VSH-1, an inducible generalized transducing

bacteriophage of *Serpulina hyodysenteriae*

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Microbiology, Immunology and Preventive Medicine
Major: Microbiology

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1996

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GENERAL INTRODUCTION

The spirochete *Serpulina hyodysenteriae* is the causative agent of Swine Dysentery. The organism inhabits the large intestine of infected animals and produces disease symptoms which often include large amounts of mucus and blood in the infected animals feces. The disease is the source of significant economic loss in swine herds throughout the world.

There have been reports of bacteriophages associated with cells of *Serpulina hyodysenteriae* in cultures of the organism. However, there is little knowledge available concerning these bacteriophages or bacteriophages from other spirochetes. The goal of this research project initially was to determine whether or not *Serpulina hyodysenteriae* has bacteriophages associated with its cells and if it did isolate and characterize these bacteriophages. Furthermore, attempts would be made to determine whether or not any *Serpulina hyodysenteriae* bacteriophages play a role in the pathogenesis of the organism. After isolation and characterization of a bacteriophage (VSH-1) was completed, it became apparent that the phage was not typical of most bacteriophages and linking it directly to pathogenesis could prove to be impossible. VSH-1 did not replicate in cells and evidence suggested that VSH-1 particles packaged large amounts of host chromosomal DNA. Therefore, the second research goal was changed to determining whether or not VSH-1 was a generalized transducing phage, capable of transferring host cell genes between *Serpulina hyodysenteriae* cells.

Thesis Organization

This thesis is written in an alternate thesis format and includes two manuscripts. The first manuscript has been published in the journal FEMS Microbiology Letters and was written in the format used by the Federation of European Microbiological Society. The second manuscript will be submitted to the Journal of Bacteriology and has been written in the format used by the American Society for Microbiology. In addition, the literature review which precedes the manuscripts is also written in the format used by the American Society for Microbiology. A general summary and discussion follow the manuscripts. References cited in the literature review follow the summary and discussion. The candidate, Samuel B. Humphrey, was the principal investigator for these studies.

LITERATURE REVIEW

Swine Dysentery and *Serpulina hyodysenteriae*

Swine dysentery is a diarrheal disease primarily affecting younger pigs during the growing-finishing period. It causes a severe mucohemorrhagic diarrhea in infected animals, from herds throughout the world, leading to significant economic losses for producers (28). In 1971 and 1972 an anaerobic spirochete was shown to be the causative agent of swine dysentery (24,26,93). Harris and colleagues characterized and named the organism *Treponema hyodysenteriae* (26). It has since been renamed *Serpulina hyodysenteriae* (84,87).

Transmission of *S. hyodysenteriae* occurs through ingestion of feces from an infected animal. In naturally exposed pigs the incubation period for swine dysentery can vary but usually occurs within 10 - 14 days after exposure of the animals. In experimental challenges incubation may be reduced to as little as 24 hours (28). Once ingested the organism colonizes the animals colon and produces lesions in the epithelial lining. *S. hyodysenteriae* has been shown to be attracted to gastric mucin by chemotaxis and is effective at moving through mucus (38,58). The organism can therefore associate with epithelial cells and invasion may not be necessary for the formation of lesions (25). The entire pathogenesis of this organism has been tied to the formation of these enteric lesions (44). Lesion formation leads to the failure of the epithelial transport mechanisms resulting in fluid loss (colonic malabsorption) (7). In severely infected animals death may result from dehydration, acidosis, or

hyperkalemia. Up to 50% mortality in experimentally infected animals has been reported (28). Animals that survive swine dysentery and show no clinical signs can carry *S. hyodysenteriae* and transmit it to other animals for up to 70 days (79).

Swine dysentery usually spreads slowly through a herd. Clinical signs may vary in intensity but in most animals the sequence is the same. Infected animals start out with loose yellow to gray stools and then large amounts of mucus and flecks of blood become evident in their feces. As the disease progresses watery bloody stools become more apparent as the pig becomes gaunt and emaciated. A characteristic arching of the back can be seen in most infected animals. The disease can be cured by treatment with a number of therapeutic agents (28).

Proper diagnosis of swine dysentery depends upon the detection of *S. hyodysenteriae*. Detection methods often rely on the isolation of the organism from rectal swabs on selective growth media. Trypticase soy agar supplemented with 5% bovine blood and 400 µg/ml spectinomycin hydrochloride is an effective, commonly used medium (80). *S. hyodysenteriae* will grow with a characteristic pattern of strong beta-hemolysis when plates are incubated at 39°C under an anaerobic atmosphere. A problem with this selective culture method is variable sensitivity since culturing is dependent on the number of organisms being shed by the animal in their feces. Carrier pigs do not always shed organisms at detectable levels (27). In addition, other nonpathogenic spirochetes (*Serpulina innocens*) inhabit the gastrointestinal tract of swine and produce a weakly hemolytic pattern of growth on trypticase soy blood agar under the same culture conditions (42). The distinction between a strong hemolytic pattern and a weak hemolytic pattern is not easily made every time; therefore, *S. innocens* can be confused

