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For the Major Program
To my parents, Charles and Faye and my wife, Theresa
for their enduring support and encouragement
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CHAPTER 1. GENERAL INTRODUCTION

*Brachyspira hyodysenteriae* is an anaerobic spirochete and an important pathogen of swine. *B. hyodysenteriae* cells harbor VSH-1, a mitomycin C-inducible prophage that mediates generalized transduction between *B. hyodysenteriae* strains (40). VSH-1 virions package random fragments of *B. hyodysenteriae* chromosomal DNA rather than a viral genome, which has complicated genetic investigations (40). N-terminal amino acid sequences generated for proteins from purified VSH-1 whole virions and tailless heads facilitated the identification of VSH-1 structural genes and allowed their assignment as head or tail associated. It remained uncertain however, as to whether or not additional genes important to virion production existed within the prophage sequence. While mitomycin C-induction of the VSH-1 prophage resulted in cell lysis pointed to bacteriophage genes for lytic growth, the identity of these genes was unknown. The ability of VSH-1 virions to mediate horizontal gene transfer in *B. hyodysenteriae* populations coupled with the recent observation that VSH-1 was common among *Brachyspira* strains suggested that VSH-1 might play an important role in *Brachyspira* ecology (99).

This dissertation is a continuation of research on VSH-1 to understand the biology of this bacteriophage. In the following studies, additional VSH-1 genes were identified including an endolysin involved in VSH-1 escape from *B. hyodysenteriae* cells. The organization of genes for head and tail structures and lytic functions was found to be similar to other bacteriophage operons. Characteristics of VSH-1 transcription were investigated, leading to the identification of an alternative to mitomycin C as VSH-1 inducing agent.
Dissertation Organization

This dissertation has 5 chapters. Chapter 1 contains a general introduction and literature review. Chapter 2 represents this author's continuation of research initiated by Thad B. Stanton, M. Greg Thompson, Samuel B. Humphrey, and Richard L. Zuerner toward a manuscript on the identification of VSH-1 genes and the development of a VSH-1 genome map. Chapter 3 and Chapter 4 are independent manuscripts. Chapter 3 reports on the identification and characterization of a VSH-1 muralytic enzyme and Chapter 4 reports on characteristics of VSH-1 transcription. Chapter 5 contains general conclusions and recommendations for future research.

Literature Review

Bacteriophages

The simplicity of bacteriophages as biological systems initially led to research focusing on understanding bacteriophage processes as models for more complex systems in higher organisms. Moreover, bacteriophages provided many of the genetic tools that gave rise to the field of molecular biology and are still invaluable genetic tools. A shift in bacteriophage research is reflected in the theme of a recent conference on bacteriophage biology entitled "New Phage Biology" (American Society of Microbiology Conference on the New Phage Biology, Aug 2004, Key Biscayne, FL), which focused on research directed toward understanding bacteriophage biology from the standpoints of diversity, function and ecology of bacteriophages themselves.
As genomic sequences have become available, it is clear that bacteriophages have genomes assembled from a vast pool of nucleic acid. Hendrix and colleagues proposed the modular theory of bacteriophage evolution, suggesting that genes and gene clusters of related function are readily exchanged among bacteriophages from disparate host organisms (35-37). Horizontally acquired bacteriophage DNA that can appropriately complement the function of another bacteriophage gene allows productive recombination and leads to genetic mosaicism of bacteriophage genomes. Evidence for both homologous and non-homologous recombination exists in bacteriophage genome sequences (61). The complex and tightly controlled regulation of bacteriophage gene expression undoubtedly provides selective pressures to maintain genome organization and this can be observed in bacteriophages greatly divergent at the level of nucleotide sequence that share similar genomic arrangements.

Extensive population studies of bacteriophages in the oceans and in other aquatic environments have led to estimates of the earth's bacteriophage population size at more than $10^{30}$ bacteriophage particles, making bacteriophages by far the most abundant biological entity on earth (4, 18). More staggering is the notion that given the bacteriophage diversity already recognized in the very limited sample of sequenced phage genomes, bacteriophages likely provide the earth's most abundant source of unexplored nucleotide sequence information that can all move in and out of bacterial cells during the course of the bacteriophage life cycle. Accounting for the frequency of transduction in many known bacteriophage systems (once in every $10^8$ infections) Chibani-Chennoufi et al. predicted that upwards of 20 million billion transduction events could occur per second in aquatic environments (18). Transduction has been documented in natural environments and is not
limited to aquatic milieu. In addition to transduction observed in lakes, rivers, and oceans, transduction has been experimentally measured in soil, on surfaces of plants and in animals (67). Zeph and colleagues demonstrated that bacteriophage P1 could transfer resistance genes for mercury and chloramphenicol between *E. coli* strains inoculated into soil and that the frequency of transduction could be affected by altering the soil composition (115). Saye and colleagues observed transduction of chromosomal loci between *Pseudomonas aeruginosa* lysogens of bacteriophage F116L in chambers containing sterilized lake water (85). In their experiment the authors recorded horizontal gene transfer frequencies of $10^{-8}$ to $10^{-6}$ transductants per colony forming unit. Bacteriophage F116L was also demonstrated to transduce selectable markers between *P. aeruginosa* strains on bean and soy bean leaf surfaces of plants inoculated with lysogens of F116 (55).

The mechanism of bacteriophage-mediated gene transfer can be divided into specialized and generalized transduction. Specialized transduction necessitate a state of lysogeny in the host bacterium and thus, only temperate bacteriophages mediate this type of exchange. In temperate bacteriophages the phage genome can integrate into the bacterial chromosome and therefore its replication is synchronous with bacterial division for many generations under appropriate conditions. Specialized transduction results from abnormal excision of the phage genome from the bacterial chromosome when the lytic cycle is induced. In phage $\lambda$ and related bacteriophages, transduction occurs when the bacteriophage $att$ sites that flank the bacteriophage genome do not align by homologous recombination during excision of the phage from the bacterial chromosome. Excision is instead initiated by non-homologous recombination at a site on the bacterial chromosome. The intervening
bacterial DNA is then replicated along with the circularized phage genome and packaged by the phage particles.

Limitations on the amount and type of DNA that is packaged also differentiate specialized transduction from generalized transduction. First, the amount of DNA required for maturation of the virions (a maximum of 109% of the normal size in λ) limits the amount of bacterial DNA that can be packaged (6). Secondly, any bacterial DNA gained is balanced by bacteriophage DNA lost during packaging, resulting in potential loss of viral function. These two limitations dictate that only bacterial genes adjacent to the att sites will result in a bacteriophage capable of specialized transduction.

Both temperate and lytic phage can mediate generalized transduction. Lytic phages do not incorporate into the bacterial chromosome and their replication is self-governed and always results in lysis of the host cell. Generalized transducing particles are produced during viral packaging of DNA rather than excision of a lysogenized bacteriophage genome. The head-full mechanism of packaging bacteriophage DNA also allows the production of virions containing bacterial DNA. In P22 and similar bacteriophages, rolling circle replication forms concatemers of linear duplex DNA. Packaging of the bacteriophage genome is initiated at nucleotide sequences that are recognized and cut by phage enzymes. The first site that is cut is called the pac site and initiates the packaging of a genome equivalent of DNA into the bacteriophage capsid. The end that is formed on the remaining length of the DNA concatemer initiates the packaging of another genome equivalent into an empty capsid and the process is repeated.

Generalized transducing particles are formed when bacterial DNA sequences are recognized and cut by bacteriophage enzymes, creating an artificial pac site on the bacterial
chromosome. Headfull packaging proceeds and bacterial, rather than phage DNA is packaged into virions. Unlike specialized transducing particles, generalized transducing bacteriophage particles do not necessarily contain any bacteriophage DNA and therefore can transfer a larger quantity of bacterial DNA from potentially any location on the bacterial chromosome. Although DNA fragments larger than the amount of DNA packaged by the phage particle will not be transferred, the cumulative amount of DNA carried by many bacteriophage particles released from an infected bacterial cell have the potential to transfer the entire bacterial chromosome to a population bacteria (67).

**Spirochete Biology**

Spirochetes are long, narrow bacteria and can be immediately distinguished by their elegant helical morphology. All known spirochetes are motile and are especially adept at moving through viscous environments (81). The mechanism of motility is unique to spirochetes among bacteria and is driven by periplasmic flagella that attach near the cell poles, wind around the protoplasmic cylinder and sometimes overlap near the center of the cell (Fig. 1). Cell dimension, helical amplitude and the number and arrangement of flagella vary among spirochete species. Flagellar rotation about the protoplasmic cylinder causes the bacterial cell to flex and gyrate, driving translational movement (63). Spirochete phylogeny based on 16S rDNA sequences groups spirochetes into a single, deeply branching phylum distantly related to other bacteria, indicating that spirochetes have diverged away from other bacteria over a long evolutionary time scale (74).
FIG. 1. Transmission electron micrograph of a *B. hyodysenteriae* B78 cell stained with phosphotungstic acid. 23,700 X mag., Bar = 2.5 µm. A) periplasmic flagella, B) outer membrane (sloughing away from cell).

Both free-living and host-associated spirochetes exist and several spirochete genera include members that are important pathogens of humans and animals. Research on spirochetal pathogens is extensive and only a few examples will be briefly mentioned here.

*Borrelia* species are associated with disease in humans, mammals and birds. *Borrelia burgdorferi* is spread by a tick vector and is the causative agent of Lyme disease in humans. The genome of the *B. burgdorferi* B31 type strain was sequenced and determined to consist of a 910 bp linear chromosome, 12 linear plasmids and 9 circular plasmids (16, 27).
Recently, Stewart and colleagues developed a mutagenesis system for *B. burgdorferi* based on a broad host range *mariner* transposon cloned into a suicide vector along with a transposase gene driven by a strong *Borrelia* promoter (101). The system allowed transient expression of the transposase enzyme, which led to random insertions of the transposon into the *B. burgdorferi* chromosome and extra-chromosomal elements.

The first evidence for a bacteriophage associated with *B. burgdorferi* was reported by Hayes and colleagues in 1983 who observed bacteriophage particles associated with isolates of the *Ixodes dammini* spirochete (the etiological agent of Lyme disease later named *B. burgdorferi*) (34). Eggers and Samuels, were the first to characterize a bacteriophage from *B. burgdorferi* and found that the bacteriophage virions packaged the cp32 plasmid (23) and later demonstrated that the prophage (designated φBB-1) encoded on the cp32 plasmid was able to mediate transduction of a selectable marker between *B. burgdorferi* strains (22). Two genes (*blyA* and *blyB*) located on the cp32 plasmid previously thought to constitute hemolysis system, instead encoded a holin-like protein and an accessory protein, respectively (20). The authors demonstrated that the BlyA protein shared structural features with known holins, was strongly expressed during induction of φBB-1 prophage and complemented the function of a holin-defective λ prophage.

*Leptospira* species can be either free-living or host-associated. *Leptospira interrogans* causes leptospirosis in animals and zoonotic disease in humans. The *Leptospira* genus is complex, containing many species and more than 200 serovars (9). The *L. interrogans* genome is the largest spirochete genome described and consists of a 4.3 Mb large (CI) and a 350 kb small (CII) circular chromosome (117-119). The *L. interrogans* serovar *Lai* was sequenced by Ren *et al.* in 2003 (77) and recently Nascimento *et al.* reported the genome
sequence of *L. interrogans* serovar *Copenhageni* and compared the genome features of these two sequenced serovars revealing a large chromosomal inversion and variations in insertion sequence element distribution and numbers (69).

Three bacteriophages were isolated from sewage waters near Paris, France in 1990 that were lytic for *L. biflexa*, a saprophytic member of the *Leptospira* genus (83). The bacteriophages named LE1, LE3, and LE4. Although the genome size of LE1 (50 kb) differed in size from LE3 and LE4 genomes (60 kb each), the bacteriophages had identical morphology with polyhedral heads and contractile tails (83). The LE1 bacteriophage was found to replicated as a plasmid in *L. biflexa*, a property which led Saint Girons and colleagues to incorporate a 2.2 kb fragment of the LE-1 genome that contained the LE-1 origin of replication into an *E. coli* vector (82). The resulting shuttle vector replicated in both *E. coli* and *L. biflexa* and imparted a kanamycin resistance phenotype on *L. biflexa* cells.

*Treponema pallidum* subspecies cause a variety of human diseases including venereal syphilis, endemic syphilis and yaws (70). Consistent with obligate parasitism, *T. pallidum* possesses a small genome and has not been continuously cultivated in vitro (84). The 1 Mb circular chromosome of *T. pallidum* is among the smallest prokaryotic genomes (70, 111) and was sequenced in 1998 by Fraser and colleagues (28). The recently completed genome sequence of *Treponema denticola*, which is associated with periodontal disease in humans allowed a comparison between these two genomes (88). The genome size of *T. denticola* was found to be significantly larger (2.8 Mb) than that of *T. pallidum* and differed in %G+C content, rRNA sequences, and gene order indicating that the two spirochetes had diverged from a common ancestor over a long evolutionary time scale (88).
Pathogenic species of *Brachyspira* include *alvinipulli*, the agent of avian spirochetosis, *pilosicoli*, the agent of spirochetal colitis in swine and birds and *hyodysenteriae*, the agent of swine dysentery. Further discussion will focus on *B. hyodysenteriae*.

**Swine dysentery**

Swine dysentery is a severe and sometimes fatal mucohemorrhagic diarrheal disease of pigs (32). The disease is marked clinically by diarrhea with varying degrees of severity and is accompanied by mucus and flecks of blood in the feces. As the disease progresses, diarrhea worsens and large amounts of blood, mucus and mucofibrinous exudates are expelled in watery stools, and pigs appear gaunt and dehydrated. Pathologically, mucosal lesions and effacement of colonic microvilli are observed in the large intestine without involvement of the small intestine. There is a marked increase in the number of neutrophils observed in and around capillaries and lamina propria of the large intestine in dysenteric pigs. Although peracute manifestation of swine dysentery occasionally results in death, mortality in most pigs infected with swine dysentery is associated with dehydration as a result of colonic malabsorption, acidosis and hyperkalemia (32). While swine of all ages can be affected, the disease is especially prominent in post-weaning and early fattening periods (2, 32), which presents a risk for disease in high-density hog confinement facilities.

The first link between a spirochete and swine dysentery was made by Blakemore and Taylor who documented that spirochetes were associated with colonic lesions (7). Later, Taylor and Alexander isolated a β-hemolytic spirochete from a dysenteric pig and
demonstrated that pure cultures containing the spirochete reproduced swine dysentery disease and that the agent could again be isolated from infected animals (104).

At Iowa State University, Harris and colleagues simultaneously confirmed the reports of Taylor and in 1972, named the etiologic agent of swine dysentery Treponema hyodysenteriae (29, 33). Since that time and with the progression of molecular and phylogenetic techniques, T. hyodysenteriae was reclassified. Based on partial 16S ribosomal RNA sequences and DNA-DNA reassociation, Stanton et al. established the genetic relatedness of T. hyodysenteriae and T. innocens and their distinctiveness as spirochete genera. As a result they grouped them into a new genus, Serpulina (93, 95). More recently, members of the genus Serpulina and Brachyspira were unified based on DNA-DNA hybrid analysis, and Serpulina was dropped in favor of Brachyspira as the genus name (71).

Ecology of Brachyspira hyodysenteriae

B. hyodysenteriae is an anaerobic bacterium that is tolerant of oxygen. Although not required for growth, a low level of oxygen (1%) in the initial atmosphere of broth cultures stimulates B. hyodysenteriae growth (96). When switched from an anaerobic atmosphere to an aerobic atmosphere, actively growing B. hyodysenteriae cells rapidly consume the oxygen. An NADH oxidase expressed by B. hyodysenteriae provides the major mechanism for oxygen consumption and NADH oxidase mutants are both less oxygen tolerant and less virulent than wild type strains (94, 98).

It is not unexpected that B. hyodysenteriae possesses a mechanism for managing the effects of oxygen and that NADH oxidase mutants would have reduced virulence. Although the lumen of the swine colon is anaerobic, B. hyodysenteriae cells likely encounter an inward
oxygen gradient near the colonic mucosal epithelium. It has been shown that \textit{B. hyodysenteriae} cells are chemotactic toward mucin \((54, 68)\). \textit{B. hyodysenteriae} cells and are intimately associated with the colonic mucosa and are capable of colonizing the mucus layer, luminal epithelium, and crypts of the large intestine \((44, 53)\).

The ability of \textit{B. hyodysenteriae} cells to withstand oxygen likely contributes to their survival outside the host. Survival of \textit{B. hyodysenteriae} in soil, soil mixed with swine feces and in feces alone was demonstrated to last up to 10, 78, and 112 days, respectively \((8)\). In the same study \textit{B. hyodysenteriae} DNA was detectable by PCR for up to 112 days for each of the conditions. The ease of transmissibility, owing in part to the long duration of survival of \textit{B. hyodysenteriae} in soil and feces and the tendency for some individuals in herds of swine to become chronic carriers \((92)\), makes swine dysentery difficult to eradicate from infected farms.

