 using a BTX Electro Cell Manipulator 600® (100 ng plasmid DNA, 2.5 kV, 50 uF, 129 ohms, 0.2-cm gap length cuvette). The infection and selection procedure was initiated with *S. typhimurium* transformants SA3394f*lux*10 and SA3552f*lux*1. However, each of these strains were found to be resistant to productive infection with Felix O1. Two more Fels-2-cured strains

of *S. typhimurium* LT2 were obtained from the *Salmonella* Genetic Stock Centre (SA3046 and SA3047); strains advertised as susceptible to Felix O1 infection at 30°C. The ability of Felix O1 to produce plaques on these strains was confirmed before preparing competent cells and electrotransforming. Once plaquing ability at 30°C was confirmed, these cells were successfully electrotransformed using the same parameters successful for strains SA3394 and SA3552. Transformants were selected and confirmed by the same colony selection, restriction analysis and PCR used for SA3394f*lux*10 and SA3552f*lux*1. Felix O1 wild-type infection of pUCf*lux*12 transformants SA3046-2-2 and SA3047-2-3 was performed according to the modified methods of Loessner *et al.* (1996).

Southern blots were used in order to detect any non-expressing recombinants. Digoxigenin-labeled DNA probes were produced for the *rII*B region (the region that should be deleted upon recombination) and for another segment of the Felix genome that should remain for use a positive control (the 0.5-kb product of genomic walking primers 86L2 and 97U3). A Boehringer-Mannheim DIG Labeling and Detection Kit® (#1093657) was used to generate DNA probes from PCR products. These probes were to be used to screen for *rII*B deletion mutants from plates using a plaque-lift assay. In order to test performance of the probes, and hopefully detect a recombinant, a semi-promising sample (based on RLU output slightly higher than background) was plated on *S. typhi*. A suspension of Felix O1 wild-type was also plated similarly for use as a positive control. Plates were incubated overnight at 37°C, and the plaques were immobilized on

a nylon membrane support (Boerhinger-Mannheim Nylon membranes, #1699075). Membranes were prepared and probed according to manufacturer's directions. All plaques (in the wild-type positive control and in the sample tested) probed with both the *rII*B and control oligonucleotide. (One would hope for an *rII*B deletion mutant that would probe with the control oligonucleotide but not with the *rII*B oligonucleotide). This screen was not pursued further since the entire approach was abandoned for the four-fragment ligation approach.

2. Four-fragment ligation approach

Generation of more sequence data surrounding *rII*B in the Felix genome enabled design of longer flanking sequences for the plasmid design in this second approach. This rationale is more in keeping with Loessner *et al.* (1996) who successfully produced a *lux*AB-expressing listeriophage A511 using a plasmid with *V. harveyi lux*AB inserted in the middle of 2.1 kb of phage DNA (Loessner *et al.*, 1996). There was no large segment of Felix O1 DNA on a plasmid that included *rII*B and the flanking regions. Instead, the new design entailed separate amplification of each of the three fragments to be inserted into the plasmid, e.g., the 1.0-kb Felix-homologous segment upstream of *rII*B (fragment 1), the 2.1-kb *lux*AB amplicon (fragment 2) and the 0.3-kb Felix O1-homologous sequence downstream of *rII*B (fragment 3). The plasmid with which these fragments were ligated could itself be considered a fragment to be included in the ligation reaction with the other three, ergo, its reference as the four-fragment ligation.

The specific decisions for what would comprise fragments 1 and 3 were based on sequence data available, optimization of primer Tm's, and amenability of primers to inclusion of restriction endonuclease recognition sequences. The primers used to amplify all three fragments were each designed with compatible, unique restriction sites (sites that did not exist in the fragments to be amplified). The initial step was to amplify each fragment separately with its respective primer set, put each amplicon into a separate plasmid vector and then clone each separately into *E. coli*. The plasmids containing the separate fragments were to be extracted from their respective host strain, at which time the fragments excised using the appropriate restriction enzymes to produce compatible ends. Following gel purification, the three fragments would be combined with an appropriately cut plasmid vector in one ligation reaction.

The primers used to amplify fragments 1, 2 and 3 are given in Figure 4.

Fragment 1 upper (Tm=53.7°C):

5’ – **GTCGAC**TGTAGGCCGCAATCAGA – 3’

Fragment 1 lower (Tm=48.9°C):

5’ – **GCTAGC**CCAAAAACTTACTCACTT – 3’

Fragment 2 upper (Tm=56.3°C) :

5’ – **GCTAGC**TTGGAGGAATATAATATGAAATTTGGAAACTTC – 3’

Fragment 2 lower (Tm=52.9°C):

5’ – **GGATCC**AGTTTAACGTTACGAGTGGT – 3’

Fragment 3 upper (Tm=55.9°C):

5’ – **GGATCC**ATGTCATCGTTGGCACTGC – 3’

Fragment 3 lower (Tm=54.8°C):

5’ – GGTA**GAGCTC**TAGCTGCTAGAGTAATAC – 3’

Figure 4. Primers used for the four-fragment ligation approach. The listed primers were used to amplify fragments 1, 2 and 3. Primers were designed using DNAStar PrimerSelect. Restriction endonuclease recognition sequences were either added to the 5’ ends or modifications were made within the primer to achieve a desired restriction site. Restriction sites are shown in bold print. Fragment 1 upper primer has a *Sal*I site. Fragment 1 lower and fragment 2 upper primers contain a *Nhe*I site. Fragment 2 lower and fragment 3 upper primers have a *BamH*I site added, while fragment 3 lower contains a *Sac*I site.

The compatibility of the fragments after restriction digest can be seen from the primer design in Figure 4. Cutting each fragment out of its respective plasmid using the restriction enzymes for which sites have been engineered into the primers would have theoretically produced compatible ends for ligation in a single reaction. For example, digesting fragments 1 and 2 from their respective vector using *Nhe*I should allow for compatible ligation of fragments 1 and 2 by virtue of the *Nhe*I 4-base 5' overhangs.

Each fragment was amplified according to the thermocycling protocol given in figure 5 using a Hybaid Omnigene® thermocycler.

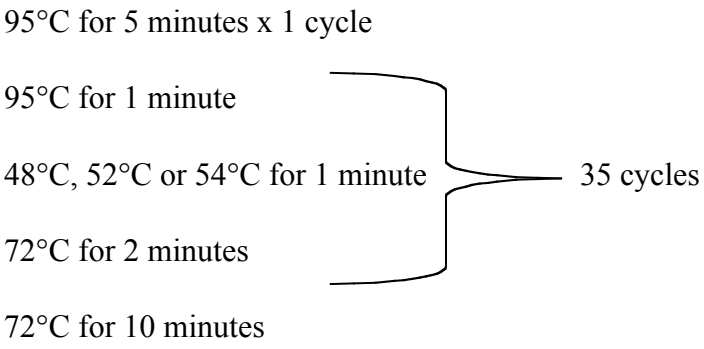

Figure 5. Thermocycling conditions for the four-fragment ligation approach. These conditions were used for amplification of fragments 1, 2, and 3. Annealing temperature used for fragments 1, 2, and 3 are 48, 52 and 54°C, respectively.

PCR reactions (25-ul) were run using Perkin-Elmer Amplitaq and GeneAmp® core reagents. The template used for the Felix-homologous amplifications (fragments 1 and 3) was a 1:100 dilution of a Qiagen Lambda® preparation of Felix O1 genomic DNA, whereas the template for fragment 2 amplification (*lux*AB) was an undiluted Qiagen plasmid preparation of 3046-2-2 which contained *lux*AB on plasmid pUCf*lux*12.

The PCR products were cloned without modification directly into a system designed for products which contain a single 3' adenosine overhang that results from amplification with *Taq* polymerase. PCR products were used directly in ligation reactions with plasmid PCR2.1® according to manufacturer's directions (Invitrogen), followed by transformation of TOP10 competent cells with the ligation mixtures. Positive transformants were obtained in the first attempt, and representative isolates for each fragment are stored frozen at -80°C. The appropriate size insert was confirmed for tranformants resulting from each of the three fragments using restriction digestion followed by gel electrophoresis. PCR (with the same primers used to produce the respective fragments) was also used to confirm the inserts, and the following isolates were stored frozen following insert confirmation: 1-1, 1-2, and 1-3 (each containing fragment 1), 2-6, 2-8 and 2-14 (containing fragment 2) and 3-2 (containing fragment 3).

Each fragment was prepared for the four-fragment ligation by extracting the plasmid from isolates 1-3, 2-8 and 3-2 and excising each fragment from its plasmid vector using the appropriate restriction enzymes (enzymes whose recognition sites were

engineered into the primers, see Figure 4). In the case of fragment 2, the *Nhe*I and *BamH*I digests could be performed simultaneously using Promega Multicore® buffer, but the other fragment digestions had to be performed in a specific order so as to prevent cutting too close to a desired restriction site with the other enzyme. After each plasmid (1-3, 2-8 and 3-2) was double-digested, the appropriate fragment was extracted from a preparative low-melt agarose gel using a QiaEx II® kit. The plasmid into which the fragments would be ligated (pUC18 and later pBluescript II SK +/-®) was cut with *Sac*I and *Sal*I (to produce compatible ends for restricted fragments 1 and 3) and was also extracted from a gel. Gel extraction was necessary to prevent erroneous ligation events with remaining plasmid pieces (that would also have the same compatible ends as the fragments). At first, the fragments were gel extracted separately, and the fragments were added separately to ligation reactions (20-ul ligation reactions using 40 units of NEB ligase per reaction). After several failures, gel slices containing the three fragments were combined and extracted together, thus allowing for higher concentrations of each fragment in ligation mixtures.

IV. Results

A. Hybrid primer design

In light of repeated failures with the BTX unit despite modulation of DNA concentration and resistance, electrotransformations of *S. typhi* were attempted using a BioRad GenePulser II®, in order to duplicate precisely the recommended parameters given by O'Callaghan et al (1990), including the lower capacitance of 25 uF. Even the exact recommended conditions for electrotransformation of *S. typhi* were

unsuccessful. Altering gap length (using an electroporation cuvettes with a gap length other than 0.2 cm and changing the voltage to achieve the same field strength) did not remedy the situation either. Likewise, using more plasmid DNA (200 ng versus 100 ng) did not improve results.

The four SA strains of *S. typhimurium* LT2 listed in Table 1 were successfully electroporated with pUC18 containing the 2.1 kb *lux*AB genes flanked upstream and downstream by 17 and 20 bp of Felix DNA (the hybrid PCR primer construct). This pUC18-derived plasmid was first successfully cloned into *E. coli* (Stratagene XL10 Gold Ultracompetent®) and is called pUCf*lux*12. The electrical parameters that facilitated the successful *Salmonella* transformations were achieved using the BTX Electro Cell Manipulator 600® with BTX 0.2-cm gap length cuvettes. In each successful case, 1.0 ul of a Qiagen preparation of plasmid DNA (about 100 ng) was used in the transformation, and settings were 2.5 kV, 50 uF capacitance and 129 ohms resistance. Successful transformants were screened for ampicillin resistance and were confirmed using restriction analysis (*Xba*I & *Sac*I) and PCR confirmation. At least one successful transformant of each strain was preserved at –80C. Table 1 gives the strain transformed, the name of a representative transformant saved, and the actual voltage and pulse length achieved when pUCf*lux*12 was inserted into each by electoporation.

Table 1. pUCflux12 Electrotransformants of *S. typhimurium* LT2 and pertinent electrical parameters.

Strain of *S. typhimurium* LT2 transformed	Name of transformant	Actual voltage achieved (kV)	Actual pulse length (ms)
SA3552	SA3552f*lux*1	2.37	5.39
SA3394	SA3394f*lux*10	2.37	5.41
SA3046	3046-2-2	2.36	5.47
SA3047	3047-2-3	2.36	5.43

The copy number of this plasmid in each *Salmonella* strain is probably not as high as it is in *E. coli* (as evidenced by lighter bands obtained on an agarose gel following restriction digestion of plasmid preparations). However, the plasmid does exist in sufficient numbers to produce visible bands on an agarose gel, and exists in high enough number to produce adequate penicillinase for selection on ampicillin-containing media. PCR amplification of plasmid extracts from the four transformants listed in Table 1 was performed using the same hybrid primers employed to produce pUCf*lux*12 in the first place. All PCR-amplified plasmids produce the desired 2.1-kb amplicon. The plasmid not only exists in each strain of *Salmonella*, but in one case there is apparent low-level expression of luciferase, despite the fact that pUCf*lux*12 is not an expression vector. Serial dilutions of SA3046-2-2 produced titratable luminescence with a relative luminescent unit (RLU) output different from background with as few as 10^5 cfus.