with *S. hyodysenteriae* as can a number of other uncharacterized spirochetes which have been

isolated from swine (43,46,50,94). Once a spirochete is isolated a number of methods can be

employed to determine whether or not it is *S. hyodysenteriae*. These methods include

ribotyping, growth inhibition tests, enzyme analysis, protein comparisons, 16s rRNA sequence

comparisons, and DNA-DNA reassociation (32,35,48,87,89). Methods like these can be

costly and time consuming. However, a dependable method for quick direct detection of *S.*

hyodysenteriae in pig feces without culturing the organism has not been developed. A

fluorescent antibody test was developed but the specificity of the test is questionable

(31,36,53). Serological tests for swine dysentery have been developed and were found to be

useful in determining the prevalence of swine dysentery but not dependable for identifying

animals which are infected (20).

S. *hyodysenteriae* is an oxygen tolerant, anaerobic spirochete. It stains gram-negative

like all spirochetes, has a loose outer membrane covering its protoplasmic cylinder, and has

14-26 periplasmic flagella. These flagella are inserted into both ends of the periplasmic

cylinder with the free ends of the flagella overlaping in the middle of the cell. The bacteria

have an overall length of 6-8.5 μm and a diameter of 320-380 nm. They are motile and have

hemolytic activity (28). It is thought that this hemolytic activity contributes to the virulence of

S. *hyodysenteriae* in pigs. Four different hemolysins have been isolated thus far with

molecular weights of 19 kDa, 26.9 kDa, 68 kDa, and 74 kDa (40,45,59,73). The 19 kDa

hemolysin has been shown to be cytotoxic to pig epithelial cells and to various tissue culture

cells (39,52). Nine different serotypes for *S. hyodysenteriae* have been identified from isolates

from swine dysentery outbreaks throughout the world. The serotypes are based on antibody

specificity to lipopolysaccharide antigens of the outer membrane of the *S. hyodysenteriae* cells (8,51,54).

　　S. hyodysenteriae can be cultured in liquid broth. Kinyon and Harris used trypticase soy broth (without dextrose) supplemented with 10% fetal calf serum under an $H_2:CO_2$ (50:50) atmosphere to grow cells (41). Lemcke and colleages used trypticase soy broth with a supplement of 10% rabbit serum for growing cells under an $N_2:CO_2$ (50:50) atmosphere (47). It was determined that the addition of serum provided cholesterol needed for growth by *S. hyodysenteriae* cells since the spirochete cells grew in serum free media supplemented with cholesterol (49,82,85). Cholesterol is converted to cholestanol by *S. hyodysenteriae* cells and is incorporated into cell lipids (82). In addition, if the basal media is depleted of lipids, phosphotidylcholine supplements were found to be necessary for growth (85). In 1988, Stanton and Lebo examined the effects of different culture conditions on the growth of *S. hyodysenteriae* cells (88). They determined that brain heart infusion broth supplemented with 10% heat-inactivated fetal calf serum gave the highest cell yields in the shortest time when the cultures were vigorously mixed under an $N_2:O_2$ (99:1) atmosphere. The addition of oxygen to the atmosphere of cultures enhanced growth. This is possibly due to the activities of NADH oxidase (83,86). Growth substrates for *S. hyodysenteriae* include glucose, fructose, sucrose, galactose, trehalose, N-acetyl-glucosamine, glucosamine, mannose, maltose and pyruvate. During growth the organism produces H_2, CO_2, acetate, and butyrate (88).

Spirochetes and Genome Structure

The order *Spirochaetales* encompasses a diverse group of helical shaped, gram-negative motile bacteria. Representative species may be free living or host-associated with some species being pathogenic. *Spirochaetales* is divided into two families, *Spirochaetaceae* and *Leptospiraceae*. The family *Spirochaetaceae* consists of the genera *Treponema*, *Serpulina*, *Spirochaeta*, *Cristispira*, and *Borrelia*. The family *Leptospiraceae* consists of the two genera *Leptonema* and *Leptospira* (16,64). This classical taxonomy of organisms within the *Spirochaetales* is based on phenotypic traits of the organisms. In 1991, Paster and his colleagues analyzed the 16s rRNA sequences of representative organisms from each of the spirochete genera and found their phylogenetic structure to be in approximate agreement with accepted taxonomy for spirochetes (64). They concluded that the spirochete ultrastructure is definitive for the spirochete group. In addition, they concluded that the spirochetes represent a monophyletic bacterial phylum based on the analysis of specific sequence signature elements.