Although predominately associated with disease in swine, evidence exists that suggests a broader host range for \textit{B. hyodysenteriae}. In addition to infecting swine, strains of \textit{B. hyodysenteriae} have been found to colonize and cause clinical symptoms in experimentally infected mice \((45, 46)\) and have been implicated as an etiologic agent of infectious typhlitis in chickens \((1, 21, 103)\) and necrotizing typhlocolitis in rheas \((43, 80)\). \textit{B. hyodysenteriae} has also been identified in a number of different environmental sources, indicating that natural reservoirs for the pathogen might exist. In addition to swine lagoon water containing feces from infected pigs, \textit{B. hyodysenteriae} has been isolated from field mice captured on farms affected with swine dysentery \((47)\), and recently, Jannson and colleagues isolated \textit{B. hyodysenteriae} from both wild and farm reared mallard ducks \((42)\).
Because of the severity and prevalence of disease, *B. hyodysenteriae* continues to be an economically significant pathogen in many pork producing countries. Of critical importance is the emerging incidence of antibiotic resistant *B. hyodysenteriae* strains. In European countries, very few antibiotics remain that are both effective in treating cases of swine dysentery and are still approved for use in pigs raised for meat production. Tiamulin blocks protein synthesis by binding to the 50S subunit and is a commonly prescribed for treatment of swine dysentery. Repeated exposure to incrementally higher doses of tiamulin resulted in gradual onset of resistance in *B. hyodysenteriae* strains (51) and recent studies suggest that the efficacy of this and similar drugs is decreasing, as field isolates with elevated levels of resistance have been identified with increasing frequency in Germany, Sweden, and the UK (50, 51). The antibiotic resistance is emerging not from the spread of a single clone through European countries, but instead appears to be developing as a result of prolonged sub-therapeutic use of the drugs. A mechanism for horizontal gene transfer could allow susceptible strains to receive antibiotic resistance determinants from resistant strains and could hasten the emergence of multiple antibiotic resistant *B. hyodysenteriae* strains.

**Genetics of *Brachyspira hyodysenteriae***

Although the genome sequence of *B. hyodysenteriae* has not yet been published, a significant amount of information is known about *B. hyodysenteriae* genetics. At recent count, over 30 *B. hyodysenteriae* genes encoding identifiable proteins have been deposited in GenBank and much is known about the *B. hyodysenteriae* chromosome arrangement. Zuerner and Stanton determined that the *B. hyodysenteriae* type strain (B78) has a single 3.2 Mb circular chromosome and developed a physical and genetic chromosome map that
included rRNA, flagellum, and putative hemolysin genes as well as the gene for NADH oxidase and a DNA gyrase (120). Recently, Zuerner et al. expanded the *B. hyodysenteriae* map by identifying the location of more than thirty *B. hyodysenteriae* genes (121). In the same study, a similar physical and genetic map was constructed for *B. pilosicoli* and compared to the *B. hyodysenteriae* map. The authors identified differences between these bacteria at the level of nucleotide sequence, gene content, chromosome arrangement and chromosome size. The *B. pilosicoli* chromosome was found to be smaller (2.5 Mb) than that of *B. hyodysenteriae* and lacked four genetic loci found in *B. hyodysenteriae* that are potentially involved in host-bacterium interactions (121). The authors theorize that these differences in gene content along with divergence in genes flanking a β-hemolysin gene could contribute to the differences in hemolytic phenotype and the ability to cause disease observed between these bacteria.

Investigators have previously reported plasmids in preparations of *B. hyodysenteriae* DNA (19, 48), however, these observations are suspect and could be explained by the presence of a bacteriophage (40). The lack of a plasmid that replicates in *B. hyodysenteriae* cells that can be exploited for use as a genetic system has complicated genetic investigations of *B. hyodysenteriae*. Fortunately, *B. hyodysenteriae* cells can be transformed by electroporation and gene knockouts have been produced by homologous recombination and allelic exchange. Insertional inactivation of the *B. hyodysenteriae* genes *nox* (98), *flaA* or *flaB* (52), and *tlyA* (41) have identified NADH oxidase activity, flagellum production, and hemolytic activity, respectively, as important factors for *B. hyodysenteriae* virulence. Although *tlyA* is associated with β-hemolytic activity in *E. coli* (105, 106), it is not the *B.*
*hyodysenteriae* β-hemolysin gene (*hlyA*), which has more recently been identified, cloned and characterized (38).

**VSH-1**

The first report for a bacteriophage possibly associated with *B. hyodysenteriae* was made by Ritchie and Brown in 1971 (78) who observed spirochetes infected with large bacteriophage particles in a sample taken directly from a colonic lesion of a dysenteric pig. Shortly after *B. hyodysenteriae* was identified as the etiologic agent of swine dysentery, Ritchie and colleagues surveyed a culture collection of *B. hyodysenteriae* isolates from around the world and found a single morphological type of bacteriophage produced in cultures of both pathogenic and non-pathogenic isolates (79). The bacteriophage was much smaller in size from one originally observed in swine lesions, having a head diameter of only 45 to 50 nm, and a simple non-contractile tail with dimensions of 9 to 11 x 65 to 70 nm. Although the bacteriophage particles were reported to have appeared spontaneously in *B. hyodysenteriae* cultures, the conditions under which the *B. hyodysenteriae* cells were cultured was not reported.

Humphrey and colleagues did not detect spontaneous appearance of bacteriophages in *B. hyodysenteriae* cultures, however they found that mitomycin C treatment induced virion production, which was accompanied by cell lysis (39). The bacteriophage particles were identical in size and morphology to those observed by Ritchie and colleagues (Fig. 2). In addition to bacteriophage production, mitomycin C treatment of *B. hyodysenteriae* cultures was associated with the appearance of a 7 to 8 kb band of extrachromosomal DNA (39). The
The DNA band was similar in size to an extrachromosomal band of DNA previously attributed to a plasmid and frequently observed in *B. hyodysenteriae* cultures (19, 48).

The bacteriophage of *B. hyodysenteriae* was purified and characterized by Humphrey and colleagues who named it VSH-1 (*Virus of Serpulina hyodysenteriae*) after the host bacterium so named at that time (40). Although cell culture lysis had been observed after mitomycin C-treatment, purified VSH-1 virions were not lytic for *B. hyodysenteriae* cells in suspensions and did not form plaques on *B. hyodysenteriae* lawns grown on agar plates. The nucleic acid contained within the capsid was studied by restriction endonuclease analysis and Southern hybridization, revealing that VSH-1 packages 7.5 kb random fragments of *B. hyodysenteriae* chromosomal DNA rather than a viral genome. When added to *B. hyodysenteriae* cell cultures, purified VSH-1 virions were found to mediate transfer of antibiotic resistance determinants between *B. hyodysenteriae* strains. This finding demonstrated that although apparently defective in certain bacteriophage functions, VSH-1 virions could mediate horizontal gene transfer by generalized transduction and were similar to other bacteriophage-like particles called gene transfer agents (40, 97).

As VSH-1 virions package a random assortment of DNA, VSH-1 genes could not be identified by sequencing the DNA contained within the virion structures. Humphrey and colleagues used an indirect approach to identify VSH-1 genes by first determining the N-terminal amino acid sequences of several VSH-1 structural proteins (Unpublished). By separating proteins comprising VSH-1 whole virions as well as VSH-1 tailless head structures, they were able to differentiate proteins that comprised head structures from those that comprised tail structures (40). Initially, a reverse genetic approach using degenerate primers with nucleotide sequences based on the N-terminal amino acids of VSH-1 proteins
facilitated the PCR amplification and nucleotide sequencing of VSH-1 genes. A primer walking strategy determined additional VSH-1 nucleotide sequences and open reading frames were classified as head-associated or tail-associated according to the translated amino acid sequences (Chapter 2, this work).

FIG 2. Transmission electron micrograph of VSH-1 particles stained with phosphotungstic acid. 169,000 X mag., Bar = 50 nm.

Gene transfer agents.

Although notably smaller than many archetypal phages that contain double stranded DNA, gene transfer agents are morphologically identical to bacteriophage particles with simple (non-contractile) tails, and thus are members of the Siphoviridae (long-tailed) or Podoviridae (short-tailed) taxonomic phage groups. Like many bacteriophages, gene transfer agents have been shown to mediate generalized transduction. The significant difference
between gene transfer agents and other generalized transducing bacteriophages is that gene transfer agents appear incapable of self-reproduction through any type of lytic cycle as no bacterial hosts as susceptible to lytic infection by the gene transfer agent particles have been identified. All gene transfer agents described to date package double-stranded DNA consisting of randomly generated fragments of the host chromosome. The length of the DNA fragments packaged appears shorter than the amount expected to encode a fully functional temperate bacteriophage (15). Thus, the function of gene transfer agents appears to be only the transduction of bacterial genes.

In addition to VSH-1, three other gene transfer agents have been described (summarized in Table 1). The first to be identified, and most thoroughly characterized, is GTA of *Rhodobacter capsulatus*. In 1975, Solioz and colleagues (91) demonstrated that bacteriophage-like particles produced by *R. capsulatus* cultures entering stationary phase growth were capable of mediating transfer of rifampicin resistance and tryptophan prototrophy markers at high frequencies. The authors noted that no viral activities were associated with the particles and later characterized the packaged nucleic acid as random 4.5 kb fragments of bacterial chromosomal DNA (90). Wall and colleagues extended the observations of Solioz by demonstrating that GTA production was widespread among strains of *R. capsulatus* (112).

The GTA structural and regulatory genes have been identified and mapped (60). The GTA structural genes are clustered within a 15 kb region of the *R. capsulatus* chromosome and are transcribed as an operon. Transposon mutagenesis identified two genes more than 10 kb upstream from the structural genes that were required for GTA production. The genes were part of a bacterial two-component signal transduction system, a histidine kinase (CckA)
and response regulator (CtrA), and were involved in upregulating both motility and GTA production at the level of transcription during the shift from logarithmic to stationary growth phase in *R. capsulatus* cultures (58, 59). GTA-mediated transduction is influenced by a quorum-sensing signal. Shaefer and colleagues identified an *R. capsulatus* gene (*gtal*) responsible for the synthesis of an acyl-homoserine lactone molecule that acts as a *R. capsulatus* quorum-sensing signal and demonstrated that inactivation of *gtal* reduced the ability of *R. capsulatus* cells to produce GTA during the transition into stationary growth phase (89).

**TABLE 1.** Properties of gene transfer agents. Adapted from Lang and Beatty (59)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Host bacterium</th>
<th>Nucleic acid packaged in virions</th>
<th>Head diameter, tail length</th>
<th>Conditions that induce production</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSH-1</td>
<td><em>Brachyspira hyodysenteriae</em></td>
<td>7.5 kb linear dsDNA</td>
<td>45 nm, 64 nm</td>
<td>mitomycin C</td>
</tr>
<tr>
<td>GTA</td>
<td><em>Rhodobacter capsulatus</em></td>
<td>4.5 kb linear dsDNA</td>
<td>30 nm, 50 nm</td>
<td>stationary growth</td>
</tr>
<tr>
<td>Dd1</td>
<td><em>Desulfovibrio desulfuricans</em></td>
<td>13.6 kb linear dsDNA</td>
<td>43 nm, 7 nm</td>
<td>unknown</td>
</tr>
<tr>
<td>VTA</td>
<td><em>Methanococcus voltae</em></td>
<td>4.4 kb linear dsDNA</td>
<td>40 nm, 61 nm</td>
<td>unknown</td>
</tr>
</tbody>
</table>

A small bacteriophage, Dd1, of *Desulfovibrio desulfuricans* was discovered to package random, 13.6 kb fragments of *D. desulfuricans* chromosomal DNA and transfer antibiotic resistance determinants between strains (76). The authors noted that they were unable to identify conditions under which increased production of Dd1 could be induced and that cell-free filtrates containing the virions appeared incapable of plaque formation on
several strains of *D. desulfuricans*. However, no further investigations of Dd1 have been reported.

The bacteriophage-like particle, VTA, of the methanogenic archaeabacterium, *Methanococcus voltae* packages random, 4.4 kb fragments of the *M. voltae* chromosome and mediates transfer of selectable markers (5, 24). Although the fragments contained within the virions were mostly random in nature, a 900-bp band of DNA present in preparations of VTA but not in *M. voltae* DNA suggested preferential packaging of the fragment. This might indicate that a portion of the prophage replicates prior to DNA packaging, however, like other gene transfer agents, VTA was found to be incapable of plaque formation on *M. voltae* cells.

**Potential ecological implications of VSH-1.**

If gene transfer agents cannot self-replicate through lytic infection, why are they not lost from the genomes of their bacterial hosts? Surveys of bacterial genomes have revealed extensive uptake of laterally transferred DNA (73). The constant influx of DNA into bacterial chromosomes must be balanced by an equally high deletion rate to prevent ever-increasing genome sizes. This inward and outward DNA flux potentially serves the purpose of removing pseudogenes that no longer hold function for the bacterium while affording the ability to uptake potentially useful genes and providing an efficient mechanism for inactivating prophages and other genetic parasites (62, 72, 73).

Several lines of evidence indicate that VSH-1 has shared a relatively long evolutionary history with *Brachyspira* suggesting that selective pressure has maintained its existence as an inducible prophage despite the likelihood that VSH-1 induction is lethal to
the host cells. VSH-1-like agents are widely distributed in Brachyspira species. Calderaro and colleagues carried out electron microscopic surveys of weakly beta-hemolytic (non-\( B. \) hyodysenteriae) spirochetes of human and animal origin. The authors identified bacteriophage particles spontaneously produced in a culture of a spirochete isolate of human origin (\( B. \) pilosicoli strain HRM 18) and were able to induce the production of bacteriophage particles in cultures of isolates from a broiler chick (strain 1380) and four isolates of human origin (strains HRM 5, 7, 18, and 22) (13, 14). In all instances the particles observed were identical in size and morphology to VSH-1. Recently, Stanton and colleagues surveyed Brachyspira species for the presence of a VSH-1 major head structural gene by Southern hybridization and detected a hybridization signal in DNA digests of 27 spirochete strains representing six species of Brachyspira (\( B. \) hyodysenteriae, \( B. \) innocens, \( B. \) pilosicoli, \( B. \) murdochii, \( B. \) intermedia, \( B. \) alvinipulli) (99). The nucleotide sequences of VSH-1 genes shared a similar low \%G+C content with \( B. \) hyodysenteriae genes (Chapter 2, this work). Trott and colleagues analyzed the genetic diversity of more than two-hundred \( B. \) hyodysenteriae isolates by multilocus enzyme electrophoresis and found evidence for substantial genetic recombination, consistent with frequent horizontal gene transfer among strains (107). As VSH-1 is the only natural mechanism of gene transfer identified for Brachyspira, VSH-1 appears to have influenced the genetic diversity and population structure of \( B. \) hyodysenteriae.

Other bacteriophages that have apparently lost the ability to propagate through lytic infection retain bactericidal properties. Strains of \( B. \) subtilis produce a non-infectious bacteriophage-like particle called PBSX (87). The virions adsorb to bacterial cells and kill \( B. \) subtilis species that harbor heterologous phages, but do not kill strains carrying homologous
phages (64, 113). The occurrence of PBSX appears to be common among Bacillus species. In addition to B. subtilis, B. amyloliquefaciens, B. licheniformis and B. pumilus all produce non-infectious bacteriophage particles called PBSX-like defective phages (100) although much of the research on these phages has focused on PBSX from B. subtilis. Production of PBSX is inducible with mitomycin C. The repressor of PBSX has been identified and shown to bind four operator sequences upstream from the PBSX late operon resulting in repression of gene transcription (65, 114). Similar to gene transfer agents, PBSX virions package random 13 kb fragments of the host chromosome (3), however the bacteriophage particles have not been reported to mediate transduction of bacterial genes. The bactericidal activity of PBSX-like bacteriophages has been proposed as a possible ecological advantage for host cells carrying these type of entities (102). The lysis is associated with PBSX escape in mitomycin C-induced B. subtilis cells and PBSX lysis genes have been identified (57) suggesting that any benefit afforded by the bactericidal activity of PBSX-like agents would likely be conferred on a population level rather than at the level of the individual cell.

Irrespective of a possible selective advantage afforded the host cell by maintaining a gene transfer agent, the ability of VSH-1 to mediate gene transfer very likely holds a significant advantage to populations of B. hyodysenteriae cells and may play a role in disease. VSH-1 has been shown in vitro to transfer antibiotic resistance determinants, which would hold an obvious and immediate advantage to recipient antibiotic-sensitive B. hyodysenteriae cells under antibiotic selective pressure. Gene transfer has also been shown important in the acquisition of virulence determinants and broadening of host range specificity (17, 25, 26, 66).
A relevant example of horizontal gene transfer contributing to bacterial evolution and potential for pathogenicity can be drawn from another spirochete. Recently, Haake and colleagues undertook an extensive investigation of the molecular evolution and genetic mosaicism of *Leptospira* outer membrane protein genes. Using a DNA sequence modeling approach, the authors compared phylogenetic relatedness of outer membrane protein genes and gene segments from 38 isolates representing six *Leptospira* species from a variety of mammalian hosts or environmental sources (31). In addition to finding evidence for extensive intragenic recombination, they identified outer membrane protein genes that were acquired by horizontal gene transfer.

The *Leptospira* example presents an intriguing possibility for genetic shuffling within a bacterium and dispersal of mosaic genes by horizontal transfer to *Leptospira* populations. As these outer membrane proteins are potentially recognized by host-immune cells or may be important in tissue colonization, variability of the expressed proteins might serve to evade immune detection or broaden the host range.

A parallel can be drawn between the genetic mosaicism in *Leptospira* and the 32-kb circular plasmids (cp32s) of the spirochetal agent of Lyme disease, *Borrelia burgdorferi*. The cp32s are a family of homologous plasmids that contain regions of variability in sequences that encode genes for outer membrane lipoproteins. Like the genes encoding outer membrane protein of *Leptospira*, those of *B. burgdorferi* have undergone extensive sequence recombination by both endogenous and exogenous sources. Moreover, as described above, the cp32 plasmids are packaged by the *B. burgdorferi* bacteriophage φBB-1 and are capable of mediating genetic transduction (22).
Bacteriophage induction and bacterial pathogenesis

Non-phage genes encoded within bacteriophage genomes (called morons) are thought to indirectly enhance the replication of temperate bacteriophages residing in bacterial chromosomes by providing a function that enhances the fitness of the host bacterial cell in its current ecological niche, allows the bacterium to adapt more rapidly than non-lysogens to changing environmental conditions, or allow the bacterium to better compete for a new niche (12, 18). Many of these bacteriophage morons both enhance bacterial survival and contribute to the virulence of pathogenic bacteria (10, 12, 116).