Felix O1 wild-type was able to productively infect the tranformants at 30°C (just as it was able to produce plaques on the parent strains). Several attempts were made to expose the wild-type Felix to the plasmid-containing host according to the infection and selective enrichment described, but none of the phage progeny (potential recombinants) tested from these experiments exhibited luminescence significantly above the background threshold. A few samples seemed to show slight promise (111 RLU versus 48-66 for negative control samples), but no significantly increased RLU outputs were seen upon sub-propagation of these samples. To give a few examples of the magnitude of luminescence one might expect with true luciferase expression, strong constitutive expression of luciferase in a bacterium (as is seen with *E. coli* JM109 pJE202, kindly provided by Ann Stevens) produces RLU values on the order of 10^7.

Tranformant SA3047-2-3 (from these experiments) produces some luciferase, though the construct was not deliberately cloned for expression. A 1.0-ml suspension of SA3047-2-3 containing 10^7 cfus produced an RLU reading of 1.9×10^4. Loessner found with phage A511::*lux*AB that using phage A511::*lux*AB at concentrations of 10^8pfus/ml yielded RLU values in the 10^5 range, and values between 80 and 100 were typical for negative controls, e.g. attributable to background (Loessner *et al.*, 1996).

B. Four-fragment ligation

Despite repeated attempts to ligate the three fragments into pUC18 and pBluescript II SK +/-® cloning vectors, using DH5α® competent cells as well as Stratagene XL10 Gold Ultracompetent® cells, no correct transformants were produced. Although some apparent positives did result from some transformations, their extracted plasmids either contained no insert or one of inappropriate size. This latter condition indicated either that the insert present was too small to detect or that the plasmid religated following degradation of the cut ends.

Subsequently, attempts were made to achieve the desired construct in a stepwise fashion, versus relying on simultaneous ligation of all four fragments. New restriction sites were researched in the plasmids that already housed the separate fragments, in hopes that the other two fragments might be placed (in two separate steps) into PCR2.1® which already held one of the fragments. There are indeed enough unique restriction sites to achieve this. For example, fragment 1 (cut from its plasmid with *Nhe*I and *Sal*I) could be ligated with a *Nhe*I/*Xho*I-cut plasmid from isolate 2-8. (*Sal*I restriction makes a four-base overhang compatible with *Xho*I-cut DNA). This ligation would place fragment 1 upstream of fragment 2 in the desired orientation. This was tried, but

unfortunately the attempts were plagued by restriction digest problems, apparent religation of fragments, and plasmid degradation. However, the possibility still exists for this to work. Had fragment 1 been successfully inserted into isolate 2-8 upstream of *lux*AB, fragment 3 could have been subsequently added to the plasmid downstream of *lux*AB in the same fashion using other restriction endonucleases.

V. Discussion

A. Electroporation of *S. typhi* phage type Tannanarive

At least three reasons are possible for the repeated failures with *S. typhi*. Perhaps the plasmid DNA failed to enter the cells in the first place (due to inadequate electrical parameters). It is also conceivable that the plasmid entered the cells, but did not achieve an adequate copy number to be detected nor to produce the selectable marker (ampR, ampicillin resistance). The third and most likely possibility is that the plasmid entered cells but was destroyed by host restriction/modification defenses (most notably, *Salmonella hsd* gene products). Subsequent electroporation of other *Salmonella* strains with the BTX 600 using parameters that had been tried with *S. typhi* proved successful. This indicates that the host was the likely reason for lack of success with *S. typhi*. This possibility is further supported by the fact that all successful electroporations resulted with strains mutant in *hsd* and *rec*A genes (*S. typhimurium* LT2 strains SA3394, SA3552, SA3046 and SA 3047). Specifically, all four strains contain mutations in *hsd*L, *hsd*SA and *hsd*SB genes and are phenotypically deficient in restriction activity associated with these three genes.

B. Hybrid primer approach

As failures were resulting from the enrichment and screening for recombinant phages with the first approach, more sequence data surrounding the *rII*B region was being generated. One shortcoming of the hybrid primer design approach is the short flanking sequences of Felix O1-homologous DNA contained on plasmid pUCf*lux*12. Some mechanisms allow for recombination between short segments of phage DNA, but longer homologous sequences enhance the chances for a recombination event to occur (Mosig, 1994). It was feared that the low frequency of double-crossover recombinants in the hybrid primer design approach would mean an inordinate number of attempts would be necessary to isolate the desired phage. Therefore, the decision was made to abandon this hybrid primer design approach for one with longer Felix O1-homologous flanking sequences. Revisiting pUCf*lux*12 unaltered as a means for producing recombinants would probably not be advisable, in light of the short Felix-homologous flanking sequences. However, since this plasmid contains *lux*AB (and apparently expresses luciferase), and has been successfully cloned into *Salmonella*, it could be changed to include longer Felix O1 flanking sequences and tried again.

The *lux*AB genes contained on pUCflux12 were promoterless. If this construct had been successfully recombined with the Felix O1 genome as planned, any and all *lux* expression would have first depended on *in situ* Felix promoter activity (transcription) and subsequently on translation facilitated by the primer-contained ribosomal binding site and 7-mer spacer. Upon initial consideration, it might have seemed logical to put a strong exogenous *Salmonella* promoter in front of a gene whose expression was desirable. It is important to note, however, that phages alter the host RNA polymerase soon after infection such that recognition

and use of phage promoters predominates (Stitt & Hinton, 1994). The selective expression of middle and late phage genes (including *rII*B) over host genes is largely attributed to the ADP ribosylation of host RNA polymerase. For this reason, incorporation of a *Salmonella* promoter may not have facilitated transcription. Also, the promoter region for *rII*B in T4 is the prototype for transcription of classical middle phage genes. Insertion of a promoterless *lux*AB construct into the region was designed to employ the endogenous *rII*B promoter(s), and to test its/their strength.

An expression-based assay for isolating a *lux*AB-containing Felix O1 recombinant assumed that the DNA construct would not only be present in the phage genome, but that it would exist in a way to allow for transcription, translation, and production of a functional protein. The possibility did exist that a recombination event may have indeed taken place in the first exposure of Felix wild-type to the pUCf*lux*12-containing *Salmonella*, but the recombinant was unable to express luciferase and was therefore undetectable using Loessner's enrichment method. The DNA probe-based plaque lift assay would have been useful for detecting non-expressing recombinants, though the pursuit of this type of screening method would need to be evaluated in terms of the usefulness of a non-expressing recombinant phage.

C. Four-fragment ligation approach

Repeated failures in the four-fragment ligation approach probably stem from one or both of the following reasons: fragment incompatibility and/or technique. It is conceivable that the three fragments to be placed together, in their current form, simply cannot be successfully ligated with one another. The lack of positive results is somewhat confounding in light of the many attempts made and parameters varied in order to try to

achieve this. One way to determine if one of the fragments is problematic would be to continue in the vein of stepwise ligation and cloning, as opposed to attempting simultaneous ligation of 4 separate fragments.

Regarding technique, there are several issues that could be addressed/tested for their significance in these experiments. For example, presumptive transformants did result from some four-fragment ligation attempts, and some even contained inserts (albeit the wrong size or composition). This indicates that preservation of fragment and plasmid compatible ends as well as ligation efficiency should be addressed more closely. Control ligations in some cases indicated ligation of fragments that should not join. This may be attributed to non-specific ligation (due to too high a concentration of ligase) of fragment ends that could have been damaged during the restriction/gel extraction steps. Again, stepwise ligation of the desired fragments would probably lend itself to identifying technical problems more than would continued attempts to ligate four fragments of DNA all at once.

D. Other Approaches to mutagenesis

Consideration of other approaches to mutagenesis should be guided by the primary goal of mutagenizing phage Felix O1. The approaches described above were designed to simultaneously produce a recombinant more applicable as an intervention and also applicable as a detection method for *Salmonella*. Perhaps this was overly optimistic. The constructs would have possibly been designed differently if these two goals had been kept separate.

If design of a detection method using *lux*AB genes is the main goal, these genes could probably be placed elsewhere in the Felix O1 chromosome

using methods that have worked before (Loessner *et al.*, 1996). Now that the entire chromosome has been sequenced, studies could be undertaken to find genes that are strongly expressed. These loci would be logical choices for placement of *lux*AB genes, so that strong expression would be expected. Loessner's A511::*lux*AB construct (1996) places luciferase after the *cps* promoter, a strong late promoter. This promoter is also employed by Chen & Griffiths in their *Salmonella* detection phage system (using relatively uncharacterized phages; Chen & Griffiths, 1996). If a region was found to be strongly expressed, it is possible that there is a clone (from one of the Felix O1 genomic libraries) that contains the Felix O1 DNA of interest on a plasmid already. A plasmid from the *Hind*III or randomly-sheared library could be employed for insertion of *lux*AB genes using the methods of Loessner (1996). In fact, this method would have probably been used for insertion of *lux*AB into the *rII*B region of Felix O1, had a plasmid containing the area of interest been available. Unfortunately, only two genomic library clones (FR338 from the random library, FH137 from the *Hind*III library) contained DNA associated with *rII*B. Neither plasmid contained both the upstream and downstream regions desireable for insertion of *lux*AB genes. However, both genomic libraries contain clones that cover several other regions where *lux*AB insertion could be tried, including *nrd*A, *nrd*D and *gp*37 (tip of tail fiber) homologues.

If, on the other hand, virulence is the main goal of mutagenesis, other approaches may be needed. Though the entire genomic sequence is known, there are still no obvious loci whose deletion would be expected to increase virulence. By the same token, there are no regions whose increased expression would be expected to enhance salmonellacidal abilities. Modulating (increasing) expression of the endogenous lysozyme

homologue may seem like a good choice, but overexpressing lysozyme by itself does not necessarily result in better lysis.

Choosing to place something foreign into the genome (something toxic) is an option. Toxic components of plasmid addiction modules (toxin/antedote pairs such as enterocidin B, *maz*EF or *par*DE) would be a logical choice, but may be difficult to work with and clone into *E. coli* and *Salmonella* (Bishop et al., 1998, Jensen et al., 1995). There are systems for cloning toxin/antitoxin pairs which would be worthy of exploration, especially if cloning in members of the Enterobacteriaciae is necessary for mutagenesis (Lutz and Bujard, 1997).

Random chemical mutagenesis, with selection for mutants possessing desirable qualities, is yet another approach. The biggest disadvantages to this approach include the unknown nature of changes produced and stability of mutation. However, if *rII*B is to be revisited, proflavin is a chemical mutagen that induces frameshifts and tends to selectively mutate *rII* genes. There are published protocols for proflavin mutagenesis of T4, easily adaptable for use with Felix O1 (Wiberg, 1994). One advantage of this method is that a variety of mutations is produced, and researchers are able to choose from several mutants based on functional criteria. It is conceivable to choose a mutant with a more rapid killing dynamic, or one that performs better at lower temperatures. Chemical mutagenesis enables the researcher to pick from a large group of candidates, versus just one that would result from a successful site-directed mutagenesis approach.

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Chapter 4: Bacteriophage Felix O1: A naturally long-circulating bacteriophage in mice

I. Abstract:

The dynamics of bacteriophage Felix O1 in the circulatory system were studied in a mouse model involving parenteral administration of phage. The wild-type phage was passaged nine times in mice in order to promote selection for a persistently-circulating variant phage. Clearance determinations for the wild-type and 9^{th} serial passage phage indicate that serial passage and propagation on a non-mutator strain of *S. typhi* did not improve circulatory longevity. The 24-hour $\log_{10}$ plasma counts for wild-type were 6.5±0.48 pfus/ml versus 4.9±1.7 pfus/ml for the 9^{th} passage following intraperitoneal injection of 5-6 x 10^9 pfus of either phage isolate. Clearance of phage from plasma does not appear to fit a linear model. Sequestration of wild-type and 9^{th} passage Felix O1 in the spleens supports previous reports of the reticuloendothelial system as an important mechanism in clearance of phage from circulation. Wild-type Felix O1 phage, by virtue of its ability to remain detectable in plasma for up to 48 hours, remains a useful candidate for treatment of septicemic *Salmonella* infections.