In spite of their ultrastructures and phylogenetic relatedness spirochetes vary greatly in genomic structure. *Borrelia burgdorferi* has a linear chromosome of approximately 1,000 kb and several extrachromosomal elements varying in size and conformation; *Leptospira interrogans* has a genome comprised of a 4,400 kb circular chromosome and a 350 kb circular chromosome; and *Treponema pallidum* possesses a single circular chromosome of approximately 900 kb (75,98,102). In addition, an extrachromosomal plasmid of 2.6 kb has been isolated and characterized from another member of the *Treponema* genus, *Treponema denticola* (34). In 1994, Zuerner and Stanton examined the physical and genetic makeup of

the *S. hyodysenteriae* genome. They reported that *S. hyodysenteriae* has a single circular chromosome of 3,200 kb (103). Even though plasmids in *S. hyodysenteriae* have been reported, their studies did not confirm this (see below).

The extraction of extrachromosomal nucleic acid from *S. hyodysenteriae* and *S. innocens* cultures was reported in 1986 by Joens and his colleagues and then again in 1992 by Combs and his co-workers (18,37). The band of extrachromosomal nucleic acid migrated in agarose gels to a position corresponding to 6-8 kb for that of linear dsDNA. Joens and his colleagues preliminary reports indicated that the extrachromosomal DNA could be cut with some restriction endonucleases. Furthermore, Combs and his colleagues determined that after digestion of DNA samples with restriction endonucleases, samples which contained the plasmid DNA were indistinguishable from those samples that did not contain any plasmid DNA when the samples restriction endonuclease patterns were examined by agarose gel electrophoresis. In 1994, Adachi and his colleagues reported the isolation of 3 plasmids from *S. hyodysenteriae* - like and *S. innocens* - like spirochetes (3). The plasmids they identified were 1.6 kb, 2.6 kb, and 40 kb in size. The isolates that were like *S. hyodysenteriae* were beta-hemolytic and produced lesions in the ceca of CF1 mice.

Natural gene transfer systems for spirochetes have neither been identified nor characterized. However, the existence of DNA filled membrane vesicles on the surface of *B. burgdorferi* cells has been reported and indirect evidence of lateral gene transfer has been obtained for *Borrelia* (22,55). Transformation of *S. hyodysenteriae* cells by electroporation to form stable recombinants has been accomplished (71,95). In electroporation experiments involving *S. hyodysenteriae* recombinants have been obtained at a frequency

of 5 recombinants per 500 ng DNA when electroporating 10^{10} cells.

Bacteriophages

General

The literal translation of the word bacteriophages is "bacteria-eaters". They are a diverse group of entities commonly referred to as bacterial viruses (66). There are fourteen families of bacterial viruses varying greatly in morphology and nucleic acid content (2). The double-stranded DNA containing phages represent the most studied groups of phages and have particles that are composed of 6 to 40 structural proteins (17). Three basic theories on the evolutionary origin of bacteriophages have been developed (66). The first is that bacteriophages are specialized descendants of the earliest forms of life. The second one is that bacteriophages represent degenerate organisms. The third and most widely accepted theory is that bacteriophages are a result of various cell genes that became infectious. The third theory envisions bacteriophage genes originating from chromosomal segments, plasmids, and transposons. This view has gained support based on the potential of recombination mechanisms for creating DNA fusions (15).

Bacteriophages have been extensively studied in relatively few bacterial species. Successful phage infections may result in immediate production of progeny virions followed by cell lysis or the infecting bacteriophage may produce what is termed a temperate response. During the temperate response the lytic cycle is suppressed and phage DNA (prophage) is replicated along with host cell DNA at each cell division. Usually, the phage DNA integrates

into the host chromosomal DNA. This lysogenic state may or may not be maintained in infected cells. Spontaneous reversion of a prophage to a lytic state may occur naturally at a certain rate or the prophage may be induced to convert to a lytic state at a higher rate by treating cells with chemical mutagens, UV irradiation of cells, or thymidine starvation (6,10). In *Escherichia coli* these treatments cause DNA damage which leads to increased *recA* protease activity resulting in the cleavage of the λ phage repressor molecule. Both lytic phage and temperate phage in a lytic state are capable of transferring bacterial DNA from one cell to another (transduction) and are therefore considered to be involved in natural gene transfer in bacterial populations (10).

Transduction can be either specialized or generalized. Specialized transduction occurs when a bacteriophage undergoes a temperate response and incorporates its DNA at a specific site in the host DNA. When reactivated, bacterial DNA adjacent to the prophage is excised, replicated and packaged into virions along with the phage DNA. When these virions infect another cell the bacterial DNA is then transferred. Generalized transduction occurs during lytic infections or after temperate phages have been reactivated. During generalized transduction phage packaging mechanisms attach to and package bacterial chromosomal DNA instead of phage DNA. Once released from lysed cells the particles containing bacterial DNA then transfer this DNA to other cells. In generalized transduction any bacterial gene may be transferred since DNA packaging is often random (10). Transduction frequencies for generalized transducing phages (Table 1) range from 10^{-4} to 10^{-9} transductants per infective phage particle depending on the phage and the marker being transduced .

Table 1. Transduction frequencies for generalized transducing phages.