The direct influence of bacteriophage morons on the progression of disease is can be illustrated by toxin encoding phages. More than just providing the genes that encode proteins with potent cytotoxic properties, bacteriophages have been shown to govern the regulation of toxin expression. The relationship between bacteriophages and disease is often tentative because artificial conditions (such as mitomycin C) are used to demonstrate that toxin production coincides with bacteriophage induction. There are examples, however that directly point to the role of bacteriophages in disease, demonstrating that phages can be induced to express and release cytotoxins by factors produced by mammalian cells.

Group A Streptococci can colonize the human upper respiratory tract and cause pharyngitis. They carry with them a repertoire of genes encoding potent cytotoxic proteins such as streptococcal pyrogenic exotoxin C (SpeC) (49). The gene encoding SpeC of Streptococcus pyogenes is encoded on a prophage carried by streptococcal lysogens. Broudy and colleagues found that production of SpeC is induced when S. pyogenes cells are cocultured with human pharyngeal cells. Toxin production is associated with the release of small, tailed bacteriophage-like particles into the culture medium (11). Pharyngeal cell
culture supernatant had similar inductive properties, and the authors detected a small, uncharacterized molecule produced by pharyngeal cells that mediated bacteriophage induction and the subsequent production of SpeC. The soluble phage-inducing factor (SPIF) was also produced by human cancer cell lines, suggesting it is a common extracellular molecule (11).

Shiga toxin-encoding *E. coli* (STEC) infections are particularly severe, causing hemorrhagic colitis and hemolytic uremic syndrome (30, 75). The severity of the disease is related to toxin production by the STEC. In STEC strains, the Shiga toxin genes are often associated with prophages of the λ phage family (56, 86). In the case of *E. coli* H-19B, transcription of Shiga toxin 1 genes (*stxJA* and *stxJB*) is driven by both an iron responsive promoter and a bacteriophage promoter. Under conditions of iron deprivation, Stx1 is expressed and is not associated with phage induction. However, a stronger level of expression is driven by an SOS responsive bacteriophage promoter located far upstream from the *stxAB* genes (110). In addition to turning on transcription of *stxAB* genes, SOS induction results in prophage excision and replication, which in turn amplifies the toxin gene copy number. Wagner and colleagues have demonstrated that human neutrophils induce phage-mediated expression of Shiga toxin genes in a clinical isolate of *E. coli* O157:H7 (108). Inhibiting neutrophil oxygen-derived free radical production made neutrophils incapable of inducing the bacteriophage. This result indicated that reactive oxygen intermediates produced by immune cells might serve as an environmental inducing agent of Stx-encoding phage.

*E. coli* H-19B cells release far more toxin if expression is driven by bacteriophage induction rather than by low iron conditions (109). This is because *stxAB* genes are located
upstream from genes for bacterial lysis. The lysis genes, S and R encode a holin and a
lysozyme, respectively. Holins polymerize in the bacterial inner membrane, forming
multimeric protein channels that allow lysozymes to reach the periplasm. Lysozymes then
hydrolyze the peptidoglycan glycosidic bonds, causing the bacterial cell to rupture. A
transcriptional terminator is located between the toxin genes and lysis genes, preventing
expression of lysis genes when the iron responsive promoter drives transcription. An
antitermination protein (Q) is expressed when the bacteriophage is induced and allows
transcription to proceed through the terminator, resulting in lysis gene expression. The
subsequent disruption of the bacterial cell wall releases the large amount of toxin contained
within the cells. Wagner and colleagues have demonstrated that human neutrophils induce
phage-mediated expression of Shiga toxin genes in a clinical isolate of *E. coli* O157:H7
(108). Inhibiting neutrophil oxygen-derived free radical production made neutrophils
incapable of inducing the bacteriophage.

The relationship between bacteriophages and their hosts appears to be extensive.
Bacteriophages are more than just pathogens of bacteria. Bacteriophages have been shown to
influence ecology, survival and evolution of bacteria. Given the diversity of bacteriophages
and the likelihood that this diversity represents merely a small cross sample of total
bacteriophage diversity, it is likely that additional gene transfer agents exist.
REFERENCES


CHAPTER 2. IDENTIFICATION OF GENES OF VSH-1, A DEFECTIVE BACTERIOPHAGE AND GENE TRANSFER AGENT OF BRACHYSPIRA HYODYSENTERIAE

Author’s contribution to a paper to be submitted to the Journal of Bacteriology

THAD B. STANTON, ERIC G. MATSON, M. GREG THOMPSON, SAMUEL B. HUMPHREY, AND RICHARD L. ZUERNER

ABSTRACT

*Brachyspira hyodysenteriae* is an anaerobic spirochete that harbors the mitomycin C-inducible prophage, VSH-1. VSH-1 is defective in replication and viral packaging. Instead of a viral genome, VSH-1 virions package random 7.5-kb fragments of *B. hyodysenteriae* chromosomal DNA and mediate gene transfer between *B. hyodysenteriae* cells. VSH-1 structural genes were identified based on matches to N-terminal amino acid sequences determined for several VSH-1 head and tail-associated proteins. The structural genes were sequenced and mapped to a 14.3-kb region of the *B. hyodysenteriae* chromosome. The VSH-1 sequence was delimited upstream and downstream by open reading frames with a high degree of translated amino acid sequence homology to bacterial genes. The VSH-1 structural genes were closely grouped with head morphogenetic genes preceding tail morphogenetic genes. An adjacent open reading frame with homology to bacteriophage endolysins was identified. The VSH-1 gene arrangement is consistent with the organization of bacteriophage late operons.
INTRODUCTION

The spirochete *Brachyspira hyodysenteriae* is an aero-tolerant anaerobe and enteric pathogen of swine (16, 36). *B. hyodysenteriae* cells harbor the mitomycin C-inducible prophage, VSH-1(18). Unlike many lysogenic bacteriophages, production of VSH-1 particles is not associated with replication of VSH-1 genes (unpublished data) and VSH-1 virions package random 7.5-kb fragments of *B. hyodysenteriae* chromosomal DNA rather than a viral genome (19). Moreover, VSH-1 particles mediate gene transfer between *B. hyodysenteriae* cells (19). VSH-1 is thus a defective bacteriophage, incapable of self-propagation through a lytic cycle and functions instead as a host-gene transfer agent similar to the defective bacteriophage gene transfer agents of *Rhodobacter capsulatus* GTA (26), *Methanococcus voltae* VTA (3), and *Desulfovibrio desulfuricans* Dd1 (32). Of these gene transfer agents, the bacteriophage genome is known only for GTA of *R. capsulatus* (27).

In an effort to identify VSH-1 structural genes, VSH-1 virions were purified from mitomycin C-treated cultures of *B. hyodysenteriae* cells and the N-terminal amino acid sequence of several VSH-1 structural proteins was determined (unpublished data). Degenerate primers based on amino acid sequences were used to generate PCR amplicons from *B. hyodysenteriae* genomic DNA. A partial genome map containing VSH-1 structural genes was assembled from sequencing data generated from the PCR amplicons and from clones identified in a lambda ZAPII library of *B. hyodysenteriae* genomic DNA. VSH-1 structural genes were localized to a 12.4-kb region of the *B. hyodysenteriae* chromosome that were delimited upstream and downstream by open reading frames with homology to bacterial genes. However, further investigation into the transcriptional organization of VSH-1 revealed a region within a VSH-1 tail-associated gene (*svp101*) that could not be PCR-
amplified from *B. hyodysenteriae* genomic DNA. Additionally, the translated amino acid sequence of *svp101* encoded a theoretical protein of 53 kDa as compared to the actual gene product, a 101-kDa protein as estimated by SDS-PAGE (19), suggesting a truncated *svp101* nucleotide sequence.

Taken together, these observations pointed to an error in the previously determined sequence of VSH-1 and allowed for the possibility that additional VSH-1 genes were located immediately downstream from *svp101*. This sequence error was subsequently shown to result from an accidental recombination within the clone (Cl-7) that contained *svp101*. A λ ZAPII library of *B. hyodysenteriae* genomic DNA was used to obtain additional VSH-1 gene clones to complete the VSH-1 nucleotide sequence.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Brachyspira hyodysenteriae* strain B204 cells were cultured at 37°C with constant stirring in brain heart infusion broth supplemented with 10% heat-treated (56°C for 30 min) bovine serum under an atmosphere of 99% N₂, 1% O₂ as previously described (34). *Escherichia coli* strains XL-1 Blue MRF’ and SOLR (Stratgene, La Jolla, CA) were cultured on LB agar plates or LB broth supplemented with 0.2% (w/v) maltose, 10 mM MgSO₄. Where appropriate, ampicillin (50 µg/ml) was incorporated into agar or broth for selection.

**Primer and probe design.** Sequence-specific oligonucleotide primers and probes were designed using Oligo version 6.0 software (Molecular Biology Insights, Inc., Cascade, CO) and synthesized by the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA) (Table 1A and B). Probes were end-labeled with gamma ³²P-ATP using a T4
kinase reaction. Unincorporated label was removed by purifying the probes through a NucTrap purification column (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

**λ ZAP II library screening.** For each round of screening, *E. coli* XL-1 Blue cells were infected with a custom λ ZAP II library of cloned *B. hyodysenteriae* B204 genomic DNA (Stratagene, La Jolla, CA). The library was plated on eight NZY agar plates according to the manufacturer's protocol at a plaque density of approximately 1000 PFU/plate (providing 10-fold coverage of the B204 genome). Plaques were allowed to form overnight at 37°C and were refrigerated at 4°C for several hours. Plaque lifts were performed with Duralose-UV membranes (Statagene, La Jolla, CA). The membranes were transferred to denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 2 minutes, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 5 minutes, and rinsed for 30 seconds in 2X SSC buffer (33 mM NaCl, 30 mM Sodium citrate, pH 7.0) containing 0.2 M Tris-HCl (pH 7.5). Denatured nucleic acid was UV crosslinked (120,000 µjoules) to the membranes in a UV Stratalinker 1800 (Stratagene, La Jolla, CA).

To detect plaques containing VSH-1 sequence, membranes were soaked in prehybridization solution (Final concentrations: 6X SSC buffer, 20 mM NaH₂PO₄, 0.4% SDS, 5X Denhardt’s reagent, 500 µg sheared salmon testes DNA /ml) at 42°C while rotating in a hybridization oven. After 2 h, the prehybridization solution was replaced with hybridization solution of similar formulation but without Denhardt’s reagent. ³²P-labeled sequence-specific probe (approximately 100 ul, 10⁶ DPM/µl) was added and the membranes
were hybridized overnight at 42°C, rinsed for 1 h in 1 liter of 6X SSC, 0.5% SDS at 45°C, and exposed to X-ray film overnight.

Lambda plaques corresponding to positive signals on the X-ray film were excised from the NZY agar plates using an inverted 200-μl pipette tip and vortexed in 1 ml of SM phage dilution buffer (100 mM NaCl, 8 mM MgSO₄, and 0.01% gelatin in 50 mM Tris-HCl, pH 7.0). Plaque supernatant was amplified by XL-PCR using a primer corresponding to the T4 or T7 priming sites on the λ ZAP II vector and a second primer complementary to the cloned DNA sequence. Lambda clones that produced the longest PCR amplicons (indicating they contained a significant length of new sequence) were plaque-purified by a second round of screening and isolation to remove contaminating, non-positive clones. Where possible, positive λ ZAP II clones were converted to the pBluescript phagemid vector in E. coli SOLR cells by in vivo excision according to the Stratagene protocol (presumably toxic gene products produced by some clones prevented in vivo excision).

Nucleic acid manipulations. B. hyodysenteriae genomic DNA was purified from cells grown to mid-exponential phase as previously described (33, 40). Briefly, 10-ml cultures were grown to a cell density of 5x10⁸ to 2x10⁹ cells/ml (OD₆₂₀ = 0.8 – 1.0, 18 mm pathlength), pelleted by centrifugation and resuspended in buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA). Cell suspensions were treated with lysozyme, SDS and Proteinase K. Nucleic acid was phenol/chloroform extracted, precipitated with sodium acetate and ethanol and spooled on a glass rod. The spooled nucleic acid was dissolved in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA and RNase A-treated to remove RNA. The DNA was extracted with chloroform, precipitated as before and dissolved in nuclease-free H₂O.
PCR was performed using an AmpliTaq Gold kit (Applied Biosystems, Foster City, CA). DNA template (1 ng/µl final concentration) or 5 µl of diluted λ plaque supernatant (cored λ plaque vortexed in 500 µl of SM buffer and diluted 1 to 4 in sterile H2O) was added to PCR mix (1X GeneAmp buffer, 2.5 mM MgCl2, 200 µM each dNTP, 1.5 U AmpliTaq Gold DNA polymerase and 0.25 pmol/µl each forward and reverse primer) in a 50-µl reaction volume. Samples were amplified using a Biometra UNO-Thermoblock thermocycler (Biometra Inc., Tampa, FL) with an initial incubation of 92°C for 9 min followed by 36 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min and a final 8-min extension at 72°C.

Long-range PCR was performed using an XL-PCR kit (Applied Biosystems, Foster City, CA). Hot-start PCRs were set up with a 40-µl bottom mixture (1X XL-Buffer II, 200 µM each dNTP, 1.0 mM Mg(OAc)2, 0.6 pmol/µl each forward and reverse primer) separated by a layer of wax from a 60-µl top mixture (1x XL-Buffer II, 2 units rTth DNA polymerase, and 5 µl of diluted λ ZAP II plaque supernatant, as above). Samples were amplified with an initial incubation at 93°C for 1 min to melt the wax layer followed by 36 cycles of 93°C for 1 min, 52°C for 45 sec, and 70°C for 10 min and a final 15-min extension at 72°C. PCR products were separated by electrophoresis through 1% agarose gels in 0.5X TBE running buffer and stained with ethidium bromide to visualize the bands.

DNA Sequencing and analysis. VSH-1 sequence was obtained by cycle sequencing (13) either overlapping pBluescript phagemid clones of B. hyodysenteriae genomic DNA or XL-PCR-amplified DNA from λ clones. Phagemid DNA was extracted from cultures of E. coli cells using Miniprep spin columns (Qiagen Inc., Valencia, CA) and sequenced by the
Iowa State DNA Sequencing and Synthesis Facility. Both the coding and non-coding DNA strands were sequenced. Sequence data was aligned into contigs using Vector NTI Suite 5.5 software (Informax, Inc., North Bethesda, MD). Translated amino acid sequences for open reading frames within the nucleotide sequence were compared to sequences in the GenBank database using BLASTP (1) to identify gene homologs. Protein masses of translated amino acid sequences were estimated using the Statistical Analysis of Protein Sequences (SAPS) program (5).

**RESULTS**

**Identification of VSH-1-associated open reading frames.** Attempts to generate overlapping PCR amplicons of VSH-1 genes from *B. hyodysenteriae* genomic DNA revealed a possible sequencing error within or downstream from *svp101*. The Lambda ZAP II clone (Cl-7) was used as template to generate the sequencing data for VSH-1 genes. The primer pair, 101F3 and 7M, PCR-amplified *svp101* DNA from clone Cl-7. However, primers 101F3 and 7M failed to produce an amplicon from *B. hyodysenteriae* genomic DNA, whereas primer pairs 101F8, 101R and 101F2, 101R3 produced amplicons of the expected size (Fig. 1). The combined results localized the recombination site within approximately a 700-bp region between the priming sites for 101R and 101F2.

Primer 101F8, complementary to the 5' end of *svp101*, was used as a probe to re-screen the *B. hyodysenteriae* Lambda ZAP II library. The 101F8 probe hybridized to a clone (Cl-18) that contained the *svp101* sequence. Sequencing data obtained from Cl-18 confirmed an artifactual recombination in the original *svp101* sequence 1299 bp downstream from the start codon. This recombination likely occurred during construction of the λ library when
two, non-contiguous fragments of DNA were ligated into the same vector. The translated amino acid sequence of the full-length svp101 gene encoded a theoretical protein of 93.7 kDa, a protein mass that was consistent with the 101-kDa molecular weight of the svp101 gene product previously estimated by SDS-PAGE.

Three lambda clones were identified through consecutive rounds of screening the Lambda ZAP II library. Sequencing reads obtained from the clones were aligned and assembled into an 8.5-kb contig, which at its 5' end overlapped the point of recombination in svp101. A 31-kb nucleotide sequence was assembled by adding the new 8.5-kb contig to the previous VSH-1 genome map. The contiguity of the entire VSH-1 sequence as well as upstream and downstream sequences was verified by PCR amplification of B. hyodysenteriae genomic DNA. Primer pairs generated PCR amplicons that linked each of the known VSH-1 structural genes and overlapping amplicons for putative bacterial sequences (Table 1B and Fig. 2).

**Analysis of the VSH-1 sequence.** The 31-kb nucleotide sequence revealed 24 predicted ORFs of greater than 100 codons that were initiated by 22 putative ATG and 2 putative GTG start codons (Fig. 2 and Table 2). Six previously unknown ORFs, including one VSH-1 structural gene (ORF 19), were identified downstream of svp101. ORF 19, located immediately downstream of svp101, shared translated amino acid identity to the N-terminal amino acid sequence of a 28-kDa VSH-1 tail-associated protein and has been designated svp28. ORF 20, encoding a hypothetical 22.9-kDa protein demonstrated significant amino acid sequence identity (E value = 5e-29) to a Salmonella enterica phage epsilon 15 endolysin (24) and was confirmed to have endolytic activity (this research, Chapter 3). Gene homologs were not identified for ORFs 21 and 22, which encoded
hypothetical proteins of 34 and 14.2 kDa, respectively. The VSH-1 sequence was delimited
downstream by ORFs 23 and 24, which shared significant translated amino acid sequence
identity (E values = 9e-81 and 3e-78, respectively) to a *B. hyodysenteriae* methyl accepting
chemotaxis (Mcp) protein.