II. Introduction:

The emergence of multidrug resistant bacterial pathogens has necessitated novel approaches for treating bacterial diseases. For this reason, researchers are again studying the utility of bacteriophages for treatment of bacterial infections, a concept that was pursued in the early part of the century up until the widespread availability of antibiotics for clinical use. Recent studies have addressed the use of phage λ and P22 for the parenteral treatment of *E. coli* and *Salmonella* in mice (Merril *et al.*, 1996). With limited success, Berchieri *et al.* (1991) used an orally administered mix of relatively uncharacterized phages obtained from the environment to prevent *Salmonella* colonization in the chicken. Berchieri and his

colleagues went on to address mortality in chickens and colostrum-deprived calves suffering from K1+ *E. coli* septicemia using a K1-specific phage parenteral treatment. Phage administration dramatically reduced morbidity and mortality in chickens, and may have controlled bacterial proliferation to a certain degree in colostrum-deprived calves (Barrow *et al.*, 1998).

This research was undertaken to develop a phage-based intervention for salmonellosis in food-producing animals. To begin to achieve this goal, the *in vivo* properties of phage Felix 01 were studied. Felix O1 is a bacterial virus possessing broad host-range within the genus *Salmonella*. An initial step in this research was to determine the clearance dynamics of this phage in animals, because one property directly related to its antimicrobial potential is how long the phage will remain in circulation following parenteral administration. Clearance dynamics were studied in mice.

One shortcoming of previous attempts to use bacteriophages for parenteral treatment was the rapidity with which phage is removed from circulation by the host reticuloendothelial system (Merril et al., 1996). The methods were designed to produce, through serial passage, an isolate of Felix 01 which would remain in circulation longer than the wild-type parent . The goal of this research was to compare the *in vivo* survivability of Felix 01 wild-type with that of Felix 01 following 9 serial passages in mice.

III. Materials & Methods:

A. Bacteria and Bacteriophage Strains

Phage Felix O1-VT1 is a clonal pool of a stock kindly provided by Dr. Hans Ackermann, Universite Laval, Quebec, Canada. *Salmonella typhi*, Vi A, phage type Tananarive was used for propagation and enumeration of phage for all experiments, and was also provided by Dr. Ackermann.

B. Propagation of Phage and Serial Passage in Mice

A cesium chloride-purified, dialyzed stock of Felix O1 wild-type was prepared from plate lysates according to the methods of Ausubel et al. (1989). The methods of Merril et al. (1996) were used with modification for serial passage of Felix O1. Specifically, 5×10^9 pfus of the dialyzed product in low-salt buffer were injected intraperitoneally into a 10-week-old female Balb/C mouse. After 24 hours, plasma from the mouse was used to propagate the phage resulting from that passage. Specifically, 100-µl aliquots of serial tenfold dilutions of 24-hour plasma were used to determine the concentration of phage in plasma (see section C, Quantitation of phage in plasma and spleens). Quantitation plates that contained enough phage to produce confluent lysis were eluted using sterile saline. The resulting phage suspension was chloroform treated, centrifuged to remove residual bacterial components, precipitated with polyethylene glycol, dialyzed four times with a low-salt buffer, and filtered through a 0.22 um filter. The resulting phage was injected into the peritoneal cavity of another mouse such that each mouse received approximately 5×10^9 total pfus of phage. This serial passage technique was repeated until a stock resulting from 9 serial passages had been obtained. The serial passage was expedited by injecting subsequent mice with 100 ul of the amplified, purified stock from the previous mouse before the phage titer of this purified suspension had been determined. Subsequent titration of each passage's purified suspension revealed that mice received between 1×10^9 and 2.4×10^{10} total pfus of phage Felix 01, depending on the passage. In every case except one, there remained enough phage in the plasma at 24 hours to make a high-titer stock using the soft agar overlay method. The fourth passage attempt failed to yield any phage remaining in plasma at 24 hours, so a high-titer stock was

prepared from phage contained in the spleen instead. The phage injected for the fifth passage therefore resulted from splenic amplification.

C. Quantitation of Phage in Plasma and Spleens

Blood was collected from the orbital plexus into heparinized capillary tubes. The plasma was separated by centrifugation, and tenfold serial dilutions in saline were plated with *S. typhi* onto solid media using a soft agar overlay method. Spleens were collected following cervical dislocation and in each case were aseptically homogenized with 1 g sterile silica and 1 ml of sterile saline.

D. Experiment 1: Determination of Clearance of Wild-type Felix 01

Eight 8-week-old female Balb/C mice were used, and two mice were housed per cage. Each mouse was injected intraperitoneally at time 0 from the same stock with 5×10^9 pfus of wild-type Felix 01 VT1. Plasma phage titers were determined for two mice every 12 hours from orbital plexus blood samples until 96 hours post-injection of phage. Plasma was sampled more than once for some or all mice. At 24-hour intervals, two mice housed in the same cage were sacrificed, and splenic phage titers were determined.

E. Experiment 2: Determination of Clearance of 9^{th} Passage Felix 01

Ten 8-week-old female Balb/C mice were injected intraperitoneally at the same time with 6×10^9 pfus of Felix 01 resulting from the 9^{th} mouse passage. At 24-hour intervals, two mice from the same cage were bled (orbital plexus), sacrificed, and spleens were collected. Plasma and splenic titers were determined for each interval, and no repeated measures of plasma were made during this experiment.

IV. Results

The concentration of phage remaining in mouse plasma and spleens at 24-hour time intervals is provided in Figures 1 & 2, respectively.

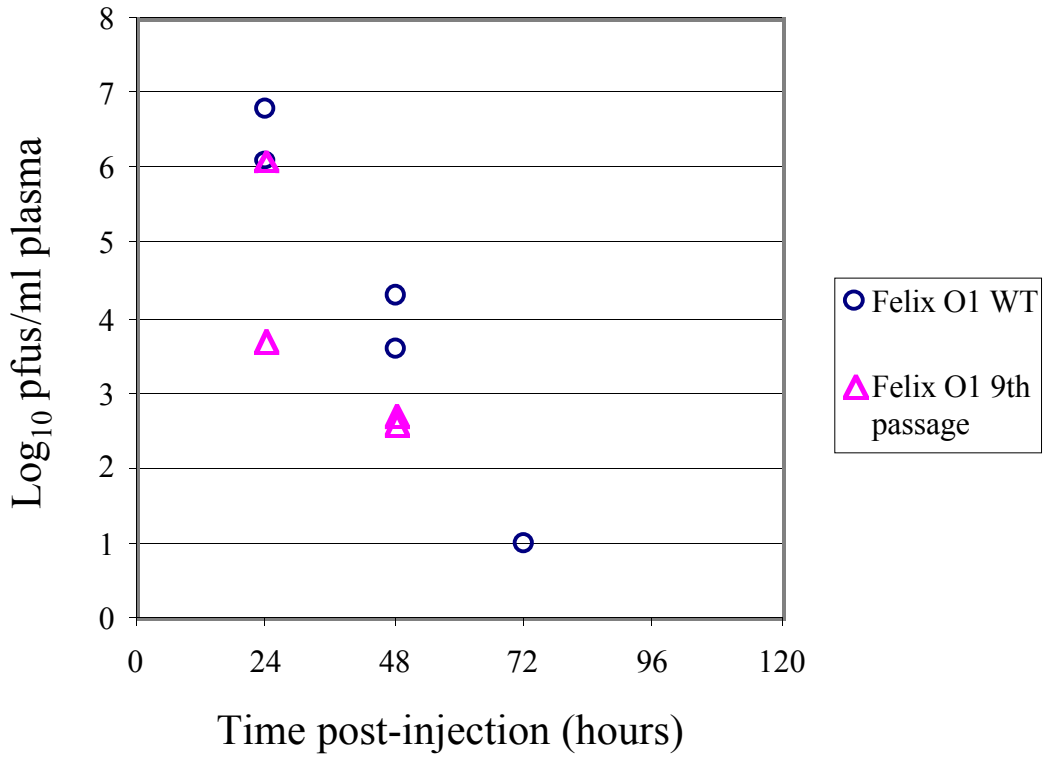

Figure 1. Plasma clearance of Felix O1 isolates. Plasma concentration of phage remaining at different time points is expressed for each mouse sampled in $\log_{10}$ pfus/ml plasma. Wild-type and 9th passage titers were determined in separate experiments. Blood was collected from the orbital plexus at 12-hour intervals for wild-type and 24-hour intervals for the 9th passage isolate following intraperitoneal injection of 5-6 x 10^9 pfus. Some animals in the wild-type-treated group were sampled more than once. Plasma was separated by centrifugation, and plasma titers were determined by soft agar overlay plating of serial tenfold dilutions of plasma.

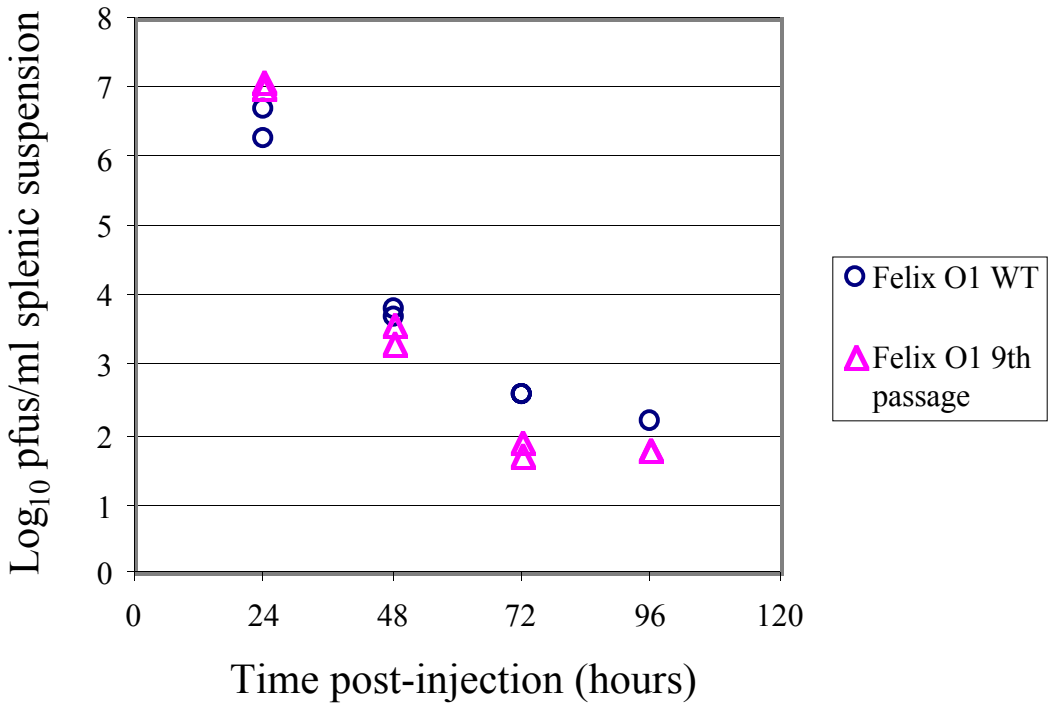

Figure 2. Splenic clearance of Felix O1 isolates. Phage remaining in mouse spleens at different sampling times is expressed in $\log_{10}$ pfus/ml splenic suspension. Wild-type and 9th passage splenic titers were determined in separate experiments. Mice were sacrificed at 24-hour intervals following intraperitoneal injection with 5-6 x 10^9 pfus of Felix O1 wild-type or 9th passage. Spleens were homogenized with 1 g sterile silica and 1 ml of 0.85% sodium chloride. Splenic titers were determined by soft agar overlay plating of serial tenfold dilutions of splenic homogenates.

The plasma phage counts fell below the limit of detection (10 pfus per ml plasma) by 72 hours for both the wild-type and 9th passage (Figure 1). However, the splenic values never went below the limit of detection for the duration of the experiment (except for one of the wild-type-injected mice at the final 96-hour sampling), and the splenic phage counts were similar for the wild-type and 9th passage experiments (Figure 2). In addition, the protein profiles of these two isolates of Felix 01 were similar, and immunoreactivity with Felix wild-type-specific rabbit antisera did not change significantly with serial passage in mice (data not shown).

V. Discussion

Though the clearance dynamics for the wild-type and the 9th passage of Felix phage were determined in separate experiments, comparison of these separate results yields some key conclusions. Serial passage of Felix 01 phage with propagation between passages on a non-mutator strain of *S. typhi* does not seem to select for a longer-circulating mutant phage. This is supported by similar counts between the two phage isolates in spleens and plasma at each 24-hour interval and the similar timing of clearance from plasma for the two experiments (Figures 1 and 2). The apparent lack of mutation may be due to the lack of impetus for mutation to occur (use of a non-mutator host strain). It is also possible that no mutations were apparent because phage was collected from the blood too soon after injection of the previous passage (thus not allowing time for selection of a longer-circulating mutant to occur). The results may also be attributed to the relative stability of Felix 01 *in vivo*, at least in terms of phenotype in circulation.