Bacteriophage	Host species	Frequency transductants/ p.f.u.	Reference
P1	*Escherichia coli*	$10^{-4} - 10^{-5}$	100
P22	*Salmonella typhimurium*	$10^{-6} - 10^{-9}$	100
T4	*Escherichia coli*	$10^{-5} - 10^{-7}$	101
SH10	*Streptomyces hygroscopicus*	$10^{-6} - 10^{-8}$	92
ΦVP253	*Vibrio parahaemolyticus*	$10^{-5} - 10^{-7}$	60

Bacteriophages can play a role in the pathogenesis of some bacteria. Virulence factors have been shown to be phage-encoded genes for *Corynebacterium diphtheriae*, *Clostridium botulinum*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Escherichia coli* (11). In these cases the virulence factor involved is an exotoxin. Other virulence factors can be difficult to define and may be part of complex multigene functions such as antigenic switching mechanisms or enzyme secretion. Therefore it is understandable that the simplest of virulence mechanisms, exotoxin production, has been the easiest to demonstrate the involvement of

bacteriophage genes.

Bacteriophages have been observed to play a role in the population dynamics of bacteria. They participate in the microbial food web as lytic agents in various bacterial populations (13). The potential for using bacteriophages to control disease exists. In 1987, Smith and his colleagues reported using bacteriophages to control experimentally induced *E. coli* diarrhoea in calves (78). They were able to cure infected calves by giving them a single dose of 10^5 phage particles or by spraying the litter in the calves' rooms with aqueous phage suspensions. Soothill demonstrated the potential benefits of having phages present during an infection of *Actinetobacter baumanii* and an infection of *Pseudomonas aeruginosa* (81). He was able to protect experimentally challenged mice from infections by administering doses of lytic phages specific for *Actinetobacter baumanii* or *Pseudomonas aeruginosa* at the time of challenge.

Defective Bacteriophages

Several bacterial species contain within their genomes genes for the production of noninfectious phage-like particles. Many of these particles have bactericidal properties (12,23). Among these particles is a well studied group of defective phages induced from bacteria belonging to the genus *Bacillus*. In 1964, Seaman et al. reported the induction of bacteriophages from strains of *B. subtilis* (77). The particles they were able to induce, designated PBSX, were capable of killing sensitive strains of *B. subtilis* but appeared to be incapable of self-reproduction either in the recipient strain or in the original host. These

PBSX particles were shown to be inducible in the absence of DNA synthesis, suggesting that

the DNA packaged inside the phage particles originated from the bacterial chromosome. To

test this hypothesis, they extracted DNA from the PBSX particles and attempted

transformation of *B. subtilis* for three randomly chosen bacterial markers. The markers,

arginine, histidine, and bryamycin were successfully transferred. Other particles induced from

strains of *B. subtilis* with similar properties to PBSX were described in 1964 and 1965

(21,33,91). Okamoto et al., in 1968, speculated that all of these particles may be identical and

attempted to better define the phage PBSX as the defective phage obtained by mitomycin C

induction of *B. subtilis* strain 168 (62,63). In addition, they further characterized PBSX.

They described the phage particle as consisting of a hexagonal head (41 nm diameter) and a

tail (192 nm x 18.5 nm) with 52 to 53 striations with a spacing of 3.5 to 4 nm. The particles

had a buoyant density in CsCl of 1.375 g/cm^3. DNA packaged within the phage heads was

determined to have a molecular weight of 8.4 x 10^6 daltons (approx. 13 kb) They confirmed

that the majority of the DNA packaged within the heads of PBSX was bacterial in origin and

not phage specific. In DNA-DNA hybridization experiments DNA extracted from phage

particles behaved identically to that of chromosomal DNA, i.e. PBSX DNA hybridized to

immobilized bacterial chromosomal DNA in the same manner as the chromosomal DNA

hybridized to itself. In the absence of DNA synthesis DNA was still packaged after induction,

and DNA obtained from the PBSX particles was used to transform bacteria for several

markers. In addition, they showed that as much as 50% of prelabeled bacterial DNA is

incorporated as uniform fragments (13 kb) into PBSX particles after induction with

mitomycin C. The killing properties of PBSX were examined. PBSX particles would absorb

to sensitive strains cell wall and kill those strains but essentially would not absorb to the cell wall of the host strain *B. subtilis* 168. In experiments similar to those of Hershey and Chase, Okamoto and colleagues demonstrated that PBSX particles were not capable of injecting their DNA into cells and were therefore not transducing particles. Some points they raised were that until their work on PBSX, phage which package host DNA were reported to do so at low levels compared to that of true phage DNA. In addition, until their work all known cases of phage replication required phage specific DNA synthesis as a prerequisite to phage production and they did not see this with PBSX.

PBSX-like defective phages, inducible with mitomycin C, have been reported for a number of strains of *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus* and *B. subtilis* (90 and references therein). However, further research into the nature of these defective phage particles has focused on PBSX induced from *B. subtilis* 168. Thurm and Garro isolated and characterized prophage mutants for PBSX (96). They were able to identify a regulatory mutant and several mutants defective for PBSX phage capsid formation and map them between *arg*C and *met*C. They also observed that there was a 10-fold increase in *met*C marker frequency present in cells after exposure to mitomycin C suggesting that derepression of PBSX may result in replication of the chromosomal region adjacent to the prophage genes.