VSH-1 structural genes are closely grouped, with head morphogenesis genes
preceding tail morphogenesis genes in the sequence, a gene organization that is shared by
many temperate bacteriophage late operons (7, 10, 11, 17). VSH-1 structural genes shared a
common transcriptional orientation and reverse transcriptase-PCR results indicated that
VSH-1-associated ORFs are transcribed as an operon (this research, Chapter 3). Within the
entire 31-kb nucleotide sequence, we identified only one ORF (ORF 20, the putative
bacteriophage endolysin) with translated amino acid sequence homology to other
bacteriophage proteins.

The %G+C content of VSH-1 genes was compared to that of other *B. hyodysenteriae*
genes dispersed throughout the genetic map of the *B. hyodysenteriae* genome (44) (*flaA1*,
*flaB1*, *flaB2*, *flaB3*, *gyrB*, *nox*, and *tly*) as well as to genes encoded by the *bit*, *blp*, and *vsp*
operons (9, 12, 14, 15, 21, 22, 28, 31, 35, 37). Consistent with the idea that the VSH-1
prophage has co-existed with *B. hyodysenteriae* over a long evolutionary time scale, the
range (20-33%) and average (28%) G+C contents of VSH-1 genes was similar to the range
(23-37%) and average (29%) G+C content of *B. hyodysenteriae* genes.

**DISCUSSION**

The genome of the archetypal temperate bacteriophage λ contains genes that are
categorized as either early or late based on their function (4). Genes that are associated with
early functions encode regulatory proteins that control whether the lysogenic or lytic cycle will be established in a newly infected cell and permit immunity to superinfection in lysogens. In addition to many regulatory proteins, the products of early-function genes facilitate integration and excision of the λ genome into and out of the bacterial chromosome and vegetative replication of the λ genome. By contrast, late functions relate strictly to production of structural components, assembly of completed virions, and escape from the host cell.

Although VSH-1 structural genes shared no homology to other bacteriophage genes, the VSH-1 sequence resembles the polycistronic late operon of λ and other bacteriophages in the organization of the genes for capsid morphogenesis, tail morphogenesis and virion escape. This result is not necessarily surprising as the sequences of similar functioning genes from unrelated bacteriophages have often diverged beyond comparison, whereas the modular organization of bacteriophage genes is highly conserved (8, 20, 42). Based on the organization of other bacteriophage late operons, we would not anticipate genes associated with early functions to be interspersed among VSH-1 structural genes and we did not find any.

In bacteriophage λ, morphogenetic and escape functions account for approximately 50% of the phage genome, suggesting that a genome size of at least 30 kb would be expected for VSH-1 if it were a functional temperate bacteriophage capable of both lytic and lysogenic lifestyles. The VSH-1 sequence appears to contain an insufficient amount of genetic information to encode the necessary genes for both early and late bacteriophage functions. Furthermore, there is no evidence for overlapping ORFs contained within VSH-1 structural
genes that might allow genes for both early and late functions to be encoded within the same sequence.

It is possible that VSH-1 early genes are located elsewhere on the *B. hyodysenteriae* chromosome and are functional under certain conditions which we have not identified. Alternatively, and perhaps more consistent with its defective nature, VSH-1 early genes may be non-functional and separated from late genes or lost all together from the *B. hyodysenteriae* chromosome with only the remnants of late operon regulators remaining to control expression of VSH-1 genes. Lang and Beatty (25) have demonstrated that virion structural gene expression for the defective bacteriophage GTA of *R. capsulatus* is coordinately regulated with bacterial motility genes through a bacterial two-component signal transduction system. An intriguing possibility that must be considered is that *B. hyodysenteriae* genes have similarly assumed control over the expression of the VSH-1 genes, linking VSH-1 production directly to *B. hyodysenteriae* physiology.

The minimum continuous length of DNA that contains all of the VSH-1 genes currently known to encode virion structural proteins is 14.3 kb. VSH-1 has been shown to package only 7.5-kb fragments of DNA and there is no evidence to suggest that VSH-1 virions package more than one fragment of DNA in a single capsid. It is more likely that VSH-1 virions are incapable of packaging the genetic information required for encoding the virion structure alone. VSH-1 is thus incapable of infecting naïve host cells, which elicits questions as to whether or not the relationship between VSH-1 and *B. hyodysenteriae* predates Brachyspiral speciation or if VSH-1 is evolving away from a temperate bacteriophage and has more recently become defective in lytic propagation.
The similar low %G+C content of VSH-1 genes compared to *B. hyodysenteriae* genes suggests that VSH-1 is an ancient entity within the *B. hyodysenteriae* chromosome. Recently, Stanton *et al.* reported the identification of VSH-1-like elements in 27 spirochete strains representing 6 *Brachyspira* species, indicating that the prevalence of VSH-1 is widespread within this genus (38). Consistent with a frequent incidence of lateral gene transfer among *Brachyspira* populations, Trott *et al.* have reported that *Brachyspira* species demonstrate a high level of genetic recombination that has shaped its population structure (41). VSH-1 is the only natural mechanism of gene transfer described for *Brachyspira*, and it is likely to have contributed to the genetic diversity of the *Brachyspira* genus.

Selective pressure for maintaining aspects of VSH-1 function might exist in cell populations, as virion structural genes have not been lost from the *B. hyodysenteriae* genome or inactivated by accumulated mutations over time as is the case for many cryptic prophages. However, it remains to be tested whether or not VSH-1 imparts a specific selective advantage to *B. hyodysenteriae* cells or cell populations growing in cultures or in their natural environment, the swine intestine.

Other defective prophages have retained the ability to produce virion structures, such as the well characterized PBSX of *Bacillus subtilis* (2, 23, 29, 30, 43). In the case of PBSX, virions package fragments of host DNA, but do not transfer the genetic material to other *B. subtilis* cells. Instead, PBSX virions have bactericidal effects on *B. subtilis* cells that harbor heterologous, but not homologous phage (29, 39). It is reported that this property is a selective advantage to the host cell populations and is the reason for the propensity of PBSX-like elements in *B. subtilis* strains (6, 43).
With the growing number of bacterial chromosomes sequenced, an increasing number of defective, cryptic, or putative prophage sequences have been identified within the genomes of diverse bacterial species. Bacteria often harbor multiple prophages and prophage genes can account for as much as 10-20% of the nucleotide sequence in some bacterial genomes such as that of *E. coli* O157, *S. pyogenes* M3, and *B. burgdorferi* B31 (7, 17). VSH-1 and similar gene transfer agents likely represent a more common mechanism for lateral bacterial gene transfer than is currently recognized and some cryptic prophage may function in a similar capacity.
TABLE 1A. Oligonucleotide primers used for identifying a svp101 sequence recombination and probes used for screening a lambda ZAP II library of cloned *B. hyodysenteriae* B204 genomic DNA. B. Oligonucleotide primers used to verify contiguity of VSH-1 and adjacent bacterial genes from *B. hyodysenteriae* genomic DNA.

**A.**

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**B.**

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*Tm calculations are based on the [2 (A+T)° + 4 (G+C)°] method.

Amplicon designations refer to Fig. 2.
TABLE 2. Predicted VSH-1 and adjacent bacterial ORFs

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<tr>
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<td>2775</td>
<td>924</td>
<td>27.53</td>
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<tr>
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<tr>
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<td>ssiT</td>
<td>1182</td>
<td>393</td>
<td>31.21</td>
<td>8e-93</td>
<td>Putative <em>F. nucleatum</em> Set/Thr sodium symporter (NP604045)</td>
</tr>
<tr>
<td>06</td>
<td><em>Gm hyp</em></td>
<td>969</td>
<td>323</td>
<td>27.00</td>
<td>1e-39</td>
<td>Predicted <em>G. metallireducens</em> Fe-S oxidoreductase (ZP00080881)</td>
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*aMinimum ORF size set at 100 codons beginning with either ATG or GTG (ORF 8 and ORF 13).

*bE values ≤ 1e-02 as returned by BLAST are reported as possible gene homologs.

*cPutative gene function assignments are based on identity to N-terminal amino acid sequences of purified VSH-1 structural proteins or to protein homologs reported in GenBank.
FIG. 1. PCR amplicon pattern for the *svp101* region. The striped arrow denotes the *svp101* ORF contained within clone Cl-7. Primer pairs and their expected amplicons (based on PCR products generated from clone Cl-7) are shown below. Solid lines between the primers indicate that PCR amplification from *B. hyodysenteriae* genomic DNA was successful or dashed lines, unsuccessful. The vertical line indicates the exact point of sequence recombination identified within the Cl-7 clone containing the original *svp101* sequence.
FIG. 2. Genetic map of VSH-1 and adjacent ORFs. Numbers above ORFs refer to Table 2. Shaded arrows denote predicted bacterial ORFs. Striped arrows denote VSH-1 structural genes identified by N-terminal amino acid sequence. The solid arrow denotes a putative VSH-1 endolysin gene. Open arrows denote predicted open reading frames of unidentified function. The direction of arrows represent transcriptional orientation. ORFs that begin with a putative GTG initiation codon are marked (*). Solid lines below the map represent PCR amplicons generated to verify sequence contiguity. The lowercase letters above the amplicons refer to primer pairs listed on Table 1B.
REFERENCES


23. Krogh, S., M. O'Reilly, N. Nolan, and K. M. Devine. 1996. The phage-like element PBSX and part of the skin element, which are resident at different locations on the *Bacillus subtilis* chromosome, are highly homologous. Microbiology 142:2031-2040.


CHAPTER 3. CHARACTERIZATION OF AN ENDOLYSIN OF VSH-1, A DEFECTIVE BACTERIOPHAGE AND GENE TRANSFER AGENT OF 

**BRACHYSPIRA HYODYSENTERIAE**

A paper to be submitted to the Journal of Bacteriology

ERIC G. MATSON AND THAD B. STANTON

ABSTRACT

VSH-1 is a mitomycin C-inducible prophage and host-gene transfer agent of *Brachyspira hyodysenteriae*. The small, lambda-like VSH-1 virions package random 7.5-kb fragments of *B. hyodysenteriae* chromosomal DNA and mediate horizontal gene transfer, but appear unable to self-replicate. The release of VSH-1 virions from mitomycin C-treated *B. hyodysenteriae* cultures correlates with cell lysis. This lysis suggests that VSH-1 employs a mechanism for breaching the cell wall to facilitate escape. Analysis of the recently sequenced VSH-1 genome revealed an open reading frame that is located immediately downstream from VSH-1 structural genes and is transcriptionally active in mitomycin C-treated *B. hyodysenteriae* cells. The open reading frame encodes a theoretical protein of 22.9 kDa with homology to bacteriophage hypothetical endolysins. The amino acid sequence, however, was not significantly homologous to enzymes for which muralytic activity has been biochemically demonstrated. We cloned the putative VSH-1 endolysin gene, which we have termed *lysV*, with the addition of a hexa-histidine tag and expressed the gene in *Escherichia coli*. Over-expression of *lysV* resulted in *E. coli* cell lysis. The purified LysV protein
digested peptidoglycan isolated from *E. coli* and *B. hyodysenteriae* cells. Treatment of purified *B. hyodysenteriae* peptidoglycan with LysV resulted in the production of reducing acetylamino sugars, a characteristic of glycosidic enzymes that cleave the polysaccharide chain of peptidoglycan. Thus, *lysV* encodes a VSH-1-associated glycosidase that likely facilitates virion escape from *B. hyodysenteriae* cells.

**INTRODUCTION**

The final step in a bacteriophage lytic cycle is the release of virions from the host cell. The rigid peptidoglycan sacculus that provides structural integrity to bacterial cells also poses a formidable barrier against the escape of bacteriophage particles. To facilitate their own release, bacteriophages employ a variety of mechanisms, many of which involve muralytic enzymes, broadly termed endolysins (30). The specific classification of an endolysin depends on the covalent bond within the peptidoglycan molecule that is cleaved by the enzyme (Fig. 1). N-acetylmuramidases or N-acetylglucosamidases cleave the polysaccharide chains of peptidoglycan and are grouped as together as glycosidic enzymes. An N-acetylmuramyl-L-alanine amidase cleaves the amide linkage between the polysaccharide and polypeptide chains. Finally, endopeptidases are a broader category of enzymes that cleave a number of different bonds within the polypeptide chains that cross-link the peptidoglycan polysaccharide chains.

VSH-1 is a mitomycin-C- inducible defective prophage and host-gene transfer agent of *Brachyspira hyodysenteriae*, a pathogenic spirochete of swine. VSH-1 packages random 7.5 kb fragments of *B. hyodysenteriae* chromosomal DNA rather than a viral genome (14) and appears unable to self-replicate (unpublished results). The release of VSH-1 virions
from *B. hyodysenteriae* cells occurs 5 hours after cultures are treated with mitomycin C and is associated with cell lysis (13). Although VSH-1 is apparently defective with respect to replication and packaging, this lysis indicates that a mechanism for virion escape is intact.

The recently completed sequence of VSH-1 structural genes (Chapter 2, this work) led to the identification of an open reading frame (ORF) with significant translated amino acid sequence homology to putative bacteriophage endolysins and other glycohydrolases (Table 1). The ORF, which we have designated *lysV* was located immediately downstream from the VSH-1 tail-associated gene, *svp28* and was transcribed following mitomycin C-treatment of *B. hyodysenteriae* cells (Chapter 3, this work). Although we identified protein homologs of LysV in the GenBank database, muralytic activity had not been biochemically demonstrated for these putative enzymes.

In this chapter, we describe cloning of *lysV* with the addition of a hexa-histidine tag, expression of the gene in *Escherichia coli*, and the purification of active LysV protein. Data are presented to demonstrate that *lysV* encodes a muralytic enzyme with activity for *B. hyodysenteriae* and *E. coli* peptidoglycan. LysV is defined as a glycosidase, cleaving the polysaccharide glycosidic linkages of peptidoglycan.

**MATERIALS AND METHODS**

**PCR-amplification and cloning.** Oligonucleotide primers for cloning the VSH-1 endolysin were designed using Oligo version 6.0 software (Molecular Biology Insights, Inc., Cascade, CO) and synthesized by the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA). Primer RF2F (5' CACCAATCAAGGAGTTTAATAAT4TGAT 3', Tm = 58°C) and primer RF1R (5' ACCTTGTAATATTTAAGAATAAT 3', Tm = 56°C)
were used to PCR-amplify the full-length VSH-1 endolysin gene. A 5’ sequence on primer RF2F (underlined), complementary to a 3’ overhang on the plasmid vector, was added to facilitate directional cloning. A putative Shine-Dalgarno sequence is shown in bold and the start of \( \text{lysV} \) is italicized on the RF2F primer sequence.

The VSH-1 endolysin gene was PCR amplified using a high-fidelity proofreading polymerase, \( \text{PfuTurbo} \) (Stratagene, La Jolla, CA). \( B. \text{hyodysenteriae} \) strain B204 genomic DNA (1 ng/\( \mu \)l final concentration) was added to the PCR mix (1X \( \text{Pfu} \) buffer, 200 \( \mu \)M each dNTP, 2.5 U \( \text{PfuTurbo} \) DNA polymerase and 0.25 pmol/\( \mu \)l each, forward and reverse primer) in a 50-\( \mu \)l reaction volume. Samples were amplified using a Biometra UNO-Thermoblock thermocycler (Biometra Inc., Tampa, FL) with an initial incubation of 95°C for 2 min followed by 35 cycles of 95°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min and a final 10-min extension at 72°C. PCR products were ethanol precipitated and suspended in nuclease-free water. Samples of the PCR products were electrophoresed through an agarose gel, stained with ethidium bromide, and quantified by comparison with DNA molecular weight standards.

The plasmid expression vector, pBAD102/D-TOPO (Invitrogen, Carlsbad, CA), was used to clone the VSH-1 endolysin gene in-frame with a V5 monoclonal antibody epitope and hexa-histidine tag. Gene expression was controlled with the arabinose-inducible promoter located within the vector sequence immediately upstream from the cloning site. A TOPO cloning reaction was performed by mixing PCR product with the pBAD102/D-TOPO vector in a 5:1 molar ratio. 1 \( \mu \)l (7 ng) of PCR product was mixed with 1 \( \mu \)l (10 ng) of vector, 1 \( \mu \)l of salt solution (1.2 M NaCl, 60 mM MgCl\(_2\)), and 2 \( \mu \)l of nuclease-free water.
The reaction was incubated at room temperature for 15 min to allow the TOPO reaction to proceed. *E. coli* One Shot TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) were transformed with 3 µl of the TOPO reaction for 15 min on ice followed by a 42°C heat shock for 30 sec. The cells were recovered in 250 µl of SOC medium (Invitrogen, Carlsbad, CA) at 37°C for 30 min. Dilutions of the transformants were plated on LB agar plates containing 50 µg/ml ampicillin and incubated over night at 37°C. Colonies containing the vector with the cloned VSH-1 *lysV* gene (pBAD102/D/lysV) were identified by PCR using primers RF2F and RF1R. The vector insert was sequenced by the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA) using plasmid DNA extracted from an *E. coli* clone as template.

The plasmid pBAD102/D/lacZ (Invitrogen, Carlsbad, CA) was transformed into chemically competent *E. coli* One Shot TOP10 cells as before and used as a positive control for protein expression and purification and as a negative control for muralytic activity assays.