Merril *et al.* (1996) rate the circulatory persistence of λvir (called W60), and mutants Argo1 and Argo2 according to regression analysis of plasma titers over a 24-hour period. The clearance results for Felix do not appear to fit a linear model,

as is Merril's interpretation of W60 clearance data, but the 24-hour titers indicate that Felix wild-type remains in circulation longer than the pre-passage wild-type phage (W60) used by Merril *et al*. (1996). The titer of Felix WT at 24 hours post-injection with 10^9 pfus is between 10^6 and 10^7 (Figure 1), versus between 10^4 and 10^5 for W60 following injection of 10^{10} pfus. The 24-hour Felix plasma titer is, however, lower than the long-circulating mutants of W60 (Argo1 and Argo2), whose titers at 24 hours are between 10^8 and 10^9. However, the importance of this comparison may be tempered by the fact that Merril *et al.* injected mice with 10 times as much phage as was used for the Felix trials. Injection of the 9th serial passage did not improve the Felix titer at 24 hours, and in fact produced a titer somewhere between 10^3 and 10^7 pfus/ml plasma (Figure 1).

Success of parenteral phage therapy for septicemia depends, in part, on ability of the phage to evade the host's immune system and remain in circulation (Merril *et al.*, 1996). The 24-hour post-injection Felix O1 wild-type titers are higher than Merril's W60 phage before serial passage, but lower than the 24-hour titers of his long-circulating Argo1 and Argo2 mutants. Comparison of our results with Merril's P22 phage isolates is difficult because the dosing regimens were different. Nevertheless, based on the circulatory longevity of the parenterally-applied phages in Merril's studies, Felix O1 appears to be a promising candidate for parenteral therapy. It appears that the wild-type phage O1 would remain in circulation long enough to be useful in a *Salmonella* treatment, though actual efficacy in treatment of *Salmonella* infections has yet to be tested.

The longevity of Felix O1 in circulation was not altered appreciably by the protocol employed for serial passage in mice. Three reasons are possible for this. First, phage was collected from mice for propagation and subsequent passage only 24 hours after injection with the previous passage. Perhaps 24 hours was insufficient to select for a longer-circulating variant. Also, a mutator strain of

Salmonella was not used for propagation between passages. This would have been expected to increase the incidence of mutation affecting circulatory longevity over the incidence expected from serial passage alone. Third, it is possible that wild-type Felix O1 remains in circulation as long as is possible for the phage, and serial passage will likely not improve the circulatory attrition seen with wild-type phage. This third possibility could potentially be verified by another study employing longer times between injection and collection as well as propagation on a mutator strain of bacteria. A repeat experiment should be designed to include concurrent testing of wild-type and serial passage isolates, so as to avoid potential confounding factors associated with isolates being tested at different times.

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Chapter 5: Bacteriophage Felix O1: suppression of *Salmonella* growth by wild-type (WT) and large-plaque (LP) isolates in liquid culture and on poultry frankfurters

I. Abstract

Salmonella typhimurium DT104 is an emerging foodborne pathogen that has been isolated from several food animal production systems, including poultry. Bacteriophage Felix O1, a member of the Myoviridae, is specific for and possesses broad host-range within the genus *Salmonella*. A poultry field isolate of this pathogen was found to be susceptible to productive infection and lysis by phage O1. This work explores a Felix O1 phage-based intervention for *S. typhimurium* DT104, an intervention potentially applicable at several stages during animal production and processing. From an experiment involving *in vivo* serial passage of Felix O1 in mice, a variant of Felix O1 was obtained that produces a larger, clearer plaque phenotype (LP) on *S. typhi* than the wild-type Felix O1 (WT), not unlike *r* mutants of phage T4. LP exhibited slightly greater overall suppression of *S. typhi* in BHI broth, based on culture turbidity (OD600). Both WT and LP were tested for their ability to suppress *S. typhimurium* DT104 growth in BHI broth and on poultry frankfurters. Both phage isolates suppressed log-phase cultures containing 8.2×10^6 cfus/ml of *S. typhimurium* DT104. A multiplicity of infection (MOI) of one was effective for WT and LP, whereas increasing the MOI to 5 did not increase suppression. WT and LP were tested for their ability to suppress *Salmonella* growth on chicken frankfurters contaminated with 300 cfus of *S. typhimurium* DT104, by comparing *Salmonella*-contaminated, untreated frankfurters with treated samples (at MOI of 1.9×10^4). Suppressions of 1.8 and 2.1 $\log_{10}$ were achieved by WT and LP, respectively ($p=0.0001$), but there was no difference between the performance of either isolate ($p=0.5088$). A 2 x 2 factorial design was used to study the impact of available *Salmonella* host cells on the concentration of phage recovered. Presence of *Salmonella* increased phage recovered from WT-treated frankfurters ($p=0.0011$) but not from LP-treated

frankfurters (p=0.4416). These results suggest that phage-based bacterial intervention may be useful for combating *S. typhimurium* DT104 in a processed poultry product.

Abbreviations used:

BHI: Brain Heart Infusion broth, Difco®

cfus: colony forming units

DNA: deoxyribonucleic acid

LP: large-plaque variant of Felix O1 wild-type phage

MOI: multiplicity of infection

OD600: optical density at 600 nm

pfus: plaque-forming units

SEM: standard error of the mean

TB: Tryptone Broth, Difco®

TSA-$CaCl_2$: Trypticase soy agar (Difco®) with 0.5 mM calcium chloride

WT: Felix O1 wild-type

II. Introduction:

Bacteriophage O1 (also called phage Felix O1, 01 or 0-1) is a member of the A1 group of the Myoviridae (Ackermann, 1973). It was first referenced by Felix and Callow and is the anti-O phage used in the original scheme for the identification and typing of *Salmonella typhi* (Felix & Callow, 1943). Phage O1 is fairly unique among *Salmonella* bacteriophages by virtue of its broad host range within and its specificity for the genus. Of 15 serogroups tested in one study, all but 2 included members that could be productively infected by phage O1 (Kallings, 1967). Furthermore, Kallings found that in general, other gram-negative enteric bacteria are resistant to lysis by phage O1.

Salmonella typhimurium DT104 is an important, emerging, multidrug-resistant pathogen that has been isolated from several food animal production systems, including poultry (Centers for Disease Control and Prevention, 1997). Foods of animal origin are considered an important source of human disease caused by this pathogen (Centers for Disease Control and Prevention, 1997). Felix O1 productively infects a poultry field isolate of this organism, and that the titer of a given phage suspension propagated on *S. typhimurium* DT104 is comparable to that obtained on *S. typhi* phage-type Tannanarive, the strain used for propagating phage O1 in this study (less than one $\log_{10}$ lower). Based on *S. typhimurium* DT104's susceptibility to Felix O1, it was felt that this phage might be useful as a biological intervention for this important human pathogen.

Serial *in vivo* passage of Felix O1 wild-type (WT) in mice resulted in isolation of a variant of Felix O1 that produces a larger, clearer plaque on *S. typhi* than that produced by WT (Chapter 4). We have designated this variant "LP". The LP variant exhibits the same plaque phenotype as do *r* (rapid-lysis) mutants of phage T4 (Hershey, 1946). T4 particles mutant in *r* genes are free of the mechanism known as lysis inhibition, a delay of host cell lysis that occurs when multiplicity of infection (MOI) is high or when superinfection by other phages occurs (Abedon, 1994). It is possible that LP is an *r* mutant of some kind, potentially free of the lysis inhibition mechanism. An ideal phage-based anti-*Salmonella* intervention would have predictable infection and lysis properties, regardless of MOI. A rapid lysis variant might be more desirable for a food processing application, since there may be little practical control over actual MOI and presence of superinfecting phage particles in a processing setting.

The following experiments include initial observations of phage Felix O1 in liquid *Salmonella* culture and an applied component addressing efficacy of phage in suppression of *Salmonella* growth on a poultry product. In addition to testing

the efficacy of Felix O1 WT for suppression of *Salmonella* growth, the comparative efficacy of WT and LP was studied in a broth system as well as on chicken frankfurters. This study was designed to exploit Felix O1 for its salmonellacidal abilities and seeks to develop a biological, phage-based application for reducing *Salmonella* contamination during poultry processing and production. The following experiments illustrate success in suppressing *Salmonella* growth in broth and on poultry frankfurters using two isolates of phage O1.

III. Materials and Methods:

A. Bacteriophages and Bacterial Strains

Bacteriophage Felix O1 wild-type (WT) used in these experiments was amplified from a clonal derivative of a stock obtained from Dr. Hans Ackermann, Université Laval, Quebec, Canada. The large-plaque isolate (LP) was obtained by *in vivo* serial passage of WT in mice (Chapter 4). WT and LP were propagated on trypticase soy agar containing 0.5 mM $CaCl_2$ (TSA-$CaCl_2$) using *Salmonella typhi* phage-type Tannanarive as the bacterial host. Phage treatments were prepared by harvesting 30 Felix O1 plaques from propagation plates, eluting in 3 ml of tryptone broth (TB, Difco), followed by chloroform treatment. The titers of plaque-pick suspensions were determined by counting plaques from serial tenfold dilutions of suspensions applied to quadrants of *S. typhi* spread plates that were subsequently incubated overnight. The concentration of each suspension was adjusted by diluting with the appropriate volume of tryptone broth. *Salmonella typhimurium* DT104 used in these experiments was a poultry field isolate. This isolate was grown in brain heart infusion (BHI) broth for use in the spectrophotometric comparison of WT and LP Felix O1 as well as for the frankfurter contamination and treatment experiments.

B. Spectrophotometric comparison of Felix WT and LP

Suspensions of Felix O1 wild-type (WT) and large plaque variant (LP) were prepared as described above. The day before the experiment, a single colony of *S. typhi* or *S. typhimurium* DT104 was used to inoculate a tube of brain heart infusion (BHI) broth which was grown overnight at 37˚C without shaking. The overnight culture was diluted 1000-fold in prewarmed BHI broth and was grown for 3 hours in a 36˚C-water bath with reciprocal shaking (speed=200 per minute). The 3-hour culture was then diluted 20-fold in prewarmed BHI, and 5-ml aliquots were dispensed into glass tubes. One ml of adjusted phage suspension or tryptone broth placebo was added to designated tubes. Tubes were shaken in a reciprocal water bath at 36˚C (speed=200), and optical density of samples at 600 nm was determined at 20-minute intervals for 4 hours in a Bausch & Lomb Spectronic 20 (Rochester, New York). The concentration of bacteria just prior to phage infection was determined so that actual MOI could be determined. In each case, graphically-depicted data represents one repetition of the experiment with a sample size of 2, 3 or 5 for each phage isolate.

C. Frankfurter contamination and treatment

Chicken frankfurters were chosen because of their potential as a source of foodborne bacterial disease. They were also used because their homogeneous nature facilitated reproducible results in our applied efficacy studies. Chicken frankfurters from a single lot were purchased from a local grocery store. Frankfurters were removed from packages such that the majority of the liquid in the package was left behind. Cylindrical samples (approximately 10 grams each) were excised from frankfurters excluding the ends (five from each), and the samples were placed in

separate stomacher bags which were held closed and refrigerated until use. These weighed, bagged samples were used within 4 days. Samples were randomly allocated to one of six treatments in a randomized complete block design in which experimental day was the block (Table 1). Ten total blocks of the experiment were performed. On each experimental day, frankfurters were held at room temperature for 30 minutes to 1 hour before use.

Table 1. Group assignments for frankfurter experiments

Group	Inoculum[a]	Treatment[b]
1	0.85% NaCl	Tryptone broth
2	*S. typhimurium* DT104	Tryptone broth
3	*S. typhimurium* DT104	Felix O1 WT
4	0.85% NaCl	Felix O1 WT
5	*S. typhimurium* DT104	Felix O1 LP
6	0.85% NaCl	Felix O1 LP

[a]Frankfurters were given either a 0.85% NaCl sham inoculum, or approximately 300 cfus of *S. typhimurium* DT104 in a volume of 250 µl.

[b]Frankfurters were treated immediately after inoculation with either a tryptone broth sham or 5.25×10^6 pfus Felix WT or LP in a total volume of 250 µl.