Anderson and colleagues were able to clone a 5.4 kb DNA fragment from the region of the PBSX prophage which allowed for self replication of the construct in *B. subtilis recE*⁻ cells (5). They believed that this fragment contained the origin of replication for the PBSX prophage. When this plasmid construct was put into *recE*⁺ cells homologous recombination took place at the prophage site. However, if the plasmid construct was put into *recE*⁺ cells

which contained a deletion for the prophage region believed to code for the origin of replication the construct was maintained as a self replication plasmid.

Anderson and Bott focused on DNA packaging of bacteriophage PBSX (4). They confirmed that PBSX particles package 13 kb fragments of mostly chromosomal DNA. However, packaging was not completely random as enhancement of some bands of DNA was detectable by ethidium bromide staining. Also genetic data they collected suggested some preferential packaging of some regions of the chromosome. DNA from the chromosome site of prophage PBSX DNA was packaged less frequently than other regions on the chromosome. Based on evidence obtained packaging occurred by the headful mechanism involving a phage associated nuclease. It was suggested that the recognition system for PBSX packaging was possibly deficient in its ability to recognize specific sequences therefore explaining why PBSX is capable of packaging so many types of molecules (different genome segments). If plasmids were present at the time of induction unusual replication intermediates of the plasmid molecules (concatemers) were packaged within the phage heads as 13 kb fragments. This form of the plasmid was packaged in small proportions compared to a monomeric form of the plasmid. It was suggested that the appearance of these concatemers relied on a rolling circle replication structure involving some function of the PBSX prophage which was deleted in one of the strains they used in their studies.

Insight into the number of proteins necessary for *B. subtilis* cells to make for the production of mature PBSX particles was obtained in 1984 by Mauel and Karamata (57). Analysis of the purified PBSX particles revealed a total of 26 polypeptides, 3 belonging to the head and the rest to the tail structure. In, addition, the synthesis of 11 additional proteins was

detected after treatment of cells with mitomycin C, seven of which were shown to be under control of the phage repressor. They hypothesized that if all these peptides were necessary for replication of the phage the DNA packaged within the phage heads would need to be approximately 54 kb, four times the actual amount packaged (13 kb), for the phage to be complete (nondefective). This, they explained, could be the reason for the defective nature of PBSX. Wood et al. cloned a region (33 kb) of the *B. subtilis* chromosome from the *met A-metC* region (PBSX mapped position) and analyzed it by insertional mutagenesis with an integrative plasmid (99). Their work suggested that the minimal size of an operon encoding PBSX structural proteins would be 19 kb. In addition, they concluded that the minimum PBSX genome size would be at least 22 kb based on their mutagenesis studies, thus giving support to the hypothesis that PBSX phage head capacity explains the defective nature of the phage.

Recently a defective phage from a strain of *B. natto* has been induced with mitomycin C (97). This phage, PBND8, had the same morphology as phage PBSX but had a head diameter (39 nm) which was smaller than that of PBSX. Unlike the PBSX-like phages, PBND8 packages 8 kb fragments of host chromosomal DNA not 13 kb fragments. PBND8 exhibited killing activity when plated with other *Bacillus* strains. The host chromosomal DNA appeared to be randomly packaged since no difference could be distinguished between restriction enzyme digestion patterns for phage DNA and Chromosomal DNA below 8 kb. Hybridization results supported the conclusion that DNA is packaged randomly within PBND8 heads and represents the entire chromosome.

Bacteriophages and Spirochetes

The first report of a bacteriophage associated with spirochetes in cases of swine

dysentery was in 1971 (68). Ritchie and Brown surveyed the contents of the colon and swabs

from a dysenteric pig under the electron microscope. They observed a "Borrelia-like"

organism which appeared to be actively infected by a bacteriophage. The bacteriophage they

reported had a icosahedral head with a diameter of 100 nm and a contractile tail 180 nm by 18

nm. In 1974, Saheb reported the existence of bacteriophages in a pure culture of a spirochete

isolated from the colon of a dysenteric pig (72). Saheb considered this spirochete a species of

Treponema. The phage particles were detected in a 14-day old deep agar culture after agar

plugs were prepared for electron microscopic examination. The phage particles had isometric

heads of 106 nm in diameter and contractile tails 65-130 nm by 34 nm. In both of these two

studies (68,72) it is not clear whether or not the spirochetes were in fact the causative agent of

swine dysentery, *S. hyodysenteriae*, and whether the phage originated from the spirochete in

question. However, in 1978 Ritchie et al. searched for bacteriophages in pure cultures of *S.*

(Treponema) hyodysenteriae obtained from J.M. Kinyon's world-wide collection (70). They

observed one common phage type in 18 different cultures of *S. hyodysenteriae*. Some of these

strains, 4/71 and B256, would later be reclassified into a separate species, *S. innocens* (42).