**Expression and purification of His-tagged proteins.** *E. coli* cells harboring plasmid pBAD102/D/lysV or pBAD102/D/lacZ were grown at 37°C with shaking (200 rpm) in 1 L volumes of LB broth containing 50 µg/ml ampicillin. Expression of the hexa-his-tagged proteins was induced with L-arabinose (0.003% w/v final concentration) when cultures reached an OD_{600} of 1.0 U (pathlength = 18 mm). After induction, the cultures were incubated for an additional 2.5 h before the cells were pelleted at 6,000 x g for 15 min and resuspended in 10 ml of phosphate buffered saline (pH 7.5). Cells were disrupted by three passages at 18,000 psi through a French Press. The crude cell extract was centrifuged at 48,000 x g for 2 hours at 4°C in an ultracentrifuge to sediment the insoluble cell fraction.
The soluble protein fraction was applied to a column with a 5-ml bed volume of His-Select Nickel affinity gel (Sigma, St. Louis, MO) with a flow rate of 4 column v/h. 5-ml volumes (total of 40 ml) of equilibration buffer (0.5 M NaCl, 25 mM imidazole) were passed through the resin at a flow rate of 15 column v/h to remove weakly bound proteins. The His-tagged proteins were eluted with three 5-ml volumes of elution buffer (0.3 M NaCl, 250 mM imidazole) at a flow rate of 4 column v/h The 15-ml volume of eluted proteins was concentrated to approximately 1 ml in a Centriprep-10 (10 kDa molecular weight cut off) centrifugal filtration unit according to the manufacturer’s protocol (Millipore, Bedford, MA). The buffer was exchanged by two repeats of diluting the sample with 9 ml of Tris buffer (50 mM Tris-HCl, pH 7.0) and concentrating as before. Purified protein concentrations were assessed using the Lowry protein assay.

**SDS-PAGE and Western blots.** The purity of His-tagged proteins was assessed by SDS-PAGE with 12% (w/v) polyacrylamide gels stained with Coomassie blue to visualize protein bands. For immunoblot analysis, gels were transferred to nitrocellulose membranes (BioRad, Hercules, CA) for 2 h at 70 V in a Mini Trans-Blot cell (BioRad, Hercules, CA) using protein transfer buffer (25 mM Tris base, 192 mM glycine, 15% methanol (v/v), pH 8.2). After transfer, the membranes were dried, rinsed thoroughly with distilled water and blocked with 5% bovine serum albumin (BSA) in 100 ml of TBS buffer (0.9% NaCl, 20 mM Tris base, pH 7.4) at 37°C for 1 h with gentle agitation. Membranes were washed three times, 5 min each in 75 ml volumes of TBS buffer with 0.1% BSA. The membranes were incubated with a 1:5000 dilution of Anti-V5-HRP (Invitrogen, Carlsbad, CA) in 10 ml of antibody incubation solution (0.9% NaCl, 20 mM Tris base, pH 7.4 with 1% BSA and 0.05% Tween 20) at room temperature for 1 h with shaking. The membranes were rinsed
thoroughly with TBS and developed with a peroxidase reaction prepared by mixing reagent A (60 mg of 4-chloro-1 naphthol in 20 ml of ice-cold methanol) and reagent B (60 μl of ice-cold 30% H₂O₂ in 100 ml of TBS buffer) and adding the solution to the membranes. The membranes were developed for 4 h at room temperature before the reaction was stopped by washing the membranes in distilled water.

**Turbidometric Assays.** For turbidometric assays, *E. coli* protoplasts were prepared according to the method of Caldentey and Bamford (7). A 1-liter mid-exponential growth phase (OD₆₀₀ = 1.0, 18 mm pathlength) cell culture of *E. coli* was pelleted (6,000 x g at 4°C for 15 min) and resuspended in 50 ml of phosphate-buffered saline (pH 7.5). Three ml of chloroform was added to the cell suspension, and the mixture was incubated at room temperature for 30 min with occasional shaking. The chloroform was removed and the cells were washed twice with 50 mM Tris-HCl buffer (pH 7.0), resuspended at an OD₆₀₀ of 1.0 in Tris-HCl buffer with 0.1% Triton X-100 and held on ice until needed. Purified protein was added to the chloroform-treated cell suspension and cell lysis was monitored as a reduction in optical density.

For hypo-osmosensitivity assays, EDTA-treated *E. coli* cells were prepared according to the method of Marvin and Witholt (22) with the following modifications: *E. coli* cells were grown in LB broth to early stationary phase growth (OD₆₀₀ 2.5), pelleted as before, and washed once with ice-cold PBS (10 mM sodium phosphate buffer pH 7.2, 0.85% w/v NaCl) before being resuspended in ice-cold 100 mM CaCl₂. For some samples, purified VSH-1 endolysin (10 μg/ml), purified β-galactosidase (10 μg/ml), or buffer alone was substituted for the lysozyme treatment described in the protocol. Osmosensitivity was monitored as a
reduction in optical density measured at 450 nm after 21-fold dilution of the treated cells in distilled water.

**Peptidoglycan purification.** *B. hyodysenteriae* cells were grown in a 15-liter fermenter in Brain Heart Infusion broth supplemented with 5% bovine serum as previously described (27). Peptidoglycan was purified from 12 liters (25.2 g total cell wet weight, 8.1 g total dry weight) of mid-exponential growth phase cells (OD_{620} 1.0) according to the method of Joseph et al. (15). A total of 72 mg (8.9 mg/g of cells, dry weight) of peptidoglycan was purified from the *B. hyodysenteriae* cells, which is comparable to the recovery of peptidoglycan (15 mg/g of cells) from *Treponema pallidum* reported by Umemoto et al. (28) using a similar method of purification. Peptidoglycan was purified from *E. coli* cells according to the method of Becktel and Baase (1).

**Peptidoglycan degradation assay.** The digestion of *B. hyodysenteriae* peptidoglycan was assessed under varying buffer conditions using a lysoplate assay which measures the dye-binding capacity of peptidoglycan (1) with modifications. Peptidoglycan (2 mg/ml final concentration) was incorporated into 0.8% molten agarose in water. Aliquots (50 μl) of the agarose/peptidoglycan mixture were added to 96-well microtiter plates and allowed to solidify. The agarose was equilibrated twice for 2 hours at room temperature with 250-μl volumes of either 50 mM Tris-HCl buffer (pH 5.0 to 8.5), 50 mM sodium phosphate buffer (pH 5.5 - 8.5) or 50 mM sodium citrate buffer (pH 3.5 - 7.0) in 0.5-unit pH increments. Purified LysV, β-galactosidase or hen egg-white lysozyme (Sigma, St. Louis, MO) in the same buffer was overlaid onto the agarose. The plates were sealed and incubated for 4 hours at 37°C with gentle agitation. The wells were rinsed with distilled water and stained for 4 hours with a cell wall-staining solution (0.1% methylene blue, 0.01% KOH in
distilled water). The plates were submerged in a large volume of distilled water and
destained overnight with gentle agitation. Absorbance of the dye retained by the undigested
peptidoglycan remaining in the agarose was measured spectrophotometrically at a 600-nm
wavelength with a 96-well plate reader.

Glycosidase activity assay. Samples (0.5 mg) of purified *B. hyodysenteriae*
peptidoglycan, *E. coli* peptidoglycan, and crab shell chitin (Sigma, St. Louis, MO) were
incubated with VSH-1 endolysin (20 μg/ml) at 37°C in 50 μl of 50 mM Sodium Phosphate
buffer (pH 6.5). The N-acetylmuramidase, Mutanolysin (Sigma, St. Louis, MO) as a positive
control or buffer alone as a negative control were used in similar reaction mixtures for
peptidoglycan hydrolysis. *S. marcesens* chitinase (Sigma, St. Lois, MO) was used as a
positive control for chitin hydrolysis. After digestion, the reaction mixtures were centrifuged
at 20,000 x g, and the supernatants were lyophilized and redissolved in 20 μl of 1% K2B7O4.
The appearance of reduced N-acetylamino sugars was assayed using the Morgan-Elson
reagent (p-Dimethylaminobenzaldehyde) according to the method of Ghuysen *et al.* (12).

RESULTS

Cloning and expression of *lysV*. The VSH-1 endolysin gene was cloned into vector
pBAD102/D-TOPO under control of the arabinose-inducible promoter and in frame with the
plasmid-encoded V5 epitope and hexa-histidine sequences. In the VSH-1 nucleotide
sequence, the *lysV* gene is preceded by an AGGAGTT sequence located 7 bp upstream from
the start codon, which closely matches the consensus Shine-Dalgarno sequence
(AGGAGGT) in *E. coli*. We believed that this sequence would be sufficient to initiate
translation of the insert in *E. coli* and incorporated the VSH-1 Shine-Dalgarno sequence into
primer RF2F. An *E. coli* clone harboring plasmid pBAD102/D/lys*V* was isolated and the insert was sequenced. The nucleotide sequence of the cloned *lysV* matched the previously determined gene sequence of *lysV* from *B. hyodysenteriae* genomic DNA.

*E. coli* cells harboring plasmid pBAD102/D/lys*V* grew similarly to cells containing the control plasmid, pBAD102/D/lac*Z* in LB broth. The addition of L-arabinose (0.02% final concentration) induced *lysV* expression and caused a reduction in culture optical density of cells containing pBAD102/D/lys*V* but had no detrimental effect on cells harboring the control plasmid (Fig. 2). The reduction in optical density was associated with an approximate 1.5 log (6.3x10⁷ cfu/ml) reduction in cell viability. For the purpose of purifying the His-tagged Lys*V* and β-galactosidase proteins, expression was induced with a lower concentration (0.003% w/v final concentration) of L-arabinose in mid-log growth phase cultures of *E. coli*. This treatment did not cause detectable cell lysis and allowed approximately 1 mg of Lys*V* to be routinely purified from 1 liter of cells.

**Purification of Lys*V***. Lys*V* was detected in and quantitatively recovered from the soluble protein fraction of whole-cell extracts (Fig.3 A and B). The protein band corresponding to Lys*V* had an estimated molecular weight of 28.6-kDa based on electrophoretic mobility through 12% polyacrylamide gels. The molecular weight of the recombinant protein was consistent with a 26.3-kDa predicted product containing the 22.9-kDa native Lys*V* protein plus an additional 3.4-kDa sequence comprising the V5 epitope and hexa-histidine tag.

Despite efforts to increase the stringency of the purification of Lys*V*, several extraneous proteins weakly bound to the nickel affinity column. These proteins were evident as faint bands in the polyacrylamide gels when a large quantity (>3 ug) of the Lys*V* was
loaded in a lane of the gels. However, these bands also appeared in polyacrylamide gels of purified β-galactosidase that were used as a negative control in our assays and thus were not associated with any detectable muralytic activity.

Activity of LysV on bacterial cells. When suspensions of *E. coli* cells were treated with chloroform to remove the outer membrane, the cells became susceptible to lysis by low levels (0.2 μg/ml) of the LysV enzyme (Fig. 4). Furthermore, EDTA treatment of *E. coli* cells, which produces holes in the outer membrane without killing the cells (3, 21, 22), rendered them sensitive to LysV treatment. The effect was detected as the production of osmosensitive spheroplasts and was similar to cells treated with egg white lysozyme (Table 2). Purified LysV was added to *E. coli* and *B. hyodysenteriae* cells to determine whether or not the enzyme was capable of crossing the outer membrane, eliciting an antibacterial effect. Not surprisingly, neither lysis nor loss of cell viability were observed when LysV (100 μg/ml) was added to cell suspensions nor was growth inhibition detected when LysV was overlaid onto cells spread on agar plates (data not shown).

Activity of LysV on peptidoglycan. The ability of LysV to digest purified peptidoglycan isolated from cultures of *B. hyodysenteriae* cells was tested. The peptidoglycan was incorporated into an agarose matrix (2 mg/ml) and added to the wells of a 96-well microtiter plate. This technique allowed a variety of buffer conditions to be assessed with minimal consumption of the peptidoglycan substrate. The incorporation of methylene blue as a cell-wall stain was especially relevant because we observed that *B. hyodysenteriae* peptidoglycan was translucent in aqueous solutions, which would make simple turbidometric analysis difficult.
LysV was active over a wide pH range (Fig. 5A) in 50 mM sodium phosphate buffer and 50 mM sodium citrate buffer, with highest activity between pH 4.5 and pH 6.5. The activity of LysV was found to be similar in Tris-HCl buffer over the range of pH tested (pH 5.0 - 8.5). We detected no effect on the activity of LysV over a range (100 – 500 mM) of sodium chloride concentrations (data not shown). Many bacterial and bacteriophage muralytic enzymes are conducive to renaturation (2, 17). Ten-minute incubation at temperatures above 45°C reduces the activity of LysV (Fig. 5B).

**Enzyme specificity of LysV.** Purified *B. hyodysenteriae* peptidoglycan digested with LysV yielded N-acetylamino sugars as detected by a positive Morgan-Elson reaction (Fig. 6). This activity is consistent with glycosidic enzymes that cleave the polysaccharide chains of peptidoglycan (12). When *B. hyodysenteriae* peptidoglycan was digested with mutanolysin (an N-acetylmuramidase), similar activity was observed (Fig. 6). Conversely, lysozyme, another N-acetylmuramidase, appeared less active against *B. hyodysenteriae* peptidoglycan than either LysV or mutanolysin (Table 3). Samples of *E. coli* peptidoglycan digested with LysV also yielded reducing sugars, but to a lesser extent than samples digested with either mutanolysin or egg white lysozyme (Table 3). No production of reducing sugars was detected in samples of chitin digested with LysV.

**DISCUSSION**

The sequence of VSH-1 structural genes (Chapter 2, this work) has significantly increased our knowledge and ability to study VSH-1, however, the lack of gene homologs in public databases has complicated the functional assignment of VSH-1 non-structural genes. To date, only one VSH-1 ORF had significant translated amino acid homology to sequences
in public databases. The VSH-1-associated \textit{lysV} gene encoded a hypothetical 197 amino acid protein with a calculated molecular weight of 22.9 kDa that shared translated amino acid identity to putative bacteriophage endolysins, bacterial lytic enzymes, and chitinases. This homology to putative muralytic enzymes combined with its position relative to the VSH-1 structural genes, in accordance with the organization of other bacteriophage late operons (8-10), strongly suggested that \textit{lysV} encoded a VSH-1 endolysin.

Purified recombinant LysV enzyme digested both \textit{E. coli} and \textit{B. hyodysenteriae} peptidoglycan and was active in several buffers over a wide pH range. The activity of some endolysins is affected by sodium chloride concentration and our use of NaCl during enzyme purification prompted us to test the affect of NaCl on LysV activity. In the case of \textit{Pseudomonas} phage φ6, endolysin activity was stimulated by a 150 mM NaCl concentration whereas the enzyme was inactive in 500 mM NaCl (7). The LysV enzyme was not influenced by NaCl concentrations. LysV was, however, unstable at temperatures above 45°C. Many bacterial and bacteriophage muralytic enzymes are conducive to renaturation, allowing detection of their activity in renaturing polyacrylamide gels (2, 17). Exposure to high temperatures caused immediate precipitation of the LysV enzyme and once denatured we detected no recovery of enzymatic activity even after prolonged incubation in several enzyme buffers. Repeated freezing and thawing of LysV-containing Tris-HCl buffer had a similar precipitory effect that also resulted in diminished enzymatic activity (personal observations). Our inability to induce LysV renaturation was likely the reason that our attempts to resolve LysV activity by renaturing-PAGE were unsuccessful.

A conserved domain database search (20) against the LysV amino acid sequence identified chitinase-like domains. Other bacteriophage lysins with homology to chitinase
enzymes have been reported (8) and some chitinases cleave polysaccharide chains of both chitin and peptidoglycan (5). Whereas peptidoglycan is a complex heteropolymer consisting of carbohydrate chains of alternating N-acetylglucosamine and N-acetylmuramic acid molecules interlinked by bridging polypeptides, chitin is a simpler homopolymer of β1,4-linked N-acetylglucosamine. Based on the amino acid homology to chitinase enzymes we hypothesized that the site of LysV enzymatic cleavage may be the carbohydrate, rather than polypeptide chains of peptidoglycan.

The appearance of reducing acetylamino sugars (detected as a positive Morgan-Elson reaction, Fig. 6) in LysV digests of *B. hyodysenteriae* and *E. coli* peptidoglycan confirmed that LysV hydrolyzed β-1,4 glycosidic bonds of peptidoglycan. This activity categorized LysV as a glycosidase rather than an amidase or endopeptidase. We further tested LysV for chitinase activity using the Morgan-Elson assay but detected no hydrolysis of chitin by LysV after overnight incubation (Table 3). This result suggests that although the amino acid sequence of LysV possesses a conserved chitinase domain, the LysV enzyme does not recognize and cleave the poly N-acetylglucosamine backbone of the chitin used in our assay.

The endolytic enzymes from gram-positive bacteria are very often lytic when applied to cultures of gram-positive bacterial cells, a characteristic which has been exploited for its antibiotic effect (18, 19, 24). Although antibacterial activity for gram-negative cells was reported for a bacteriophage endolysin purified from *Bacillus amyloliquefaciens* (23), in most cases the outer membrane of gram-negative cells blocks the entry of endolytic enzymes. LysV did not cross the outer bacterial outer membrane, as treatment of *E. coli* and *B. hyodysenteriae* whole-cells with LysV had no effect on cell growth or viability whereas disruption of the outer membrane followed by LysV treatment resulted in cell lysis.
We found that lysis of *E. coli* cells harboring plasmid pBAD102/D/lysV results from arabinose-induced gene expression. Bacterial lysis is a complex and tightly controlled process in bacteriophage lytic cycles. The activity of bacteriophage endolysins is dependent on their ability to cross the bacterial inner membrane. Most often, holin proteins form inner membrane channels through which bacteriophage endolysins pass. The production of endolysins precedes the formation of protein channels, thus the polymerization of holin proteins in the inner membrane controls the timing of cell lysis (4). Since *E. coli* cells expressing LysV from pBAD102/D/lysV did so in the absence of an associated holin, cell lysis was an unexpected result that suggested LysV crossed the inner membrane in sufficient amounts to lyse cells without a holin channel.