Briefly, on each experimental day, a culture of *S. typhimurium* DT104 was prepared by diluting an overnight BHI broth culture prepared from a single colony 1,000-fold in prewarmed BHI and shaking at 36°C in a reciprocal shaker (speed=200 per minute) for three hours. The three-hour culture was diluted for use with sterile, room-temperature 0.85% NaCl such that each sample would receive approximately 300 cfus in a total inoculum volume of 250 µl (groups 2, 3 and 5). Groups that were not inoculated with *Salmonella* (1, 4 and 6) were given 250 µl of 0.85% NaCl. Phage plaque-pick suspensions were diluted with tryptone broth as described in part A such that each treated sample would receive approximately 5.25×10^6 pfus in a total volume of 250 µl. Achieving the target bacterial and phage concentrations would mean a multiplicity of infection for groups 3 and 5 of approximately 1.75×10^4. Frankfurters that were not treated with phage were instead given 250 µl of tryptone broth. In each case, the liquids were added directly to the non-cut surface of the frankfurter sample, and the samples were oriented in their stomacher bags such that they would remain in contact with the liquids that were applied. After treatment, the stomacher bags were closed with clips, and the samples were incubated at 22°C (± 1°C) for 24 hours. The frankfurters were then cooled rapidly on ice and homogenized in a Tekmar Stomacher Lab-Blender 80® (Cincinatti, Ohio). The resulting suspension was immediately strained through 2 layers of cheesecloth and held on ice pending titration of *Salmonella* and/or phage. Serial dilutions of strained suspensions were plated on XLT_4 (Difco®, Detroit, Michigan), and black-centered, non-lactose fermenting colonies that resulted were used to calculate concentration of *Salmonella* per gram of frankfurter. Phage counts were determined similarly by plating serial dilutions of chloroform-treated, strained frankfurter suspensions on plated lawns of *S. typhi* phage type Tannanarive on TSA-$CaCl_2$.

D. Statistical Analysis

1. Suppression of Bacterial Growth on Poultry Frankfurters

Results from a pilot study were used to estimate sample size necessary in a randomized complete block design to ensure detecting one $\log_{10}$ difference between performance of WT versus LP in the suppression of *Salmonella* growth on frankfurters (PASS 6.0, power≥80%, α=0.05, Sigma=0.659). This sample size calculation resulted in an estimated15 blocks necessary to test the difference in performance between the two isolates. That is 15 samples would be necessary to detect a 0.5 $\log_{10}$ difference among performance of phage isolates. Log-transformed colony counts for treatment groups 2, 3 and 5 were used as response variables, and responses for each treatment across blocks were compared using one-way analysis of variance and Tukey's post-hoc comparison of least squares means (SAS Mixed Proc., version 8). Standard error of the mean for each group was corrected based on the pooled mean square error. After ten blocks were completed, the experiment was terminated because it was apparent that there was no difference between the performance of WT and LP. Residual plots were used to assess the adequacy of the model and appropriateness of the significance tests performed. Data from the 10 blocks was also used to conduct a retrospective power analysis. This analysis determined the sensitivity of the assay in terms of the smallest difference that could be detected between the performance of two phage isolates using the described methods (Two-Sample T-Test, PASS 6.0).

2. Bacteriophage yield from frankfurters with and without *Salmonella*

Data from groups 3, 4, 5 and 6 of the treatment scheme in Table 1 were used in a two-by-two factorial design addressing presence of *Salmonella* and its effect on resulting frankfurter phage counts following 24-hours incubation. Log-transformed phage concentrations per gram frankfurter were used as response variables to analyze the two main effects (presence/absence of *Salmonella* and phage isolate) and their interaction. The interaction was further defined by performing tests of simple main effects using the SLICE option (SAS Proc Mixed, version 8), and standard error of the mean for each group was corrected using the pooled mean square error.

IV. Results

A. Comparative *Salmonella typhi* suppression by WT & LP in broth

Based on the premise that turbidity can be used to roughly assess bacterial growth and viability in broth (Koch, 1981), spectrophotometric measurements of WT- and LP-infected broth cultures were used to characterize the behavior of WT and LP in liquid culture. Figure 1 shows the optical density of *S. typhi* BHI broth cultures over time following treatment with WT or LP. The more dramatic culture suppression achieved by LP versus WT treatment was typical for experiments involving *S. typhi*.

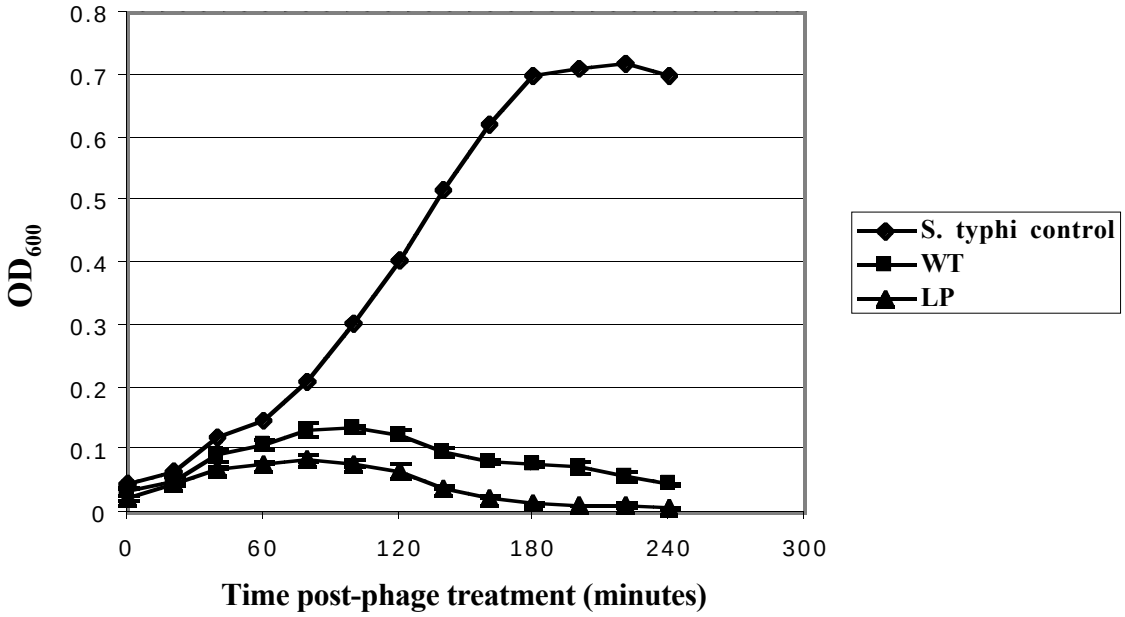

Figure 1. WT and LP suppression of *S. typhi* turbidity in BHI broth. Optical density at 600 nm was determined at 20-minute time intervals following treatment of log-phase BHI cultures of *S. typhi* (1.2×10^7 cfus/ml at time=0 minutes). MOI was 0.14 for both WT and LP. Data points represent the mean of three samples ± SEM. The *S. typhi* control data points are derived from one sample, but are typical of growth characteristics seen under these experimental conditions.

B. Suppression of *S. typhimurium* DT104 in broth, various MOI's

Optimal MOI necessary for culture suppression of *S. typhimurium* DT104 was determined for each phage isolate in broth using optical density as a measure of viability (Figures 2 and 3). MOI's close to 1 (1.2) were effective in suppressing *S. typhimurium* DT104 in BHI broth for both phage isolates tested. A higher MOI (6.1) was not significantly better than an MOI of 1.2 for suppressing culture turbidity for either phage isolate.

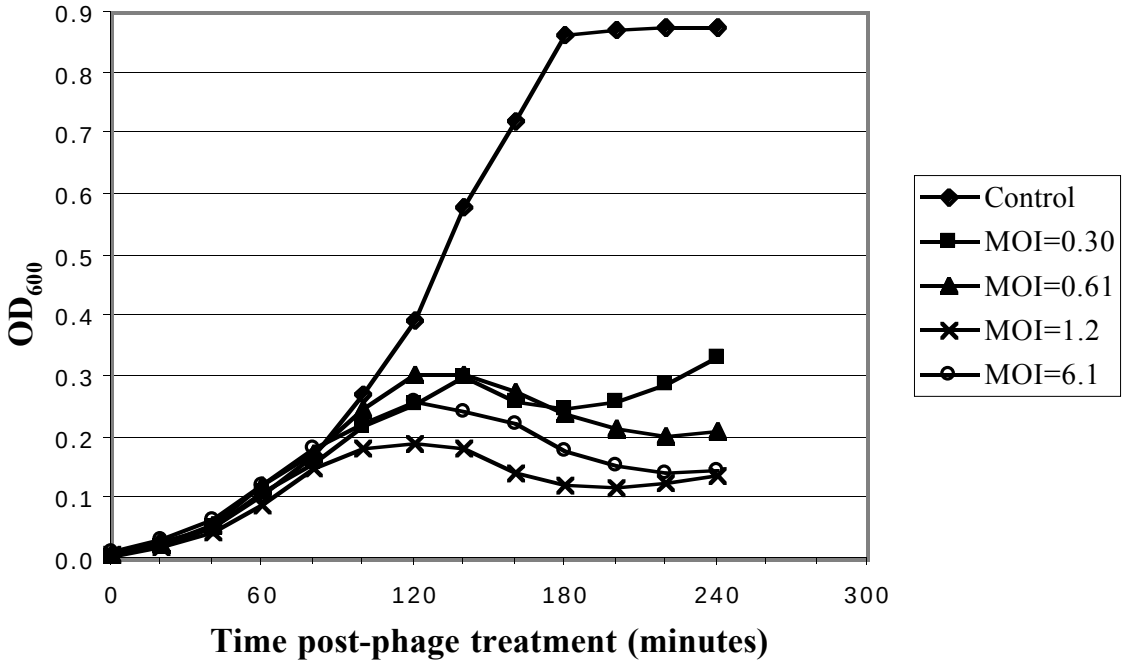

Figure 2. *S. typhimurium* DT104 suppression by WT at different MOI's. Log-phase *S. typhimurium* DT104 was treated with Felix O1 WT in BHI broth at various MOI's. Data points represent the mean optical density of 2 samples at 600 nm. Starting bacterial concentration was 8.2 x 10^6 cfus/ml.

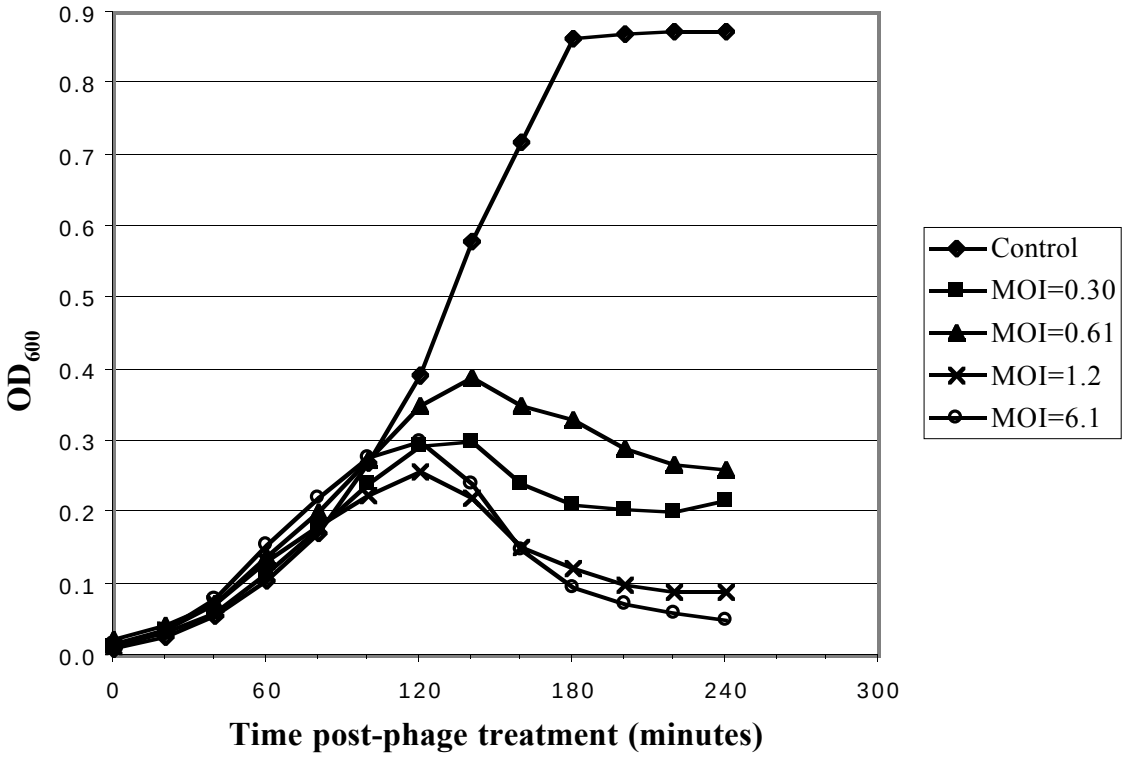

Figure 3. *S. typhimurium* DT104 suppression by LP at different MOI's. Log-phase *S. typhimurium* DT104 was treated with Felix O1 LP in BHI broth at various MOI's. Data points represent the mean optical density of 2 samples at 600 nm. Starting bacterial concentration was 8.2 x 10^6 cfus/ml.