The phage type that they described was different than the two previous bacteriophages

reported to be associated with a spirochete from a dysenteric pig. The bacteriophage had a

typically round head with a diameter measuring between 45 nm and 50 nm. The tail appeared

to be simple with the dimensions of 9 - 11 nm x 65 - 70 nm. In addition the particles had a

baseplate, which was difficult to resolve, 40 nm in diameter with 6 fine fibres that attached to the outer membrane of the spirochete cells. By direct immuno-electron microscopy they determined that the phages seen in one culture were serologically identical to phages seen in other cultures regardless of the geographical origin of the isolate. Antibodies used in the study were produced in rabbits against cultures of isolate B204. Ritchie and his colleagues went on to speculate that it was unlikely that the virulence of *S. hyodysenteriae* was controlled by the phage since both pathogenic and nonpathogenic (*S. innocens*) cultures contained the phage. This speculation was made under the assumption that the phages observed in their study were identical. Furthermore, they stated that the pathogenicity of *S. hyodysenteriae* could be affected by the phages since virus lytic growth could reduce the number of viable cells in a culture. A year later in 1979, Berthiaume and colleagues reported observing a bacteriophage-like particle in the cytoplasm of spirochetes during an electron microscopy study of spirochetes in the intestinal flora of pigs (9). They identified the spirochete as belonging to the *Treponema* genus based on arrangement of fibrils and the diameter of the cells. The bacteriophage-like particles they observed were hexagonal with a diameter between 40 - 45 nm. No extracellular structures could be identified as phage-like particles. All particles they observed were within the cytoplasm of the spirochete.

Bacteriophages observed with other spirochetes have been reported. An early observation of bacteriophages associated with a spirochete was in 1967 (30). During an investigation into the possible significance the bacterial cell wall may play in the nutrition of sheep, Hoogenraad and colleagues performed an electron microscopy survey of bacterial types present in the rumen contents of fistulated sheep. During their investigation they

detected groups of spirochetes with numerous bacteriophages attached to their cell outer

membrane. They noted that some of the phages heads seemed to be empty, containing no

nucleic acid. No isolation of the spirochete or phages was attempted.

Then in 1969, Ritchie and Ellinghausen reported bacteriophage-like entities associated

with a leptospire cell (69). These phage-like entities were observed by electron microscopy of

spirochete cells of an unclassified leptospire (isolate 3055) culture supplied by L. E. Hansen

from the University of Illinois. The viruses were seen attached to the outer membrane of the

spirochete cells, associated with cellular debris, and within the cells. For their studies 2 or 3

day old static cultures were preferred. The particles they observed resembled the phages in

the "T4-like phages" genus from the family *Myoviridae* (1). Additional electron micrographs

of these virus particles were published in 1976 by Ritchie after the organism had been

designated serotype *illini* (67).

Bacteriophage-like particles were induced from *Treponema phagedenis* biotype Reiter

cells after the cells were treated with the mutagen mitomycin C (56). The mitomycin C was

added at a concentration of 5 µg/ml to cells in the exponential phase of growth. After 4

hours of continued growth, a slow decrease in culture optical density took place. Intact cells

from the cultures were removed by centrifugation (3,000 x g, 10 min.) and the supernate was

then recentrifuged. It was from the pellet obtained from the centrifuging (23,000 x g, 30

min.) of the supernate that they observed phage-like particles. Round particles of two

different diameters (62 nm and 51 nm) were seen under the electron microscope. Neither of

these had any tail structures attached to them. Masuda and Kawata did not include electron

microscopic examination of untreated cultures as a control; therefore, it is unclear as to

whether these spherical structures exist in normal cultures of this spirochete.

Patel and colleagues observed bacteriophages attached to the cell of a thermophilic anaerobic spirochete from a New Zealand hot spring (65). These observations were made by looking at an enrichment culture containing rifampicin (10 µg/ml) from one of the hot springs included in the study. The phage particles consisted of a head with approximately a 100 nm diameter and a tail approximately 150 nm in length. Phage particles were not detected in their pure culture of isolate Rt3-BS1.

While attempting to isolate spirochetes from the equine caecum Davies and Bingham observed phage particles associated with spirochete-like organisms (19). They were unable to culture any of the spirochetes detected in the study beyond the primary isolation. The phage they observed were either tailed and attached to the outer membrane of the spirochete cell or were small (32 nm diameter) isometric virus-like particles packed within the cells.