A drop of chloroform added to arabinose-treated *E. coli* cells expressing LysV from pBAD102/D/lysV caused immediate and complete lysis of the cell cultures (data not shown). The chloroform treatment did not cause similar lysis of *E. coli* cells that harbored pBAD102/D/lysV but were not treated with arabinose to induce LysV expression nor did chloroform affect arabinose-treated or non-treated *E. coli* cells harboring pBAD102/D/lacZ. These observations argue that the ability of LysV to cross the inner bacterial membrane and not cytoplasmic production of the LysV enzyme is rate-limiting for lysis of *E. coli* cells.

Other investigators have identified bacteriophage endolysins which, when expressed in *E. coli*, cause cell lysis without a holin. These endolysins were found to possess N-terminal amino acid sequences that were recognized as signal peptides for sec-mediated transport out of the cytoplasm. This characteristic was first identified with Lys44, the endolysin of bacteriophage fOg44 that infects the gram-positive bacterium *Oenoccous oeni* (26). Here, the N-terminal signal peptide was cleaved from the preprotein after transport to
produce the active enzyme and release it into the periplasm. Recently, Young and colleagues
demonstrated that the Lyz endolysin of *E. coli* bacteriophage P1 is also externalized by the
sec translocon, but without signal peptidase cleavage (29). It is important to note that both
P1 and fOg44 possessed holin genes, which might indicate that sec-dependent transport alone
is insufficient to mediate phage release.

It is tempting to speculate that the VSH-1 LysV protein may be transported by a
bacterial secretory pathway, however, when analyzed by signal prediction algorithms (11,
25), the N-terminal amino acid sequence of LysV was not predicted to encode a signal
peptide. It is possible that strongly induced *lysV* expression from the pBAD102/D/LysV
plasmid results in the build up of LysV in the cytoplasm that has a toxic effect unrelated to its
muralytic activity or that LysV was co-transported into the periplasm with other proteins.

It is not known if LysV possesses novel properties that allow it to mediate holin-
independent lysis in *B. hyodysenteriae*. VSH-1 is most similar to the defective bacteriophage
and gene transfer agent GTA of *Rhodobacter capsulatis*. GTA production is induced during
the onset of stationary phase in *R. capsulatis* cultures and, consistent with the observation
that the release of GTA virions is not associated with cell culture lysis, genes that might
encode GTA lytic functions were not identified (16). Unlike GTA, VSH-1 is mitomycin C-
inducible and virion release correlates with cell lysis.

We have identified a small ORF downstream from *lysV* with properties consistent
with bacteriophage holins. The ORF encodes a theoretical protein of 65 amino acids, which
is within the size range (65-95 amino acids) described for other bacteriophage lysins (30).
The protein is overall slightly hydrophobic with a strongly charged C-terminus. Most
significantly, the amino acid sequence is predicted by Trans Membrane preditiction using
Hidden Markov Models (TMHMM) algorithms to form two trans-membrane helices, a hallmark of class II holins.
**TABLE 1.** Most closely matching homologs returned by a BLAST search of GenBank using the LysV translated amino acid sequence.

<table>
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*Predicted gene function from conceptual translations of nucleotide sequences, as indicated by the annotation on the GenBank submissions. *Molecular weight based on amino acid sequences and calculated by Statistical Analysis of Protein Sequence (SAPS) algorithms (6).
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**TABLE 2.** Osmosensitivity assay for peptidoglycan-degrading enzymes. Samples were diluted 21-fold in $\text{dH}_2\text{O}$ after a 1 min incubation at room temperature for each treatment and immediately measured spectrophotometrically.
TABLE 3. Substrate specificity of LysV. Samples of bacterial peptidoglycan (PGN) and chitin in 300 ul of 50 mM sodium phosphate buffer (pH 6.5) were digested overnight with 5 \( \mu \)g of enzyme. The production of reducing sugars was assayed using the Morgan-Elson reaction and scored for the relative production of chromogen.
FIG. 1. Basic structure of a peptidoglycan oligosaccharide chain shown with a peptide cross-link. Arrows indicate the bond that is attacked by N-acetylglucosaminidase (1), N-acetylmuramidase/lytic glycosylase (2), L-alanine amidase (3), and endopeptidase (4).
FIG. 2. L-arabinose induction (0.02% final concentration) of *E. coli* clones. Symbols: ○, non-induced cells harboring plasmid pBAD102/D/lysV; ●, induced cells harboring plasmid pBAD102/D/lysV; □, non-induced cells harboring plasmid pBAD102/D/lacZ; ■, induced cells harboring plasmid pBAD102/D/lacZ.
FIG. 4. Lysis of *E. coli* cells by purified LysV. *E. coli* cells treated with CHCl₃ to remove their outer membranes were subjected to treatment with 0.2 µg/ml LysV (●), 0.2 µg/ml β-galactosidase (▲), or no enzyme (■) and incubated at room temperature.
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FIG. 5. A. Activity of LysV on purified *B. hyodysenteriae* peptidoglycan. The activity of LysV was tested at different pH ranges in 1. Sodium Phosphate Buffer (50 mM, pH 5.5 – 8.5), 2. Sodium Citrate Buffer (50 mM, pH 3.5 – 7.0), and 3. Tris-HCl Buffer (50 mM, pH 5.0 – 8.5). OD$_{600}$ values (average of 3 replicates per treatment) are given below the wells in each row. B. Heat inactivation of LysV. The activity of LysV was tested after a 10-min incubation at different temperatures. OD$_{600}$ values (average of 2 runs) are given below the figure.
FIG. 6. Reducing sugar assay for peptidoglycan hydrolysis. Samples of purified *B. hyodysenteriae* peptidoglycan (0.5 mg) were incubated at 37°C with 1 μg of LysV (●), 1 μg Mutanolysin (▲), or no enzyme (■). After incubation, samples were assayed for reducing sugars by the Morgan-Elson reaction.
REFERENCES


CHAPTER 4. INDUCTION AND CHARACTERIZATION OF VSH-1

TRANSCRIPTION IN BRACHYSPIRA HYODYSENTERIAE

A paper to be submitted to the Journal of Applied and Environmental Microbiology

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THAD B. STANTON

ABSTRACT

Brachyspira hyodysenteriae cells harbor VSH-1, a mitomycin C-inducible, non-self replicating prophage. VSH-1 structural genes and genes of putative lytic functions have been sequenced and found to occupy a contiguous 15.7-kb region of the B. hyodysenteriae chromosome. Genes of apparent regulatory function were not identified within or immediately adjacent to known VSH-1 genes. As a step toward understanding the regulation of VSH-1, we investigated the organization of the VSH-1 operon and measured transcription of VSH-1-associated genes during the switch from lysogeny to lysis. RNA slot blot hybridization determined that VSH-1 transcription reached maximal levels between 3 and 4 h over a 6-hour time period following mitomycin C treatment (10 μg/ml) of B. hyodysenteriae cell cultures. By contrast, mRNA levels of an experimental control gene encoding the B. hyodysenteriae flagellar sheath gene (flaA1) declined over the same period. Reverse transcriptase-PCR amplification of VSH-1 intergenic regions and Northern blots of B. hyodysenteriae RNA indicated that VSH-1 genes share a common transcript beginning with svp45 and continuing through genes of lytic function. Multiple promoters may drive
transcription of VSH-1 genes as several transcriptional start sites were located near the beginning of the *svp45* ORF start codon. In addition to mitomycin C, VSH-1 gene transcription was induced by H$_2$O$_2$ treatment of *B. hyodysenteriae* cells, indicating that VSH-1 induction can likely be triggered by a bacterial response to DNA damage, suggesting a possible mechanism for VSH-1 production and subsequent bacterial gene transfer among *B. hyodysenteriae* cells in the swine intestinal tract.

**INTRODUCTION**

*Brachyspira hyodysenteriae* is an aero-tolerant anaerobe and an enteric pathogen that causes severe muco-hemorrhagic diarrhea in swine (6, 25). *B. hyodysenteriae* cells harbor VSH-1, a replication and packaging-defective prophage. Induction of the VSH-1 prophage results in the assembly of small, tailed VSH-1 virions (7) but does not trigger viral replication (unpublished results). The VSH-1 virions package random 7.5 kb fragments of *B. hyodysenteriae* DNA rather than a complete viral genome and have the capacity to mediate genetic exchange between *B. hyodysenteriae* strains *in vitro* (8, 26).

As a host-genome packaging and gene transfer agent, VSH-1 is similar to the gene transfer agent (GTA) of *Rhodobacter capsulatus*, the Voltae transfer agent (VTA) of *Methanococcus voltae*, and Dd1 of *Desulfovibrio desulfuricans* (2, 15, 20). Among these gene transfer agents, the regulatory mechanism is known only for GTA. Production of GTA correlates with the entry of *R. capsulatus* cells into stationary growth phase and is driven by a bacterial two-component sensor kinase regulator (14, 16). Whereas GTA production is responsive to the growth phase of its bacterial host, no such innate condition for VSH-1 induction has been identified. Spontaneous production of VSH-1-like virions has been
reported by others (21), however, the conditions leading to virion production were unknown and we have previously observed VSH-1 virions only when their production is induced by treating *B. hyodysenteriae* cultures with mitomycin C.

In functional temperate bacteriophages, expression of morphogenesis “late genes” follows the self-replicative production of the viral genome (3). This increase in bacteriophage genome copy number amplifies the number of late gene operons, allowing rapid accumulation of mRNA and consequently, virion structural proteins. Unlike many temperate bacteriophages, VSH-1 lacks a detectable replicative intermediate and thus, a single copy of VSH-1 structural genes per bacterial chromosome exists in *B. hyodysenteriae* cells. Consequently, we hypothesized that VSH-1 transcription would be strongly upregulated in response to inducing agents such as mitomycin C to compensate for a lack of multiple VSH-1 gene copies.

VSH-1 may facilitate the *in vivo* exchange of genes among populations of *B. hyodysenteriae* cells under certain environmental conditions. A lack of information about the influence of genetic and environmental factors on the expression of VSH-1 genes has hindered investigations of the regulation of VSH-1 production and ecological significance of VSH-1-mediated gene transfer. To provide a foundation for uncovering the mechanisms of VSH-1 regulation, investigations were carried out to characterize the transcriptional organization of VSH-1 and to measure the production of VSH-1-associated mRNA during the switch from lysogeny to virion production.
MATERIALS AND METHODS

VSH-1 induction. *Brachyspira hyodysenteriae* strain B204 cells were cultured at 37°C with constant stirring in brain heart infusion broth supplemented with 10% heat-treated (56°C for 30 min) bovine serum under an atmosphere of 99% N₂, 1% O₂ as described previously (24). To induce production of VSH-1, mitomycin C (10 μg/ml final concentration) (Sigma, St. Louis, MO) or H₂O₂ (300 μM final concentration) was added to *B. hyodysenteriae* cultures (OD₆₂₀ 0.54 – 0.6, pathlength = 18 mm) during early logarithmic phase growth.

RNA Extraction and Purification. *B. hyodysenteriae* cells were centrifuged (3,000 x g for 10 min at 4°C) and resuspended in ice-cold cell suspension buffer (50 mM Tris, 50 mM EDTA, pH 8.0). The cell optical density of the suspensions was adjusted with additional suspension buffer to standardize samples to an OD₆₂₀ of 0.5. The number of cells/ml in each of the suspensions was determined directly by cell counts using a Petroff-Hauser chamber. A 7-ml volume of each cell suspension was pelleted with a second centrifugation step. The buffer was aspirated and the pellets were frozen at -70°C.

For hybridization analysis, cell pellets were lysed using the three-detergent method of Syn et al. (29). Briefly, thawed cell pellets were resuspended in 0.5 ml of STT lysis buffer (2% SDS, 1% Tween 20, and 1% Triton X-100) and vortexed for 1 min at medium speed. The samples were acidified by the addition of 12.5 μl of 1 M HCl, and 50 μl of sodium acetate (2 M, pH 4.0) was added to facilitate precipitation of nucleic acids in an equal volume of 100% isopropyl alcohol. RNA was extracted twice with equal volumes of citrate-buffered phenol chloroform (4:1, pH 4.0) and once with 100% chloroform. The RNA was
precipitated in an equal volume of ice-cold isopropyl alcohol for 1 h at -20°C and pelleted at 15,000 x g for 30 min at 4°C. The RNA pellets were resuspended in 1X DNase buffer and RNase-free DNase I enzyme from Promega was added to an approximate concentration of 0.1 U per ng of nucleic acid. DNA was digested for 15 min at 37°C. RNA was chloroform-extracted once, precipitated as above and resuspended in diethyl pyrocarbonate (DEPC)-treated H₂O to determine the concentration and purity by spectrophotometrically measuring the ratio of 260/280 OD.

For reverse transcriptase PCR (RT-PCR) and primer extension analysis, B. hyodysenteriae RNA was prepared using the RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s protocol for bacterial RNA extraction. RNA samples were diluted (100 ng/μl, final concentration) in 1X DNase reaction buffer (Promega). DNase I enzyme was added to a concentration of 0.1 U per ng of nucleic acid as before, but the 37°C incubation time was extended to 30 min. The DNase enzyme was inactivated by heating to 70°C for 10 min, and the RNA concentration was determined as above.

**Primer and probe design.** Sequence-specific oligonucleotide primers and probes were designed using Oligo version 6.0 software (Molecular Biology Insights, Inc., Cascade, CO) and synthesized by the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA). Probes were end-labeled with gamma ³²P-ATP using a T4 kinase reaction. Unincorporated label was removed by purifying the probes through a NucTrap purification column (Stratagene, La Jolla, CA) according to the manufacturer’s protocol.

**Cloning VSH-1 and B. hyodysenteriae genes.** To provide RNA standards for slot blot hybridization experiments, VSH-1 genes, *svp45, svp38, svp53, svp101,* and lys*V* and a *B.
*B. hyodysenteriae* gene, *flaA1*, were cloned into separate pBlueScript II SK− plasmids (Stratagene). To facilitate cloning, the genes were PCR-amplified using primers with additional restriction sites (Table 1B). PCR was performed using an Amplitaq Gold kit (Applied Biosystems, Foster City, CA). *B. hyodysenteriae* DNA template (0.8 ng/μl final concentration) was added to PCR mix (1X GeneAmp buffer, 2.5 mM MgCl₂, 200 μM each dNTP, 1.5 U Amplitaq Gold DNA polymerase and 0.25 pmol/μl each forward and reverse primer) in a 100-μl reaction volume. Samples were amplified using a Biometra UNO-Thermoblock thermocycler (Biometra Inc., Tampa, FL) with an initial incubation of 92°C for 9 min followed by 36 cycles of 95°C for 1 min, 48°C for 1 min, and 72°C for 2 min and a final 8-min extension at 72°C. PCR products were ethanol precipitated and resuspended in nuclease-free water.

PCR amplicons and pBlueScript II SK− plasmid were double digested using the restriction enzymes Hind III and EcoR I for *svp45*, *svp38*, *svp53*, *lysV*, and *flaA1* amplicons and BamH I and EcoR I for the *svp101* amplicon in the manufacturer’s recommended buffers (Invitrogen, Carlsbad, CA). After digestion (30 min at 37°C), the PCR products and plasmids were extracted twice with an equal volume of Tris-buffered phenol chloroform, isoamyl alcohol (25:24:1, pH) to remove restriction enzymes and precipitated for 30 min at −70°C in 3 vol of ice-cold 100% ethanol, 100 mM sodium acetate (pH 5.5) and 1 μg glycogen as a carrier. The DNA was pelleted by centrifugation (15,000 x g at 4°C) and resuspended in nuclease-free water. The concentrations of the digested products were estimated by agarose gel electrophoresis and compared to molecular weight standards of known concentration.
The digested PCR products and plasmids were combined at a 2:1 insert to vector ratio (0.06 pmol PCR product, 0.03 pmol vector) and ligated for 30 min at 16°C with Invitrogen T4 DNA ligase. The ligation products were ethanol-precipitated and resuspended in nuclease-free H₂O (as above) and electroporated into OneShot TOP 10 electrocompetent *E. coli* cells purchased from Invitrogen. Transformants harboring pBlueScript II SK- vectors with cloned inserts were selected by blue/white colony screening on X-gal-containing LB agar plates (23) and verified by PCR amplification of the insert DNA using primers flanking the cloning site. Plasmids were extracted from *E. coli* clones using Qiagen MiniPrep plasmid extraction columns according to the manufacturer’s protocol.

**Preparation of slot blot standards.** pBlueScript II SK- plasmids were digested with BamHI and the linearized plasmids were purified by phenol/chloroform extraction and ethanol precipitation as described above to remove restriction enzymes and buffer salts. *In vitro* transcription was performed using a Stratagene RNA transcription kit. Individual reactions (0.4 mM each rNTP, 30 μM DTT, 10 U T7 RNA polymerase, 1 μg plasmid DNA, in 1X Transcription Buffer) for each plasmid were prepared according to the manufacturer’s protocol and transcription was carried out for 30 min at 37°C. Plasmid DNA was removed by digestion with DNase I (0.1 U/μl) for 30 min at 37°C, and the concentration of *in vitro*-transcribed RNA was determined spectrophotometrically as above.