C. Comparative *Salmonella typhimurium* DT104 suppression by WT & LP in broth

The performance of WT and LP on *S. typhimurium* DT104 was compared using MOI's close to one (Figure 4). The greater turbidity suppression effected by LP on *S. typhi* did not occur with *S. typhimurium* DT104.

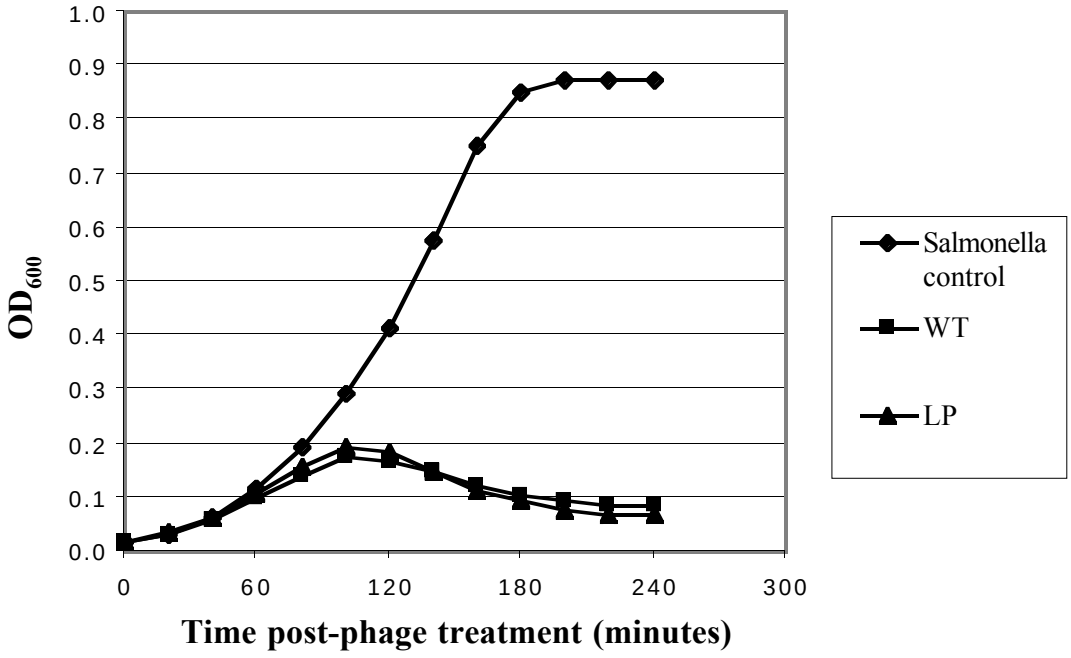

Figure 4. WT and LP suppression of *S. typhimurium* DT104 in BHI broth. Optical density at 600 nm was determined at 20-minute time intervals following treatment of log phase BHI cultures of *S. typhimurium* DT104 (9.0×10^6 cfus/ml at time=0 minutes). MOI was 1.1 and 0.96 for WT and LP, respectively. Data points represent the means of five samples ± SEM.

D. Performance of WT and LP on poultry frankfurters

Although there was no difference between Felix O1 WT and LP performance on *S. typhimurium* DT104 in broth, LP was further pursued as a potential frankfurter treatment in applied experiments. Pilot studies showed that MOI's higher than those necessary for broth experiments were necessary to affect *Salmonella* growth on frankfurters. MOI's of 100 or higher were able to suppress growth, but the lowest effective frankfurter MOI was not determined. Figure 5 depicts the ability of Felix O1 WT and LP to suppress growth of *S. typhimurium* DT104 on poultry frankfurters, based on 24-hour *Salmonella* concentrations for groups 2, 3 and 5. Significantly lower numbers of *Salmonella* were recovered from groups treated with either WT (group 3) or LP (group 5) when compared with the *Salmonella*-inoculated frankfurters in group 2 (ANOVA, $p<0.0001$). However, no difference in 24-hour *Salmonella* concentration was found between WT- and LP-treated groups ($p=0.5088$). An uninoculated control was included in every block. No naturally-occurring Salmonellae were isolated from these control frankfurters (group 1).

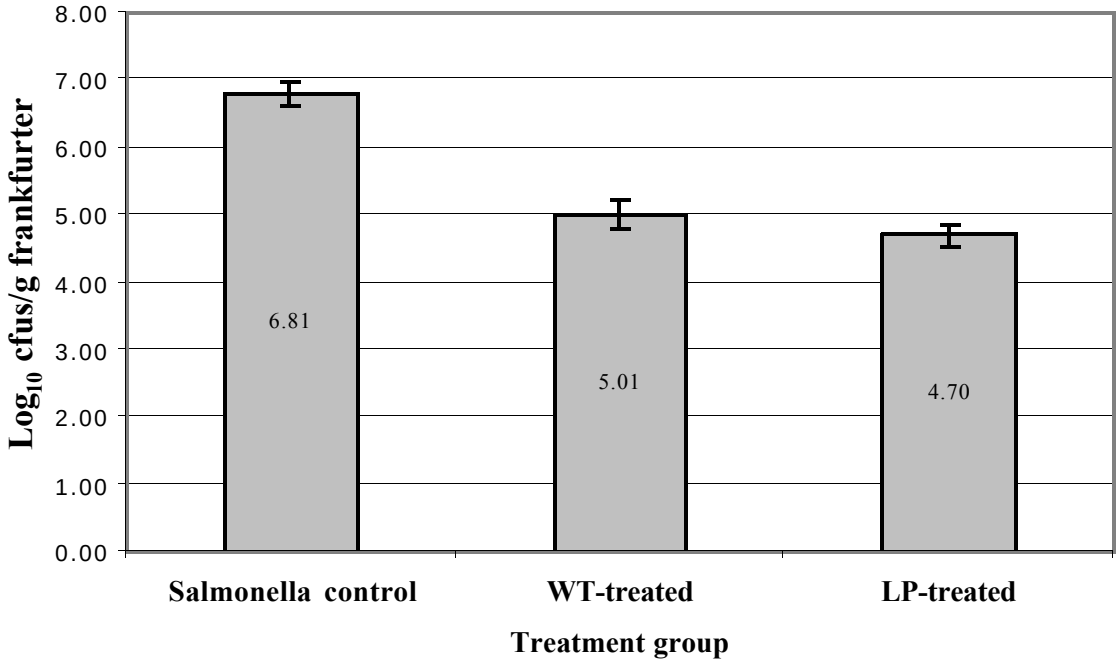

Figure 5. Suppression of *S. typhimurium* DT104 in chicken frankfurter samples by WT and LP. A random complete block design in whichexperimental day was the block was used to test comparative suppression of *Salmonella* growth on poultry frankfurter samples. Approximately 300 cfus of log-phase *S. typhimurium* DT104 were added to randomly-allocated frankfurter samples, followed by immediate treatment with tryptone broth placebo (*Salmonella* control), Felix O1 wild-type (WT-treated) or the Felix O1 large plaque isolate (LP-treated) at an MOI of 1.9×10^4. Log-transformed concentration of *Salmonella* per gram frankfurter after 24-hour incubation at 22°C is reported as mean of 10 samples ± SEM.

Analysis of phage concentrations for groups 3, 4, 5 and 6 revealed a significant interaction between *Salmonella* presence and concentration of phage recovered (Figure 6, p=0.053). However, the interaction applied to only one of the phage isolates. A test for simple main effects within the interaction showed that presence of *Salmonella* contributed to a higher number of WT phage (p=0.0011) but not a higher number of LP (p=0.4416; SAS Mixed Proc., SLICE Option). Frankfurter samples from group 2 were used to confirm that untreated samples were free of naturally-occuring *Salmonella* phages. Most of the group 2 samples did not contain any phage that could plaque on *S. typhi* phage-type Tannanarive. However, two pfus were isolated from the untreated group 2 sample on one of the experimental days. Phage from experimentally-contaminated frankfurters were enumerated from higher dilutions (10^{-2} and 10^{-3}). Therefore, it is unlikely that the spurious, naturally-occurring phage affected the results.

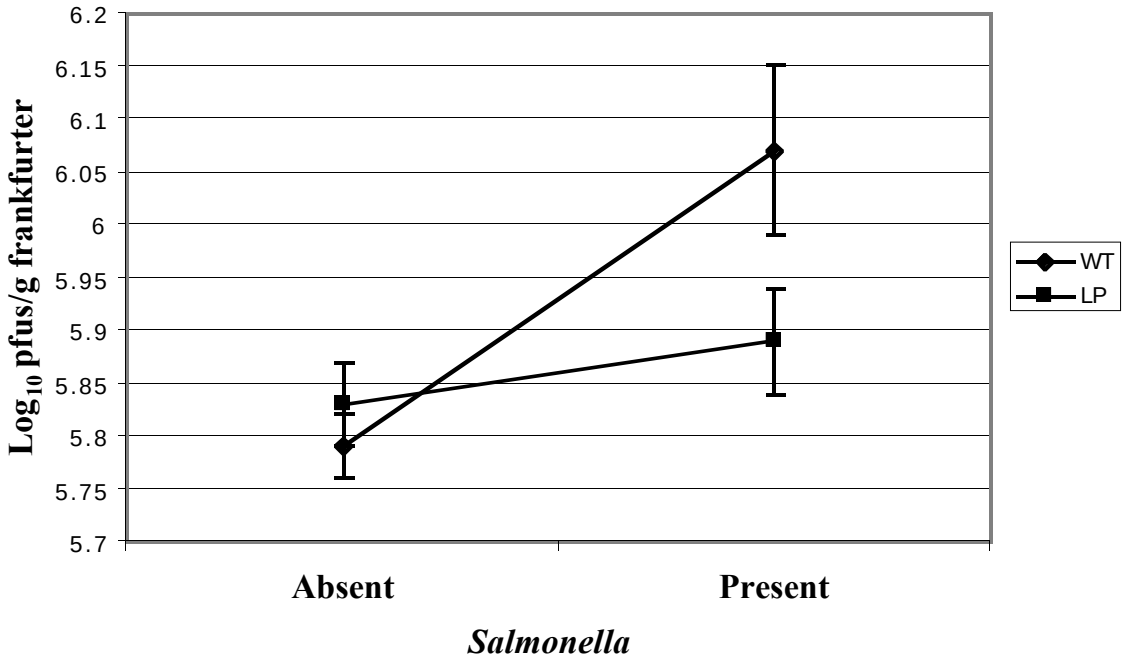

Figure 6. Presence of *S. typhimurium* DT104 as a factor in phage yield from chicken frankfurters. Interaction of phage isolate and presence of susceptible host cells was determined using a 2 x 2 factorial design. Means of 10 blocks are plotted ± SEM. Presence of *Salmonella* was found to have a significant effect on phage recovered for WT-treated frankfurters (p=0.0011).

V. Discussion

Both WT and LP isolates of phage Felix O1 show promise as an anti-*Salmonella* food application. Even under the gross temperature abuse conditions used, both effected about a 2-$\log_{10}$ suppression. The experimental inoculum (300 cfus) may be high compared to that expected in a naturally-contaminated poultry product (Whittemore, 1993). It is possible that, with fewer starting cfus, application of Felix O1 WT or LP could successfully prevent *Salmonella* from reaching an infectious dose in a temperature-abused product. It was expected that a lower starting inoculum might adversely affect the reproducibility of this assay, and might necessitate many more samples to determine the overall and comparative efficacy of phage treatment. However, studies addressing lower levels of contamination are logical in the continued study of phage-based processing intervention. In addition, the lower threshold of phage necessary to achieve suppression of *Salmonella* in different food products should be determined to minimize the cost of phage-based intervention. An MOI of 100 was used successfully in pilot studies with higher starting bacterial inocula (5×10^4 cfus). The results given in Figure 5 indicate a much higher MOI because a lower bacterial inoculum was used (300 cfus versus 5×10^4 cfus), but the phage concentration remained the same. Further study would be necessary to determine whether Salmonella growth suppression is dependent on MOI or an absolute lower threshold of phage in the food product being tested. Knowing the minimum phage concentration necessary to prevent growth of *Salmonella* in naturally-contaminated foods would be helpful for developing appropriate, inexpensive interventions.