The spirochete *Borrelia burgdorferi* is a well studied organism and has been shown to be the causative agent of Lyme disease (14). In 1983 Hayes and his colleagues reported that a bacteriophage was present inside the cells and attached to the outer membrane of cells in a culture of spirochetes shown to induce Lyme disease (29). Through transmission electron microscopy studies it was determined that this phage had an elongated head (40-50 nm long by 17-20 nm wide) and tail (50-70 nm long by 8-10 nm wide). The phage particles attached to the outer membrane of the spirochetes were observed both full of nucleic acid and devoid of nucleic acid. Neubert and colleagues, in 1993, concluded that a *B. burgdorferi* strain they were working with harbored at least two prophages after they treated actively growing cells with the gyrase inhibitor ciprofloxacin (61). The phages observed in their studies differed in

morphology from the phage reported by Hayes and his colleagues. One of the phages was composed of a isometric head 30 nm in diameter and a contractile tail 50-64 nm in length and the other was composed of a 30 nm isometric head and a long, 115-130 nm, noncontractile head. These phages were observed both extracellularly and intracellularly as whole phage particles or as heads or tails. These phages were observed at subinhibitory levels of ciprofloxacin and were never detected in untreated control cultures. Therefore, it is likely Neubert and co-workers were working with a lysogenic strain of *Borrelia*. Neubert and his colleagues speculated that the Hayes group could have observed phages in their cultures of *Borrelia* due to adverse culture conditions and that temperate phages could explain why logarithmic growth of *Borrelia* cells suddenly stop and cell numbers decrease before new growth is observed. Schaller and Neubert followed up their original observations that prophage can be induced from cells of *B. burgdorferi* with ciprofloxacin by treating two more isolates with this antibiotic (76). Both of the isolates they tested had inducible phages after cells were treated with subinhibitory levels of ciprofloxacin.

The first isolation and characterization of spirochete phages was reported by Saint Girons and colleagues in 1990 (74). They isolated and characterized three phages, from the sewage waters around Paris. The viruses where capable of lytic growth (plaque formation) in cultures of the saprophytic aquicole bacterium *Leptospira biflexa* (serovar *patoc*). No lysogenic state could be demonstrated for any of the phages since hybridization studies, probing host DNA with phage DNA, gave negative results. The three leptophages LE1, LE3, and LE4 were morphologically identical and composed of a polyhedral head (85nm diameter) and a contractile tail (100 nm by 25 nm), placing them in the A-1 group of phages. The three

phages LE1, LE3, and LE4 packaged linear DNA, 60 kb, 50 kb, and 50 kb in size, respectively. Restriction enzyme digestion patterns were distinct for each phage. The work undertaken by Saint Girons and colleagues was done in hopes that a genetic vector could be developed. Thus far, that has not been accomplished.

MITOMYCIN C INDUCTION OF BACTERIOPHAGES

FROM *Serpulina hyodysenteriae* AND *Serpulina innocens*

Sam B. Humphrey, Thad B. Stanton, and Neil S. Jensen

Abstract

A prophage was induced from cells of the pathogenic spirochaete *Serpulina hyodysenteriae* using mitomycin C. Five to seven hours after mitomycin C was added (8 μg/ml, final conc.) to *S. hyodysenteriae* B204 cultures in BHIS broth (OD_{620}=0.9) cell lysis was detected as a decrease in culture optical density. Bacteriophage particles attached to whole cells and to cell debris were detected by electron microscopic analysis of negatively stained (2% PTA, pH 7.0) bacteria harvested by centrifugation from mitomycin C treated cultures. The phage particles consisted of a head (45 nm diameter) and a tail (64 nm x 9 nm). Bacteria from untreated cultures lacked phages detectable by electron microscopy. The appearance of bacteriophage particles in mitomycin C treated cultures correlated with the appearance of extrachromosomal DNA, 7 - 8 kb in size as estimated by agarose gel electrophoresis, in DNA preparations from treated *S. hyodysenteriae* cells. When cultures of

other *S. hyodysenteriae* strains (B78, B169, A-1, B8044, B6933, Ack300/8, R-1) and *S. innocens* 4/71 in BHIS were treated with mitomycin C (8-15 µg/ml, final conc.), phages similar in morphology and size to the *S. hyodysenteriae* B204 phage were induced.

Introduction

The order *Spirochaetales* consists of a diverse group of organisms, including several which are human or animal pathogens. There is little known about the bacteriophages of spirochaetes. An early report of bacteriophages associated with a spirochaete by Hoogenraad and colleagues in 1967 [1] described virions attached to a cell of an unknown spirochaete in sheep rumen fluid. Since then bacteriophages have been observed in cultures of both free living and host-associated spirochaetes [2-9, and references therein]. Bacteriophage-like particles were observed by Katawa and Masuda after treating cultures of *Treponema phagedenis* biotype Reiter with the mutagen mitomycin C [7]. More recently, prophages were induced when *Borrelia burgdorferi* cells were treated with ciprofloxacin, an inhibitor of DNA gyrase [4,5]. In 1990, Saint Girons and colleagues described the isolation and characterization of three lytic phages of *Leptospira biflexa* [2]. To the best of our knowledge these lytic phages of *Leptospira biflexa* are the only spirochaete bacteriophages that have been isolated and characterized.