A dilution series of *in vitro*-transcribed RNA reference standards was prepared for *svp45, svp38, svp53, svp101, lysV, and flaAl*, and the molecular mass of each transcript was calculated based on the length and base composition. To minimize sample handling variation and to control for loss of RNA during extraction, the amount of transcript equivalent to 3x10^{12} copies was added to pelleted cells prepared as describe above from a non-treated
culture of *B. hyodysenteriae* during lysis in STT buffer. After extraction, a series of 2-fold dilutions of the RNA stock containing the standards was prepared and adjusted with RNA extracted from non-induced *B. hyodysenteriae* cultures to maintain the balance of total RNA (6 μg) in each dilution standard.

**RNA slot blot hybridization.** RNA samples (6 μg total RNA) or standards (adjusted to 6 μg total RNA) were diluted in DEPC-treated H₂O to a final volume of 50 μl and denatured for 20 min at room temperature in 3 vol of freshly prepared denaturation solution (2% glutaraldehyde, 1.5 μg/ml polyadenylic acid, 0.00025% bromophenol blue). After denaturation, the samples were transferred to the wells of a BioRad Bio-Dot SF microfiltration manifold and blotted to Zeta-Probe membranes (BioRad, Hercules, CA) by applying gentle vacuum (10 mbar). After the sample liquid was completely drained, the wells were filled with 400 ul of DEPC-treated H₂O and a vacuum was again applied until the wells were dry. The RNA was UV crosslinked (120,000 μjoules) to the membranes in a Stratagene UV Stratalinker 1800, and the membranes were dried overnight at room temperature.

To hybridize the RNA with transcript-specific ³²P-labeled oligonucleotide probes (Table 1c), the membranes were pre-soaked in prehybridization solution consisting of 6X SSC buffer (100 mM NaCl, 90 mM Sodium citrate, pH7.0) with 20 mM NaH₂PO₄, 2% SDS, 5X Denhardt’s reagent and 500 μg sheared salmon testes DNA/ml at 42°C while rotating in a hybridization oven. After two hours, the prehybridization solution was replaced with hybridization solution of similar formulation but with 1X Denhardt’s reagent. ³²P-labeled sequence-specific probe (approx. 100 μl, 10⁶ DPM/μl) was added to the hybridization buffer,
and the membranes were hybridized overnight at 42°C. The membranes were rinsed for 1 h in 1 liter of 6X SSC, 0.5% SDS at 45°C, and the specific radioactivity of each sample on the membrane was quantified using an Instant Imager (Packard Instrument Co., Meriden, CT). The absolute mRNA copy number was determined for each gene in samples of total RNA extracted from *B. hyodysenteriae* cultures by comparing the signal intensities of the samples to the corresponding *in vitro* transcribed mRNA reference standard. The average number of mRNA copies per cell was calculated by dividing the absolute mRNA copy number of each gene by the total number of cells in each sample.

**Reverse transcriptase PCR.** Comparative RT-PCR under non-saturating conditions was used to analyze induction of VSH-1 transcription. A single-tube Access RT-PCR kit (Promega, Madison, WI) was used to amplify cDNA from *svp38* and *flaA1* mRNA’s using primer sets *svp38F/svp38R* and *flaA1F/flaA1R*, respectively (Table 1A). The PCR cycle number and concentration of template were optimized so that the endpoint of amplification occurred prior to saturation of the PCR reactions. Thus, a 2-fold increase in target copy number was detectable as an approximately 2-fold increase in signal intensity of the PCR products in agarose gels. Fifty-μl duplex RT-PCR reactions were prepared according to the manufacturer’s protocol with 50 pmol of each forward and reverse primer and 1 ng of *B. hyodysenteriae* total RNA. The samples were incubated at 48°C for 45 min to allow first-strand cDNA synthesis and amplified with an initial 2 min incubation at 94°C followed by 23 cycles of 94°C for 30 sec, 52°C for 1 min, and 68°C for 2 min and a final 8-min extension at 68°C.
Intergenic RT-PCR was performed using a two-step process to assess transcriptional linkage of VSH-1 genes. First, 500 ng of total RNA extracted from *B. hyodysenteriae* cultures grown for 3.5 h after mitomycin C treatment was converted to cDNA using an Invitrogen ThermoScript RT system and random hexamer primers according to the manufacturer's protocol. After first-strand cDNA synthesis, the samples were heated to 85°C for 5 min to denature the reverse transcriptase enzyme, and the RNA was destroyed by RNase H-treatment (2 U) for 20 min at 37°C. For negative control samples, the reverse transcriptase enzyme was omitted. Secondly, intergenic regions were amplified by PCR from the cDNA using Invitrogen Platinum *Taq* DNA polymerase and gene-specific primers that flanked regions between open reading frames (Table 2). Separate 25-μl PCR reactions (1X Platinum *Taq* buffer, 1.5 mM MgCl₂, 200 μM each dNTP, 1 U Platinum *Taq* DNA polymerase, 5 pmol each of forward and reverse primers) for each primer set were prepared in triplicate. The first set received 1 μl of control cDNA (prepared without reverse transcriptase), the second set received 1 μl of cDNA, and the third set received 10 ng of *B. hyodysenteriae* genomic DNA. The samples were amplified with an initial incubation of 94°C for 2 min followed by 32 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 2.5 min and a final 8-min extension at 72°C. PCR products were visualized by agarose gel electrophoresis.

**Northern hybridization.** RNA extracted from *B. hyodysenteriae* cultures 3.5 h after mitomycin C treatment was denatured with glyoxal (23). Briefly, 10 μg of RNA in 2 μl of DEPC-treated H₂O were denatured for 10 min at 65°C with 10 μl of glyoxal reaction mixture (60% DMSO, 2% deionized glyoxal, 1.2X BPTE electrophoresis buffer, 4.8% glycerol, and
0.2 mg/ml ethidium bromide). The 12-μl reaction volumes were electrophoresed at 55 V for 2 h through a 1% agarose gel in 1X BPTE electrophoresis buffer (10 mM PIPES, 30 mM Bis-Tris, 1 mM EDTA pH 8.0) prepared with DEPC-treated H2O. After electrophoresis the gels were rinsed in DEPC-treated H2O and soaked for 30 min in 10 gel volumes of 10X SCC transfer buffer (1.5 M NaCl, 150 mM sodium citrate). The RNA was transferred to a Zeta-Probe membrane by upward capillary transfer for 4 h using 10X SCC transfer buffer. After capillary transfer, RNA was UV crosslinked to the membranes as described above. Glyoxal adducts were removed by submerging the membrane in 500 ml of 95°C Tris-HCl, pH 8.0, 1 mM EDTA with gentle agitation at room temperature for 1 h. The membranes were hybridized with 32P-labelled oligonucleotide probes for svp38, svp101, and lysV (Table 1C) as described for slot blot hybridizations.

**Primer extension.** To provide plasmid template for sequencing, a clone containing the VSH-1 svp45 ORF and upstream sequence was identified by screening a λ ZAP II library of cloned B. hyodysenteriae B204 genomic DNA (Stratagene) using 32P-labelled primer svp45R (5’ CTGCTCTTTCAAAATTGGCTTAT 3’, Tm = 60°C) as described previously (Chapter 2, this work). The λ ZAP II clone (pBlue/svp45) was converted to the pBluescript phagemid vector in E. coli SOLR cells by in vivo excision according to the Stratagene protocol. Plasmid DNA was purified using a Qiagen MiniPrep plasmid purification column. To prepare template for sequencing, plasmid pBlue/svp45 was denatured for 20 min at 37°C with 0.1 vol of freshly prepared denaturation buffer (2 M NaOH, 2 mM EDTA) and neutralized with 0.1 vol of 3 M sodium acetate, pH 5.5. Plasmid DNA was ethanol-precipitated, pelleted by centrifugation, and resuspended in nuclease-free H2O. One pmol of
denatured plasmid was sequenced using a Sequenase Version 2.0 DNA sequencing kit (USB Corp., Cleveland, OH) according to the manufacturer's protocol with 20 pmol of the same $^{32}$P-labelled primers used in primer extension reactions. Primer extension was carried out using a Primer Extension System-AMV reverse transcriptase kit (Promega) according to the manufacturer’s protocol. One hundred fmol of $^{32}$P-labelled primer svp45R55 or svp45R80 (Table 1a) was annealed to 1 µg of *B. hyodysenteriae* total RNA extracted from cultures after mitomycin C treatment. Extension was carried out for 30 min at 42°C. The results were verified at a higher extension temperature (30 min at 55°C) using Invitrogen ThermoScript reverse transcriptase. The primer extension products and sequencing ladders were resolved by electrophoresis at 90 W for 2 h through a 1 mm, 8 M urea, 8% polyacrylamide sequencing gel. After electrophoresis, the gel was transferred to 3 mm chromatography paper (Whatman International Ltd., Maidstone, England), dried, and exposed to X-ray film overnight to visualize the bands.

**RESULTS**

**Quantitation of VSH-1 transcripts.** Transcription of VSH-1 head morphogenesis (*svp45* and *svp38*), tail morphogenesis (*svp53* and *svp101*) and lysis (*lysV*) genes was monitored over a period of 6 h following mitomycin C treatment of *B. hyodysenteriae* cultures and in untreated controls. Northern slot blot hybridization signals of RNA extracted from *B. hyodysenteriae* cultures at 1 h intervals were compared to dilutions of *in vitro* transcribed standards by simultaneous hybridization with $^{32}$P-labelled oligonucleotide probes specific for the target sequence (Fig. 1A). Slot blot results indicated an increase in VSH-1 transcription and a decrease in *flaA1* transcription after mitomycin C treatment.
Changes in the average mRNA copy number per cell of VSH-1 genes were similar over time. Increased transcription of VSH-1 genes was detectable 2 h after cultures were treated with mitomycin C. Based on cumulative slot blot data, the VSH-1 genes reached maximal transcription levels approximately 4 h post-treatment (Fig. 2). At approximately 400 copies per cell, the maximum number of svp38 and svp53 transcripts was similar. The maximum number of svp45, lysV, and svp101 transcripts per cell varied by approximately 150, 200, and 300 copies, respectively, from svp38 and svp53. VSH-1 genes transcripts were undetectable (<30 copies per cell) over time in non-treated B. hyodysenteriae cultures.

Compared to the induced transcription of VSH-1 genes, the normally strongly transcribed B. hyodysenteriae flagellar sheath gene, flaA1 (13) demonstrated a reduction in transcription following mitomycin C treatment (Fig. 1B). The flaA1 mRNA copy number per cell declined significantly \((P < 0.05)\) from approximately 200 copies to 75 copies per cell within a period of 2 – 4 h following mitomycin C-treatment. Relative to mitomycin C-treated cultures, flaA1 transcript copy numbers in control B. hyodysenteriae cultures remained stable over time (Fig. 3).

**Transcriptional organization of VSH-1.** B. hyodysenteriae RNA extracted from mitomycin C-treated cultures was included in RT-PCR assays that targeted VSH-1 intergenic regions in order to determine whether or not VSH-1 genes are co-transcribed as a single operon. Amplicons were generated from mRNA that overlapped every adjacent VSH-1 gene, consistent with VSH-1 genes being co-transcribed (Fig. 4). As expected, the VSH-1 svp45 gene was not transcriptionally linked to the putative Ser/Thr sodium symporter gene \((sstT)\), which is located upstream and oriented in the opposite direction of transcription of svp45. However, a low level of mRNA was detected between VSH-1-associated genes and
the putative bacterial methyl-accepting chemotaxis gene (*mcpB*). This gene is located downstream from VSH-1 lysis genes and oriented in the same direction of transcription (Fig. 4, amplicon M, lane 2).

Northern blots of RNA from mitomycin C-treated *B. hyodysenteriae* cultures were probed for *svp38*, *svp101* and *lysV* by hybridization with gene-specific probes (Fig. 5). For comparison, RNA from non-treated cultures was probed for *flaA1*. Although blots that were hybridized with VSH-1 gene probes demonstrated considerable size heterogeneity of mRNA molecules with regions of higher signal intensity between 2.5 kb and 7.5 kb, the hybridization pattern of each blot was similar and the presence of high-molecular weight (> 9 kb) RNA hybridizing to VSH-1 gene probes was evident. Taken together, these data support intergenic RT-PCR data indicating that VSH-1 genes share a common multi-gene transcript. By contrast, a probe for *flaA1* resolved a more discrete mRNA band of 1.4 kb (Fig. 5, lane 4) indicating the *flaA1* mRNA is a single-gene transcript.

**Identification of VSH-1 transcription start sites.** Analysis of the VSH-1 nucleotide sequence revealed regions that lacked ORF's assignable to known virion structural components and that could instead contain promoters or transcription elements. The regions were located upstream from *svp45*, between *svp38* and *svp24*, and between *svp53* and *svp32* (Fig. 4). Primer extension was performed using oligonucleotides with binding sites located downstream from each of these regions to initiate cDNA synthesis from VSH-1 mRNA. Two possible major and two minor start sites were detected near the beginning of *svp45* (Fig. 6) but not within the other two regions (data not shown).

The transcriptional start sites were mapped to 4 adenosine bases using primer *svp45R55* to generate both a primer extension product from *B. hyodysenteriae* RNA and a
sequencing ladder from a plasmid containing a cloned copy of the svp45 gene for comparison (Fig. 6). A major start site was located 29 bp upstream from svp45 start codon and three were located within the svp45 gene at positions 11, 32, and 47 nucleotides downstream of the svp45 ATG start codon (Fig. 7). The same transcriptional start sites were identified with a second primer (svp45R80) with a binding site located downstream from that of svp45R55 and to reduce the possibility that mRNA tertiary structure contributed to false positive signals, both primers were used to verify the start sites at higher (55°C) extension temperature (data not shown). Additionally, primer extension was performed using RNA isolated from B. hyodysenteriae cultures at 0, 1.5, and 3 h time points after mitomycin C treatment. The signals generated from RNA extracted at 1.5 h post-treatment were similar to those at 3 h post-treatment, but markedly weaker in intensity. No signal was obtained using RNA extracted at the 0 h time point.

**Hydrogen peroxide induction of VSH-1.** The growth rate of B. hyodysenteriae cells was monitored after treating early log growth phase cultures with various concentrations of H$_2$O$_2$ (100 μM – 1 mM final concentration). At a concentration of 300 μM H$_2$O$_2$, B. hyodysenteriae growth was slowed but not completely inhibited, which was similar to the effect of treating cultures with mitomycin C. Unlike mitomycin C-treated cultures, H$_2$O$_2$-treated cultures did not demonstrate obvious cell lysis between 5 h and 7 h after treatment. Higher (≥ 400 μM) H$_2$O$_2$ concentrations had the effect of immediately halting cell growth whereas lower (< 200 μM) H$_2$O$_2$ concentrations had no detectable effect on growth rate.

Transcription of the VSH-1 svp38 major head protein gene and the B. hyodysenteriae flaA1 gene were assessed by duplex RT-PCR amplification of RNA extracted from cultures treated with 300 μM H$_2$O$_2$ and compared to mitomycin C-treated and non-treated control
cultures. Transcripts of *svp38* were detected in H$_2$O$_2$-treated and mitomycin C-treated *B. hyodysenteriae* cultures 2 h after treatment and were not detected in non-treated controls (Fig. 8A and B; Fig. 9B). As expected from the previous slot blot hybridization results, *flaA1* transcripts were detected for all time points in each sample and the intensity of the *flaA1* amplification signals appeared to diminish over time in the treated samples but not in the non-treated control.

As cell lysis was not observed in cultures treated with H$_2$O$_2$, we verified that induction of VSH-1 transcription with H$_2$O$_2$ also resulted in virion production by crossing *B. hyodysenteriae* strains A203 (22) (*ΔflaA1::cat*) and K19 (27) (*Δnox::kan*) in co-culture. A 1 log increase in the frequency of gene transfer occurred in the presence of H$_2$O$_2$ as measured by the formation of doubly resistant transductants compared to non-treated crosses (data not shown). This increase in gene transfer frequency was slightly lower than the 1.5 log increase measured when similar crosses were made in the presence of mitomycin C. Additionally, we observed VSH-1-like virions in electron micrographs of negatively stained (2% phosphotungstic acid) samples of H$_2$O$_2$-treated *B. hyodysenteriae* cells although the virions appeared qualitatively less abundant than in samples of mitomycin C-treated cells.

To determine whether or not *de novo* protein synthesis was required for induction of VSH-1 transcription, an inhibitory concentration of chloramphenicol (400 µg/ml) (11) was added to *B. hyodysenteriae* cultures immediately following mitomycin C treatment. Transcription of *svp38* and *flaA1* were again monitored by duplex RT-PCR. The addition of chloramphenicol after treatment with mitomycin C prevented transcription of *svp38*, but did not reduce transcription of *flaA1* (Fig. 9).
DISCUSSION

The shift from VSH-1 lysogeny to cell lysis occurs over a period of 6 h after treating
*B. hyodysenteriae* cells with mitomycin C. Rapid accumulation of VSH-1 mRNA occurred
between 2 and 4 h after mitomycin C treatment. Although cell lysis occurs approximately 5
– 6 h after mitomycin C treatment, VSH-1 transcripts rapidly decreased between 4 – 5 h after
treatment. The reduction of VSH-1 transcripts from their maximal levels at 4 h correlates
with the period of time during which significant cleavage of *B. hyodysenteriae* genomic
DNA into 7.5 kb fragments is occurring (7). The fragmentation of genomic DNA
undoubtedly results in reduced transcription of VSH-1 genes, and mRNA levels would be
expected to continue to fall as a result of mRNA loss during cell lysis 5 h after mitomycin C
treatment.

A rapid reduction in the number of *flaAl* transcripts was observed after mitomycin C
treatment of *B. hyodysenteriae* cultures as compared to the relatively stable levels in non-
treated control cultures. This reduction in transcription was not simply due to mitomycin C-
responsiveness of the *flaAl* promoter. The addition of chloramphenicol to inhibit *de novo*
protein synthesis when added in conjunction with mitomycin C had the dual effect of
preventing induction of VSH-1, as indicated by a lack of VSH-1 gene transcription while
allowing the continued transcription of *flaAl*. Taken together, these results indicate that one
or more protein factors synthesized after mitomycin C treatment of *B. hyodysenteriae* cells
are required for transcription of VSH-1 genes and that a shift from bacterial to viral gene
transcription coincides with VSH-1 induction.