The frankfurter method described is a powerful tool for determining not only the efficacy of phage treatment in general, but also comparative efficacy of different phage isolates. A retrospective power analysis using data from the current experiment showed that, had the phage isolates performed differently, as little as a

0.5-log10 difference in bacterial suppression would have been detectable (2-sample T-test, Power=90%, α=0.05, $Sigma_1$=$Sigma_2$=0.38; PASS 6.0). The 0.5 log_{10} difference criterion was used because a difference of 0.5 log_{10} or greater would be expected to impact success of phage-based food intervention. Based on this rationale, the assay is adequately sensitive to differences that would be biologically important and would likely affect performance in an applied setting.

Fewer phage were recovered from *Salmonella*-inoculated, phage-treated groups (3 and 5) than might be expected (Figure 6). In light of the low phage yield, it is important to consider the number of susceptible host cells available for infection. Had infection of host cells been productive, one round of infection would be expected to result in a 2-log_{10} increase in phage titer (expected burst of 10^2 progeny). However, the number of susceptible host cells at the beginning of the experiment was quite low, a factor that would negatively impact yield of progeny. The low phage yield from *Salmonella*-contaminated groups could be due to lysis from without (non-productive infection and lysis) or productive infection with early consumption of most of the host cells. Regarding the latter possibility, even if all 300 *Salmonella* cells had been productively infected and killed at the beginning of the experiment, a burst of 200 progeny per infected cell would only yield approximately 6 x 10^4 progeny. This increase might be inapparent since samples received 5.25 x 10^6 pfus initially. Nevertheless, presence of susceptible host cells effected a small but significant increase in WT concentration (Figure 6). This indicates that some degree of productive infection of *Salmonella* occurred, at least in the WT-treated frankfurters. Conversely, presence of *Salmonella* did not result in a significantly higher yield of LP.

The differences in phage titer found in the 2 x 2 factorial design are not dramatic. Therefore, it is difficult to draw definitive conclusions about the importance of the interaction between presence of *Salmonella* and phage isolate. There is room for

improvement in studying phage yield within the confines of the frankfurter experiments described. Additional frankfurter suspension treatments or use of a different eluent could help reduce experimental error within groups (Kennedy *et al.*, 1986a). However, the exact nature and extent of differences between WT and LP would best be determined with single-step infection studies addressing infection dynamics and burst size (Hershey, 1946). Although other experiments are indicated, the data from the frankfurter experiment described here does offer a possible explanation for the essence of the LP isolate. If the differences in yield for WT and LP found in the frankfurter experiment are indicative of true biological differences, the lower yield for LP in the presence of *Salmonella* could indicate that it is a rapid-lysis mutant. Rapid-lysis mutants of T4 produce far fewer progeny than their wild-type counterparts, as determined in single-step infections (Hershey, 1946).

Preliminary study of LP has not revealed the genetic reason for the LP phenotype, and it remains to be seen if LP is a rapid-lysis mutant. Several genes, including the *r* (rapid lysis) genes, can produce a rapid-lysis mutant in T4 (Matthews, 1994). DNA sequence comparison of WT and LP did not show a mutation in the putative *rII*B gene (data not shown), but mutations in several other genes can produce a rapid-lysis phenotype.

Phages have been found to occur naturally in meats and even further-processed foods (Kennedy *et al.*, 1986b). In fact, phages would be expected to exist wherever their bacterial hosts would be found. The appearance of a few naturally occurring phages from group 2 frankfurters illustrates their ubiquity. Nevertheless, the safety and prudence of adding large quantities of biological entities such as phage to human foods should be explored carefully. Even though phage infection may be specific for a bacterial host, infection of bacteria can have ramifications for eukaryotic systems, including humans (Waldor et al., 1996).

Some phages are able to transfer virulence properties among bacteria. Phage-mediated virulence has been shown for diseases such as cholera, in which genetic transfer by a temperate phage can turn a normally non-pathogenic organism into one that can cause disease (Waldor *et al*., 1996). Felix O1 is most likely a lytic phage rather than a temperate one. If this is the case, all cells infected by phage O1 are killed, thus precluding the opportunity for increased virulence. The entire DNA sequence of the Felix O1 genome has been determined in order to screen for genes that could increase *Salmonella* virulence. No obvious virulence genes have been found so far through homology searches. However, more extensive studies testing the abilities of Felix O1 to transfer genes among *Salmonella* should be performed.

During the sequencing of Felix O1 WT, a few cloned segments of DNA were found that showed strong homology with the Vi antigen of *S. typhi*, an important virulence factor of this human host-adapted *Salmonella* serovar. This was cause for great concern. The Felix O1 used for cloning was propagated on a strain of *S. typhi* that does carry the Vi antigen-coding DNA. It was feared that Felix O1 could carry this gene with the possibility of transfering it to other *Salmonella*. However, the apparent Felix Vi sequences could have been cloned contaminant sequences from the *S. typhi* genomic DNA. In order to localize the source of these cloned segments, Felix O1 was propagated on both Vi+ *S. typhi*, and on a Vi-negative *S. typhimurium*. Amplification of the Vi region from both phage DNA extracts was attempted using PCR primers specific to that region in *S. typhi*. An appropriately-sized product was obtained for phage DNA propagated on *S. typhi*, but not for phage propagated on *S. typhimurium* (data not shown). It was concluded that the Vi amplicon was most likely due to residual genomic DNA from the bacterial host, rather than presence of Vi antigen DNA in Felix O1.

Even if a phage-based intervention does not directly contribute to the virulence of bacteria it infects, selection of subpopulations of organisms is another important consideration for any antibacterial food treatment. Just as inappropriate use of antimicrobials can result in emergence of resistant bacteria, treatment with phage can result in selection of phage-resistant organisms. Felix O1-resistant bacteria have invariably emerged in all the applied experiments we have performed with phage O1. The implications of these phage-resistant organisms for the human host must be determined. Generally speaking, Felix O1 infects smooth strains of *Salmonella*, whereas many rough isolates are resistant. Smooth strains are typically more virulent than rough ones. However, Felix O1 susceptibility is determined by steric availability of the terminal N-acetylglucosamine in the lipopolysaccharide core, and smooth strains resistant to phage O1 have been identified (Hudson *et al.*, 1978; MacPhee *et al.*, 1975). Since a different subpopulation is expected to emerge in the face of phage-based intervention, the comparative virulence of the phage-resistant population selected should be addressed in the development of a Felix O1-based anti-*Salmonella* food application.

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Chapter 6: Conclusions and future directions

The work described in this dissertation lays the foundation for the future promise of applied bacteriophage research as well as the promise of Felix O1 as an anti-*Salmonella* remedial and therapeutic application. Felix O1 was expected to be attractive as a phage-based *Salmonella* intervention, which is why this particular phage was pursued. This phage has met or surpassed the original expectations. The potential of Felix O1 in its future application and development is highlighted by some key findings described in the previous chapters, but more work is indicated in several areas as well. The safety and environmental impact associated with release of unmodified and modified phages should be explored further. In addition, public disclosure of the specifics of phage-based interventions is another aspect important in the development of future phage-based interventions.

I. Safety of a Felix O1-based *Salmonella* intervention

The safety of Felix O1 hinges on a few key aspects of the phage's structure, life cycle and host range. Obvious criteria for safety include the presence/absence of virulence-associated genes in the phage chromosome, and the phage's ability to transfer virulence genes among bacteria. Selective pressure which may result in drug-resistance or a more virulent subpopulation of bacteria should be a concern with any antibacterial treatment, and likewise should be a concern with a phage-based intervention. The virulence of bacteria that are resistant to a Felix O1-based therapy or bacteria that replicate to occupy the niche left by those killed should be explored in detail for their impact on animal and human health. Only 0.3% of other members of the Enterobacteriaceae were found to be lysed by Felix O1 (Kallings, 1967). Nonetheless, the ability of this phage to kill other beneficial bacteria is another important concern for a phage-based intervention.

In order to address some if not all of the above safety concerns, the entire sequence of the Felix O1 genome was elucidated. This is one key step in evaluating the safety of this phage-based *Salmonella* intervention. Some bacteriophages are known to transfer virulence genes among bacteria (Waldor, et al, 1996), a feature that would complicate a phage-based intervention. Homology searches suggest that no obvious virulence genes are present in the complete Felix O1 chromosome. However, there are many regions of the genome whose functions are not known. Expression studies to determine the actual function of segments of the genome are indicated.

Selection of a more virulent subpopulation of *Salmonella* is a potential undesireable consequence of phage-based intervention. In some published studies, outgrowth of resistant bacteria has been seen as a result of phage therapy. For example, Barrow *et al.* (1998) found that bacteriophage R succeeded in preventing or diminishing severity of *E. coli* infection in colostrum-deprived calves. However, some of the target bacteria remained, only to grow at a later time (Barrow *et al.*, 1998). Greer and Dilts (1990) found that the effect of their phage-based *Pseudomonas* intervention designed to reduce spoilage organisms in beef was diminished by outgrowth of *Pseudomonas* that were not susceptible to the cocktail of phages used. The characteristics of these bacteria left behind by a phage therapy/intervention are worth studying.

By the same token, Felix O1-resistant *Salmonella* grew in every Felix O1 treatment experiment performed. Though suppression of *Salmonella* growth was achieved with Felix WT and LP, phage treated frankfurter samples still contained a relatively high concentration of cfus (at least 10^4 cfus/g frankfurter, Chapter 5). When phage O1 was propagated on plates, resistant colonies of *S. typhi* often appeared. One might deduce that these resistant bacteria are of a rough phenotype since many rough *Salmonella*e are resistant to Felix O1. Despite this fact, smooth

Felix-resistant strains have been isolated (Hudson *et al.*, 1978), and smooth *Salmonella* strains tend to be more virulent than rough ones (Sanderson & Stocker, 1987).

The characteristics of the *Salmonella* that grew despite phage treatment were not tested. Future intervention experiments should at least include evaluating these persistent *Salmonella* for their lipopolysaccharide phenotype (smooth versus rough). Subsequent work should probably also include testing these isolates for actual virulence as well. Perhaps selection of a rough subpopulation of *Salmonella* by Felix O1 treatment would not be so bad, as these isolates would be expected to be less virulent than those that would otherwise grow without Felix O1 intervention.

One aspect of safety that was not addressed in these experiments is cross-reactivity of Felix O1. Though mainly specific for the genus, this phage infects some Enterobacteriaceae other than *Salmonella* (Kallings, 1967). If normal gut flora, for instance, were killed by phage O1, the effects for an animal that ingests the phage could be detrimental. Killing essential normal enteric flora and consequent release of endotoxin could both be potentially dangerous for the host. Kallings (1967) reported that only 0.3% of other Enterobacteriaceae were affected by phage O1. If the susceptibility of normal gut bacteria were on the same order, the effects on the complex gut flora would be minimal. Nevertheless, this is a point that should be addressed before marketing a phage-based intervention for human or animal foods. The effects on important gut microflora could be tested directly by determining susceptibility of normal enteric isolates. However, exhaustive testing of susceptibility of all gut microflora isolates might be difficult. Alternatively, effects on gut flora could be ascertained by histopathological study of gut mucosa of phage-treated animals.

II. Consumer acceptance

The success of any bacterial intervention, whether it be chemical, physical or biological, is dependent in part on the market, consumer acceptance and compliance with regulatory entities. Knowledge regarding the specifics of novel bacterial interventions is an important part of this acceptance. Bacterial interventions designed for food products are especially sensitive to consumer perception, if the historic public resistance to irradiated food products is any indication (Bruhn, 1995). A more recent study indicated that some prior knowledge and understanding of irradiation, among other factors, increased the likelihood that consumers would purchase irradiated products (Frenzen *et al.*, 2000). More work addressing the safety and efficacy of a Felix O1-based intervention is obviously indicated and some specific areas worth addressing were described above. Should future studies continue to support the safety and efficacy of a Felix O1-based intervention, then educating the public on these safety issues would be an important aspect of enhancing acceptance of this novel approach to controlling salmonellosis and improving food safety. Perhaps reminding the public that many foods naturally contain a wide variety of bacteriophages would be useful in improving acceptance of a phage-based food intervention (Kennedy et al., 1986).

Though there may be promise for marketability of phage-based interventions in this country, it is difficult to say whether use of phage-based interventions will gain worldwide acceptance. Globally speaking, researchers in Russia and Great Britain have a historical interest in parenteral phage therapy (Alisky *et al.*, 1998). Pre-harvest phage-based bacterial interventions for food animals have been explored by researchers from Brazil (Berchieri *et al.*, 1991), and post-processing applications have been pursued by a Canadian research group (Greer and Dilts, 1990).