Although VSH-1 regulatory genes remain unknown, the production of an alternative
σ factor alone or in conjunction with a VSH-1 repressor would account for many of our
results. Treatment of \textit{B. hyodysenteriae} cells with DNA damaging agents such as mitomycin C would likely initiate an SOS response cascade that would include the production of an alternative $\sigma$ factor, redirecting RNA polymerase specificity toward promoters that control expression of genes for cellular repair (32). This shift would serve to down regulate \textit{B. hyodysenteriae} housekeeping genes such as \textit{flaA1} and increase VSH-1 transcription if the VSH-1 promoter was recognized by the alternative $\sigma$ factor. Furthermore, the production of an alternative $\sigma$ factor in response to DNA damage would account for chloramphenicol inhibition of VSH-1 transcription. Inhibiting \textit{de novo} protein synthesis would prevent expression of the Sigma factor. Consistent with our results, this would prevent redirection of the RNA polymerase and thus transcription of VSH-1 genes while allowing continued transcription of \textit{flaA1}. Additionally, our results would allow for a model similar to the regulation of phage lambda (3) in which a cleavable repressor protein binds an operator sequence near the VSH-1 promoter serving to increase the stringency of VSH-1 transcription.

The variation in the number of mRNA copies for several VSH-1 genes allows the possibility that independent promoters with varying strengths drive transcription of these genes. However, the results of intergenic RT-PCR analysis of VSH-1 transcriptional organization were instead consistent with VSH-1 genes being co-transcribed as an operon. Additionally, we were unable to identify palindromes within the VSH-1 sequence that could potentially form $\rho$-independent mRNA stem loop structures constituting transcription termination signals. If overlapping transcripts initiated from promoters upstream from several VSH-1 genes were not terminated, we would expect the mRNA copy numbers to be higher for genes located near the 3' end of VSH-1. Our results indicate the lowest mRNA copy number for \textit{svp101}, which is located near the 3' end of the VSH-1 sequence.
Northern blots of mRNA extracted from mitomycin C-treated cells revealed high molecular weight mRNA that encoded VSH-1 genes. Although the blots did not clearly resolve a large, 15 kb VSH-1 transcript that would be expected if VSH-1 genes were co-transcribed, the results argue against overlapping transcripts. The mRNA that hybridized to VSH-1 probes demonstrated a similar pattern for \textit{svp38}, \textit{svp101}, and \textit{lysV} genes. We would expect a significant difference in the size of mRNA that hybridizes to \textit{lysV} compared to \textit{svp38} if separate promoters drove overlapping transcription of these genes as was observed for the overlapping photosynthesis gene operons in \textit{R. capsulatus} (34).

Several factors likely contributed to the size heterogeneity of mRNA molecules observed in Northern blots. Fragmentation of mRNA during extraction and sample handling and the activity of ribonucleases contaminating the agarose gels or equipment would reduce the population of full-length transcripts. Our Northern blot results are similar to those obtained for the gene transfer agent of \textit{R. capsulatus} by Lang \textit{et al.} (16) who report that GTA genes are transcribed as an operon that is rapidly cleaved into smaller mRNA fragments. VSH-1 transcripts might similarly be degraded into smaller mRNA fragments. Furthermore, differences in the stability of these mRNAs would account for variations in the mRNA copy number observed for several VSH-1 genes in slot blots of \textit{B. hyodysenteriae} RNA. Such differences in mRNA stability have been reported for other operons and are reported to have a regulatory role in the expression of bacterial (19, 35) and bacteriophage genes (5).

Two major and two minor transcription start sites were located near the beginning of \textit{svp45} when \textit{B. hyodysenteriae} cells were treated with mitomycin C. Three of these start sites were located downstream from the \textit{svp45} initiation codon and may be important in producing truncated \textit{svp45} gene products or reducing the translation of \textit{svp45} relative to other VSH-1
genes. Multiple transcription start sites may also indicate the presence of several promoters that drive transcription of the VSH-1 operon. Seven promoters that can be independently modulated by a variety of environmental conditions have been identified for the \textit{rpsU-dnaG-rpoD} operon of \textit{E. coli} (17, 18). Three of these promoters are located internal to the \textit{dnaG} coding region and allow independent modulation of \textit{rpoD} expression. Wagner and colleagues (31) have demonstrated that shiga toxin gene expression is under the control of dual promoters. The toxin genes (\textit{stx1A} and \textit{stx1B}) are encoded within the genome of \textit{E. coli} phage H-19B. A promoter located adjacent to the genes drives expression under conditions of low iron and a separate promoter, located upstream, drives expression of the toxin genes as well as bacteriophage lysis genes in response to DNA damage. At the nucleotide sequence level, putative -10 and -35 regions compared to the relative positions of +1 transcriptional start sites revealed little information.

VSH-1 is pervasive in \textit{Brachyspira} species (28) suggesting that selective pressure has ensured its evolutionary maintenance as an inducible gene transfer agent. The ability of mitomycin C to induce bacteriophages is well known. Mitomycin C intercalates into DNA causing lesions that elicit a bacterial SOS response, making it a potent inducing agent for diverse bacteriophages (10, 12, 33). However, on an ecological level, mitomycin C and similar drugs do not represent "natural" stressors that \textit{B. hyodysenteriae} would likely encounter in the swine intestine.

Investigators have noted that DNA damage is caused by hydrogen peroxide and have demonstrated its capacity to induce bacteriophages (1, 4, 9). The finding that both H$_2$O$_2$ and mitomycin C induce production of VSH-1 lends further support to the idea that, like many bacteriophages, VSH-1 induction is tied into the bacterial SOS response. Moreover, H$_2$O$_2$ is
likely to exist in the environment inhabited by *B. hyodysenteriae*. Neutrophils are known to release H₂O₂ among other antimicrobial molecules. Harris and Lysons report that an excessive accumulation of neutrophils is commonly observed in and around capillaries near microscopic lesions on the lumenal surface of the cecum, colon, and rectum in dysenteric pigs (6).

It has recently been demonstrated that neutrophils are capable of inducing the lytic cycle of Stx toxin-encoding bacteriophages in enterohemorrhagic *E. coli* (30). By inhibiting neutrophil NADPH oxidase-dependent production of reactive oxygen intermediates and demonstrating that neutrophils inhibited in this way were incapable of inducing the Stx toxin-encoding bacteriophage, the investigators provided a specific link between bacteriophage induction and the oxygen free radicals released by neutrophils. It is possible that H₂O₂ and other reactive oxygen species produced by immune cells during the progression of swine dysentery disease could contribute to the induction of VSH-1. Such conditions would represent a "natural" source of bacterial stress that would promote VSH-1-mediated gene transfer among *B. hyodysenteriae* cells *in vivo*.

The high level of transcription identified by this study for several VSH-1 genes following viral induction suggests a strategy to exploit transcription of the VSH-1 *svp38* gene as a simple and sensitive test for inducers of VSH-1 and could allow us to test the hypothesis that VSH-1 is produced *in vivo*. 
**TABLE 1.** PCR primers and oligonucleotide probes used in these studies. A) Primers used for comparative RT-PCR of *flaA1* and *svp38* gene transcription and for identifying transcription start sites. B) Primers used for cloning VSH-1 and *B. hyodysenteriae* genes for *in vitro* transcription. C) Probes used for Northern and slot blot hybridization of VSH-1 and *B. hyodysenteriae* genes.

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*aTm calculations are based on the [2 (A+T)° + 4 (G+C)°] method.*
### TABLE 2. Oligonucleotide primers used for intergenic RT-PCR analysis of co-transcription of VSH-1 genes.

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*a Tm calculations are based on the \[2(A+T)° + 4(G+C)°\] method.

*b Amplicon designations refer to amplicons indicated in Fig 4.
FIG. 1. Northern slot blots of *B. hyodysenteriae* RNA. The membranes shown were hybridized with a probe for *lysV* (A) and *flaAl* (B). Column 1. *In vitro* transcribed RNA standards complementary to the gene specific probe. Calculated RNA copies indicated at the left of the figure. Column 2. RNA extracted from *B. hyodysenteriae* cultures at 1 h intervals after mitomycin C treatment. Column 3. RNA extracted from non-treated control cultures. RNA copies in time point samples were estimated by comparing hybridization signal intensities (column 2 and 3) with reference standards in column 1.

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FIG. 2. Transcript levels of VSH-1 genes in *B. hyodysenteriae* cells treated with mitomycin C. Symbols: *svp38* (■), *svp53* (□), *svp45* (▲), *lysV* (○), and *svp101* (●). The data represent the average VSH-1 mRNA copy number per cell determined for each gene. Error bars indicate the standard error of the mean of four measurements of copy number for each gene at each time point. * VSH-1 transcripts were not detected at these time points.
FIG. 3. Transcript levels of flaAl in B. hyodysenteriae cells treated with mitomycin C (▲) or control (△). The data represent the average mRNA copy number per cell for the B. hyodysenteriae flaAl gene. Error bars indicate the standard error of the mean of three measurements of copy number at each time point.
FIG. 4. Intergenic RT-PCR amplification of VSH-1 genes. The map of known VSH-1 structural genes (hatched arrows), the VSH-1 endolysin (solid arrow), putative holin (blank arrow), putative bacterial genes (shaded arrows) and unknown ORFs (stick arrows) is given above the figure. The direction of the arrows indicates transcriptional orientation. Amplicons generated from B. hyodysenteriae RNA extracted from mitomycin C-treated cultures, (lane 2, each image) or from genomic DNA (lane 3, each image) and their corresponding location (A – N) are given below the map. No Reverse Transcriptase controls for DNA contamination (lane 1, each image) were negative for all amplicons.
FIG. 5. Northern blots of *B. hyodysenteriae* RNA. *B. hyodysenteriae* total RNA (10 µg) was extracted from cultures 3.5 h after mitomycin C treatment and hybridized with probes for *svp38* (lane 1), *svp101* (lane 2) and *lysV* (lane 3). For comparison, total RNA extracted from a non-treated culture was hybridized with a probe for *fiaA1* (lane 4). Molecular marker sizes (kb) are indicated to the left of the figure.
FIG. 6. VSH-1 transcriptional start signals. Primer extension products from primer svp45R55 (Lane 1) were generated from total RNA extracted from *B. hyodysenteriae* cultures 3.5 h after mitomycin C treatment. The same primer was used to generate a sequencing ladder for comparison. The letters above sequencing lanes designate the dideoxy nucleotide that terminated each of the reactions. The markers to the right of the figure indicate the base (reverse complement) corresponding to each of the transcriptional start sites.
FIG. 7. Nucleotide positions of VSH-1 transcriptional start sites. The ATG start codon for *svp45* is in bold. Transcriptional start sites are in bold and underlined.
FIG. 8. Hydrogen peroxide-induced transcription of the VSH-1 svp38 gene. RNA extracted from *B. hyodysenteriae* cultures was amplified by duplex RT-PCR using oligonucleotide primers for *svp38* and *flaA1*. Amplicons are identified to the right of the figure. Lane numbers correspond to the time (h) after the addition of 300 µM (final concentration) H₂O₂ (panel A) or the corresponding time points in a control (no H₂O₂) culture (panel B). A 10-fold higher concentration of RNA was not amplified in the absence of reverse transcriptase enzyme (No RT lane), indicating no detectable DNA contamination.
FIG. 9. Chloramphenicol inhibition of VSH-1 transcription. RNA extracted from *B. hyodysenteriae* cultures was amplified by duplex RT-PCR using oligonucleotide primers for *svp38* and *flaAl*. Amplicons are identified to the right of the figure. Lane numbers correspond to the time (h) after the addition of mitomycin C and chloramphenicol (panel A) or to the corresponding time points in a control culture treated with mitomycin C only (panel B). A 10-fold higher concentration of RNA was not amplified in the absence of reverse transcriptase enzyme (No RT lane), indicating no detectable DNA contamination.
REFERENCES


Chapter 5. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

General Conclusions

VSH-1 genes have been sequenced and mapped to a contiguous 16 kb region of the \textit{B. hyodysenteriae} chromosome. Structural genes encoding both head-associated and tail-associated proteins were identified by comparing nucleotide sequences to N-terminal amino acid sequences determined for proteins from purified VSH-1 whole virions and VSH-1 tailless head structures. An endolysin, encoded by a gene (\textit{lysV}) located immediately downstream from the VSH-1 tail-associated \textit{svp28} structural gene, was shown to digest \textit{B. hyodysenteriae} peptidoglycan. The release of soluble reducing acetyl amino sugars from LysV-treated peptidoglycan indicated that LysV activity targets the peptidoglycan polysaccharide chain. A small open reading frame located downstream from the \textit{lysV} gene may encode a holin based on the hypothetical protein's size, hydrophobicity characteristics, and trans-membrane helix domains.

All of the VSH-1 genes identified to date encode typical bacteriophage late functions and share a common transcriptional orientation suggesting that these genes comprise the VSH-1 late operon. Consistent with this idea, intergenic RT-PCR, Northern hybridization, and primer extension data indicate that VSH-1 genes are co-transcribed and that transcription is initiated near the beginning of the first VSH-1 gene (\textit{svp45}). Measurements of VSH-1 transcription levels in mitomycin C-treated \textit{B. hyodysenteriae} cells revealed differences in the average mRNA copy number per cell for several VSH-1 genes and allowed for the possibility that transcription of several of the VSH-1 genes may be driven by separate promoters. Alternatively, our data would also support the possibility that differences in
mRNA stability might serve as a mechanism of post-transcriptional regulation of VSH-1 gene expression. Such a mechanism could also account for the high degree of VSH-1 mRNA size heterogeneity observed in Northern blots.

Mitomycin C-induced transcription of VSH-1 genes corresponds to a reduction in transcription of a *B. hyodysenteriae* housekeeping gene (*flaA1*). Treatment of *B. hyodysenteriae* cells with chloramphenicol in conjunction with mitomycin C inhibits transcription of VSH-1 genes as well as prevents reduction of *flaA1* transcription. These results indicate that a protein factor synthesized after *B. hyodysenteriae* cells are treated with mitomycin C is required for induced transcription of VSH-1 genes. Possibly the same factor or an additional protein factor is responsible for shifting transcription from bacterial genes to VSH-1 genes. A common element that could result in both observed changes in transcription would be an alternative RNA polymerase σ factor.

In addition to mitomycin C, H$_2$O$_2$ induced transcription of VSH-1 genes and also led to the production of VSH-1 virions and an increase in the frequency of transduction. The identification of H$_2$O$_2$ as an alternative VSH-1 inducing agent allows for the possibility that additional agents and conditions induce VSH-1 and that H$_2$O$_2$ or some other agent might induce VSH-1-mediated transduction *in vivo*. As both mitomycin C and H$_2$O$_2$ are known to damage bacterial DNA, other conditions that have similar effects on bacterial cells might induce VSH-1 production.

The studies reported in this dissertation correlated the production and release of VSH-1 virions with genes encoding virion structural proteins, transcriptional control over the production of VSH-1 virions, and a mechanism for virion escape. Further investigations will
be required to identify regulatory mechanisms such as repressors, transcription factors, and operators that control the switch from VSH-1 lysogeny to virion production.

**Recommendations for Future Research**

Previous attempts an insertional inactivation of VSH-1 genes have been unsuccessful and the reasons for this remain unclear but could be due to polar effects of the antibiotic resistance cassettes on transcription of downstream VSH-1 genes. The identification of a VSH-1 endolysin provides a new and possibly more suitable target for a gene knockout as it is located near the end of the VSH-1 transcript and is likely toxic to *B. hyodysenteriae* cells if not tightly regulated. A deleterious *lysV* mutation would allow direct assessment of the function of this gene in the release of VSH-1 virions. A *lysV* mutant created with a selectable marker that incorporates a strong transcriptional terminator might also prevent expression of holin genes. Such a strain, if defective in the ability to release VSH-1 virions, would also provide a genetic background to test what effect, if any, VSH-1 has on the survival of *B. hyodysenteriae* populations. Moreover, if induction of VSH-1 in this genetic background was non-lethal to *B. hyodysenteriae* cells, the strain could further be modified with a reporter gene linked to the VSH-1 promoter to act as an indicator strain for induction of VSH-1.

VSH-1 transcriptional start sites located near the beginning of *svp45* provide a basis for investigating cis-elements that might be involved in the regulatory circuit controlling VSH-1 transcription. Electrophoretic mobility shift experiments and nuclease protection assays could be designed to identify DNA sequences in the region near *svp45* that selectively bind to *B. hyodysenteriae* proteins.
The finding that *de novo* protein synthesis is required for VSH-1 transcription suggests that a strategy to identify proteins possibly involved in the induction of VSH-1. Incorporating radiolabel into newly synthesized proteins, if produced in sufficient quantities, would allow their detection by 2D gel electrophoresis following mitomycin C treatment of *B. hyodysenteriae* cells. Newly synthesized proteins detected in the 2D gels would provide material for amino acid N-terminal sequencing and could lead to the identification of transcription factors important to VSH-1 induction.

Finally, increased transcription of the VSH-1 *svp38* gene appears to be a sensitive means to screen for VSH-1 induction. An RT-PCR-based assay could be designed to identify additional agents or conditions that induce VSH-1 transcription. In addition to antimicrobial agents, the possibility that reactive oxygen intermediates produced by neutrophils induce production of VSH-1 could easily be tested and would lend indirect support to the hypothesis that VSH-1-mediated transduction between *B. hyodysenteriae* cells occurs *in vivo*. 
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