Despite the global effort to explore phage-based therapies and interventions, the global climate regarding genetically altered animals and foods may affect the widespread applied use of wild and recombinant phages. Russia implemented strict import regulations for genetically modified foods (Fay, 1999), and over 130 countries had adopted the United Nations International Protocol on Biosafety which regulates living modified organisms (LMO's) as of February of this year (USDA, 2000). Although this biosafety protocol does not specifically apply to food safety or processed foods, it would be expected that international regulatory entities would develop a means to regulate addition of a biologically-active phage entity to food products. The intent of the International Protocol on Biosafety is to base regulatory decisions on scientific data and risk-assessment (USDA, 2000), yet another reason to fully explore and disclose the safety and efficacy of any Felix O1-based *Salmonella* intervention.

III. Bioremedial applications

The phage Felix O1 project was pursued because exploitation of the host-parasite relationship among bacteria and their phages was long overdue. Phages coexist with their bacterial hosts in many varied settings: the environment, the gastrointestinal tract, even processed foods (Kennedy *et al*., 1986). Basically, phages are found wherever there are bacterial hosts to be infected, and sometimes they even serve to control the number of viable bacteria.

The goal was not only to explore the potential of a Felix O1-based *Salmonella* intervention, but also to make the phage even better at doing what it already did well: kill *Salmonella*. Three general ideas were considered for mutagenesis of Felix O1. Mutagenizing Felix O1 could have involved adding something bactericidal to the Felix O1 genome, taking something out of the phage genome that hindered killing, or modulating expression of something already in the genome to enhance killing. Taking something out that could perhaps hinder lysis

(*rII*B) while simultaneously inserting genes (*lux*AB) that would make the recombinant a useful detection method was ultimately tried. Unfortunately this design was unsuccessful.

As it turned out, mutagenesis of the *rII*B region of Felix O1 was more difficult and time-consuming than expected. Luckily, however, naturally-occurring phenotypic variants appeared in our *in vivo* studies (Chapter 4). One of these large-plaque (LP) isolates was employed in testing comparative abilities of two different phage treatments.

The large-plaque phenotype was particularly intriguing as well. The phenotype was consistent with rapid-lysis mutants of T4, and it may prove useful in later studies to determine the mechanisms of lysis in Felix O1. Lysis inhibition (LIN) in T4 and the exact role of the *r* genes are still not completely understood. Of the *r* genes found in T4, Felix O1 contains *rII*A and *rII*B, but apparently lacks *r*I and *r*III. This fact along with the rapid-lysis phenotype of the Felix O1 LP variant could prove useful in continued research to elucidate phage lysis mechanisms in general, including those present in T4 (Paddison et al., 1998).

Regardless of the phage genetics responsible for host cell lysis, the success of Felix WT and LP in suppressing bacterial growth in broth and on a processed food product highlights the future promise. Felix O1 was successful in suppressing growth of an important pathogenic *Salmonella* isolate (*S. typhimurium* DT104) in a further processed poultry product (frankfurters) under gross temperature abuse conditions. Many approaches to testing Felix O1's efficacy could have been tried. However, this experimental contamination system proved useful in the initial "proof of concept" necessary to decide whether to continue to pursue Felix O1 as an intervention.

Suppression of growth rather than outright reduction of *Salmonella* was pursued for a few reasons. First, experience has shown that Felix O1 replicates best on actively-growing, log-phase cells. The phage may not exhibit particularly dramatic effects on a static population of bacteria, and it was concluded that the best Felix O1 interventions would be targeted to settings that involve cells in the appropriate stage of susceptibility. In fact, the situations in which a Felix O1-based intervention would be used include times at which exponential increase of *Salmonella* is a problem. These situations include septicemic infections in animals and temperature abuse of *Salmonella*-contaminated food products. An animal experiencing a septicemic bacterial infection would have a better chance of surviving when given a phage treatment, if the treatment is able to suppress or delay the normal fulminant, exponential growth of the pathogen (Barrow *et al.*, 1998). As far as poultry food products are concerned, a product contaminated with a low number of *Salmonella* may not be a problem in terms of human infection unless the bacteria can grow to an infectious threshold concentration. A poultry carcass contaminated with *Salmonella* may contain as few as 10 cfus (Whittemore, 1993). Salmonellosis may result from consumption of fewer than 10^3 cfus, but higher concentrations of the bacterium lead to higher attack rates in *Salmonella* outbreaks (Blaser *et al.,* 1982). The advantage of the Felix O1-based post-processing food application pursued in these experiments would lie in the phage's ability to suppress *Salmonella* growth when the contaminated product is temperature abused.

Based on Felix O1's preference for rapidly-growing, log-phase cells, designed applications should focus on availability of phage when Salmonellae are growing to their disease-causing numbers. The potential applications for an intervention that is able to hold bacterial growth below an infectious dose threshold are numerous. In a parenteral therapeutic situation, this could mean successfully suppressing infectious bacteria to a level low enough to permit successful

engagement by the host's immune system. On the other hand, phage could also be applied as an adjunct along with other traditional antimicrobial therapies. Perhaps phage could be altered to act as a delivery system to the target bacteria rather than an outright killer. For instance, a phage could potentially act as an indirect chemotactic enhancer. If phage, upon infection of the bacterial host, could facilitate expression of the right antigens upon lysis or on the surface of the infected bacterial cell, perhaps more immune cells could be recruited to the site of infection.

As far as other food interventions are concerned, the possibilties are endless. Felix O1 exhibits lytic activity on *Salmonella* in liquid and on solid media, as long as the bacterial cells are susceptible and are at the appropriate stage of growth. It remains viable at refrigeration temperatures for long periods of time, and is resistant to drying. Phage O1 has a broad host-range within the target genus, a quality that other phage interventions have lacked (Greer & Dilts, 1990). These characteristics give hope for the utility of Felix O1 and provide flexibility in application. Although testing specific food products would be necessary, there is no reason to doubt Felix O1's ability to suppress growth of *Salmonella* in any contaminated food product.

This research has focused mainly on two applications for Felix O1: a parenteral therapy and a food intervention. Other aspects of the zoonotic transmission of *Salmonella* could be addressed with Felix O1 phage as well, especially in light of the importance of transmission from food-producing animals. A phage-based intervention could be targeted for the times that food animals become colonized with *Salmonella* in the first place, as was addressed by Berchieri et al. (1991). Expounding on the example in poultry, it would be conceivable to give Felix O1 in food or water, or apply it directly to the poultry environment, during the first few days of the growout phase when birds are most susceptible to *Salmonella*

colonization. Early in production as well as later on, phage might be useful in suppressing environmental *Salmonella*, or in reducing the fecal-oral transmission of *Salmonella* in poults and chicks. Since *Salmonella* from carrier birds may be shed at harvest during shipment to the processing facility, a dose of Felix O1 phage near the time feed is withdrawn could reduce the number of *Salmonella* shed. This could help reduce contamination of the feet and feathers of birds entering the slaughter facility that ultimately results in a *Salmonella*-contaminated meat product.

In conclusion, bacteriophages naturally act as an elegant system of checks and balances for their respective bacterial hosts. Despite a long history of antimicrobial and disinfectant research, in most cases pathogenic bacteria have eventually found a way to evade even our best antibacterial interventions. This work with phage O1 has helped rekindle the original hopes of Felix D'Herrelle, and others like him who shared the hope of exploiting the host-parasite relationship between bacterium and phage. This project also went beyond the original goals of phage-based therapy by exploring bacterial intervention in foods. Perhaps exploiting the natural ecological relationship among phages and their bacteria represents the future of bacterial disease intervention. Using the adaptive abilities of phage to our advantage may be the next best chance for successfully combating the pathogenic bacteria that plague us.

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VITA

Jean M. Whichard

Contact Information:

Home Address:
1720 Donlee Drive
Blacksburg, VA
24060
540-953-2742

Business Address:
Center for Molecular Medicine and Infectious Diseases
Virginia Tech
1410 Prices Fork Road
Blacksburg, VA
24061-0342
540-231-4002 (phone)
540-231-3426 (fax)
email: jwhichar@vt.edu

Education:

1995-present	Doctoral candidate	Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM), Blacksburg, Virginia; GPA: 3.88
1991-1995	D.V.M.	VMRCVM, Blacksburg, Virginia; GPA: 3.16
1987-1991	B.A.	Mary Baldwin College, Staunton, Virginia Major: Chemistry; Minors: Biology, Spanish GPA: 3.93, Summa Cum Laude with Distinction

Professional/Research Experience:

1995-present: Graduate Research Assistant, Dept. of Biomedical Science and Pathobiology, Dept. of Large Animal Clinical Sciences, VMRCVM, Blacksburg, Virginia. Responsibilities: molecular aspects of phage Felix 01 DNA sequencing, recombinant development and applied testing; lipooligosaccharide analysis using fluorescence-assisted carbohydrate electrophoresis (FACE); maintenance and instruction of personnel in use of spectrophotometers.

1995-present: Graduate Teaching Assistant, Dept. of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, Virginia. Responsibilities: assisting in laboratory teaching, grading and limited didactic teaching of second year veterinary students in Veterinary Bacteriology and Mycology course.

Summer, 1993: Research assistant, Dept. of Biomedical Sciences and Pathobiology, Dept. of Large Animal Clinical Sciences, VMRCVM, Blacksburg, Virginia. Responsibilities: Testing of *in vitro* enzymatic methods for inactivation of *Salmonella* species; oral administration of phage Felix 01 (turkeys); care, feeding and monitoring of research animals.

Summer, 1992: Transcriptionist, Adia Personnel Services, Blacksburg, Virginia. Responsibilities: transcription of radiology reports in human hospital.

1990-1991: Veterinary Assistant/Night Attendant, Veterinary Emergency Center, Richmond, Virginia. Responsibilities: patient monitoring, restraint and administration of treatments and diagnostics, client reception.

Summer, 1989: Co-head Counselor, Young Women in Science program, Mary Baldwin College, Staunton, Virginia. Responsibilities: student instruction, residence life duties, media and reagent preparation for microbiology laboratories.

Summer, 1989: Research Assistant, Mary Baldwin College, Staunton, Virginia. Responsibilities: characterization of indolic compounds using nuclear magnetic resonance, infrared, and ultraviolet spectrophotometry; thin-layer and gas chromatography. Synthesis and purification of pharmaceutically-promising indolic compounds.

1988-1991: Laboratory Assistant, Mary Baldwin College, Staunton, Virginia. Responsibilities: student instruction, reagent and equipment preparation for general, organic and quantitative analysis chemistry teaching labs.

Professional Activities:

Member, American Veterinary Medical Association (AVMA), 1995-1999
Member, Virginia Veterinary Medical Association, 1995, 1996, 1998-present
Licensee, practice of veterinary medicine, state of Virginia, 1995-present
Representative, Graduate Student Assembly (GSA), Virginia Tech, 1997-1998
Representative, VMRCVM College Council, Virginia Tech, 1997-1998
Representative, VMRCVM Research and Graduate Studies Committee, 1997-1998
Representative, Commission on Student Affairs, Virginia Tech, 1997-1998
Chair, GSA ad hoc committee on safety phone access, Virginia Tech, 1997-1998
Representative, VMRCVM Curriculum Board, 1991-1995
Member, VMRCVM Student Chapter of the AVMA, 1991-1995
Member, VMRCVM Wildlife and Exotic Animal Club (WEAC), 1991-1995
Wildlife Clinic Coordinator, VMRCVM WEAC, 1991-1993
Member, American Chemical Society Student Affiliates, Mary Baldwin College, 1987-1991
President, American Chemical Society Student Affiliates, Mary Baldwin College, 1989-1990
Representative, Educational Policy Committee, Mary Baldwin College, 1987-1991

Awards:

Outstanding Student Presentation, VMRCVM College Research Days, May, 1998.
Outstanding Student Presentation, Northeastern Conference on Avian Diseases, Cornell University, Ithaca, New York, June, 1997
Outstanding Student Presentation, VMRCVM College Research Days, May, 1997
Cunningham Fellowship (academic, Virginia Tech, 1995-1998)
Rufus Bailey Scholarship (academic, Mary Baldwin College, 1987-1991)
Ellis Ollson Memorial Scholarship (academic, Chesapeake Corp., 1987-1991)
Hillhouse Scholarship (highest GPA in class, Mary Baldwin College, 1987-1988)
Inductee, Phi Beta Kappa (academic, Mary Baldwin College, 1991)
Inductee, Omicron Delta Kappa (leadership, Mary Baldwin College, 1991)
Outstanding Chemistry Student Award (Mary Baldwin College American Chemical Society Student Affiliates, 1990)
Inductee, Iota Sigma Pi (honor society for women in Chemistry, Mary Baldwin College, 1990)
Inductee, Beta Beta Beta (biological honor society, Mary Baldwin College, 1990)

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