The spirochaete *Serpulina (Treponema) hyodysenteriae* [10] is the causative agent of swine dysentery, an economically important disease of swine [11]. Bacteriophages have been

detected by electron microscopy in cultures of *S. hyodysenteriae* cells by Ritchie and

colleagues [6]. The bacteriophages they observed in different *S. hyodysenteriae* strains were

morphologically identical and appeared spontaneously in bacterial cultures. Unfortunately,

neither the conditions necessary for the appearance of these phages in culture nor the

properties of these phages were further studied. The objective of these studies was to

determine whether or not inducible prophages are present in *S. hyodysenteriae* cells.

Materials and Methods

Bacterial strains and culture conditions

S. hyodysenteriae strains and *S. innocens* 4/71 used in these studies have been

described [12]. The *S. hyodysenteriae* strains represent seven serotypes [13]. *S. innocens* is a

non-pathogenic spirochaete from the swine intestine [10,14]. Spirochaetes were cultured in

tubes of BHIS broth (Difco brain heart infusion broth with 10% calf serum; 7ml/tube). The

cultures were stirred at 39°C beneath an initial $N_2:O_2$ (99:1) atmosphere [15].

Mitomycin C induction of prophages

Mitomycin C (M-0503; Sigma Chemical Co., St. Louis, MO, USA) was added to

BHIS broth cultures of spirochaete cells in exponential growth phase ($OD_{620} = 0.9$, water

blank, pathlength 1.8cm, approx. 1×10^8 cells/ml). For each spirochaete strain a mitomycin C

concentration (8-15 μg/ml, final conc.) was selected so that a gradual decrease in culture

optical density (0.2-0.4 units; Bausch and Lomb Spectronic 70 spectrophotometer) occurred within 5-7 hours after addition.

Electron microscopy

After a decrease of 0.2-0.4 OD units in the mitomycin C treated cultures, bacterial cells from both treated and untreated (control) cultures were harvested by centrifugation (1.5 ml; 4,000 x g, 5 min., 4°C). The pelleted cells were resuspended in 50 µl of 5 mM sodium phosphate buffer (pH 7.0) and mixed with an equal volume of phosphotungstic acid (2%, pH 7.0). Samples (10 µl) were then deposited on parlodion-coated, carbon-reinforced, 200 mesh copper grids and examined using a Philips model 410 electron microscope (80 kV). Photographs were taken on Kodak Astar thickbase 4489 film.

Electrophoresis of nucleic acid extracted from induced/ noninduced cells

Five culture tubes of *S. hyodysenteriae* B204 were treated with mitomycin C to induce prophage. At 3, 3.5, 4, 4.5, and 5 hours after the addition of mitomycin C, the tubes were removed and placed on ice. A control culture (no mitomycin C) was placed on ice 5 hours after the other tubes were treated.

Cells from 2 ml of culture were harvested by centrifugation (5,000 x g, 5 min.) and the nucleic acid extracted by using a modified Marmur technique [12,16]. After harvesting the precipitated nucleic acid by spooling with a Pasteur pipette, any remaining nucleic acid was pelleted by centrifugation (10,000 x g, 5 min., 4°C) and harvested.

Extracted nucleic acid was quantified with a spectrophotometer (1 OD_{260} = 50 µg/ml

for double-stranded DNA) and the samples were separated by electrophoresis (70V; 2.5 hrs.; 1% agarose gel) using 0.5 X TBE buffer [17]. The gel was then stained with ethidium bromide, illuminated with UV light and photographed (Fig. 3).

Results and Discussion

In preliminary studies we attempted to detect lytic bacteriophages of *S. hyodysenteriae* in water samples from a swine waste lagoon and municipal sewers using methods similar to those used for leptospire phages [2]. Additionally, we examined *S. hyodysenteriae* cultures by electron microscopy to detect the bacteriophages described by Ritchie and co-workers [6]. Phages were not detected by either method. Based on the induction of phage-like particles from *Treponema phagedenis* [7], mitomycin C was tested as an inducer of prophages in *S. hyodysenteriae* cultures. Five to seven hours after mitomycin C was added to *S. hyodysenteriae* B204 cultures in BHIS broth, cell lysis was detected as a decrease in culture optical density (Fig. 1). Effective conditions for induction depended on both mitomycin C concentration and cell density. In order to observe continued growth followed by cell lysis, indicating prophage induction (optical density decrease), 8 µg/ml of mitomycin C was found to be an effective concentration for induction when added to B204 cultures at $OD_{620} = 0.9$. If lower concentrations (<5 µg/ml) of mitomycin C were added, cell lysis was not detectable. To treat cells in early exponential growth phase a standard cell density equivalent to a culture OD_{620} of 0.9 was used for all strains.

Figure 1. Mitomycin C induction of prophage from *S. hyodysenteriae* **B204**. Mitomycin C (8 µg/ml) was added to

cultures in BHIS broth 19 hours after inoculation (open triangles). Growth of control cultures (no mitomycin C added) in

BHIS broth also indicated (closed triangles).
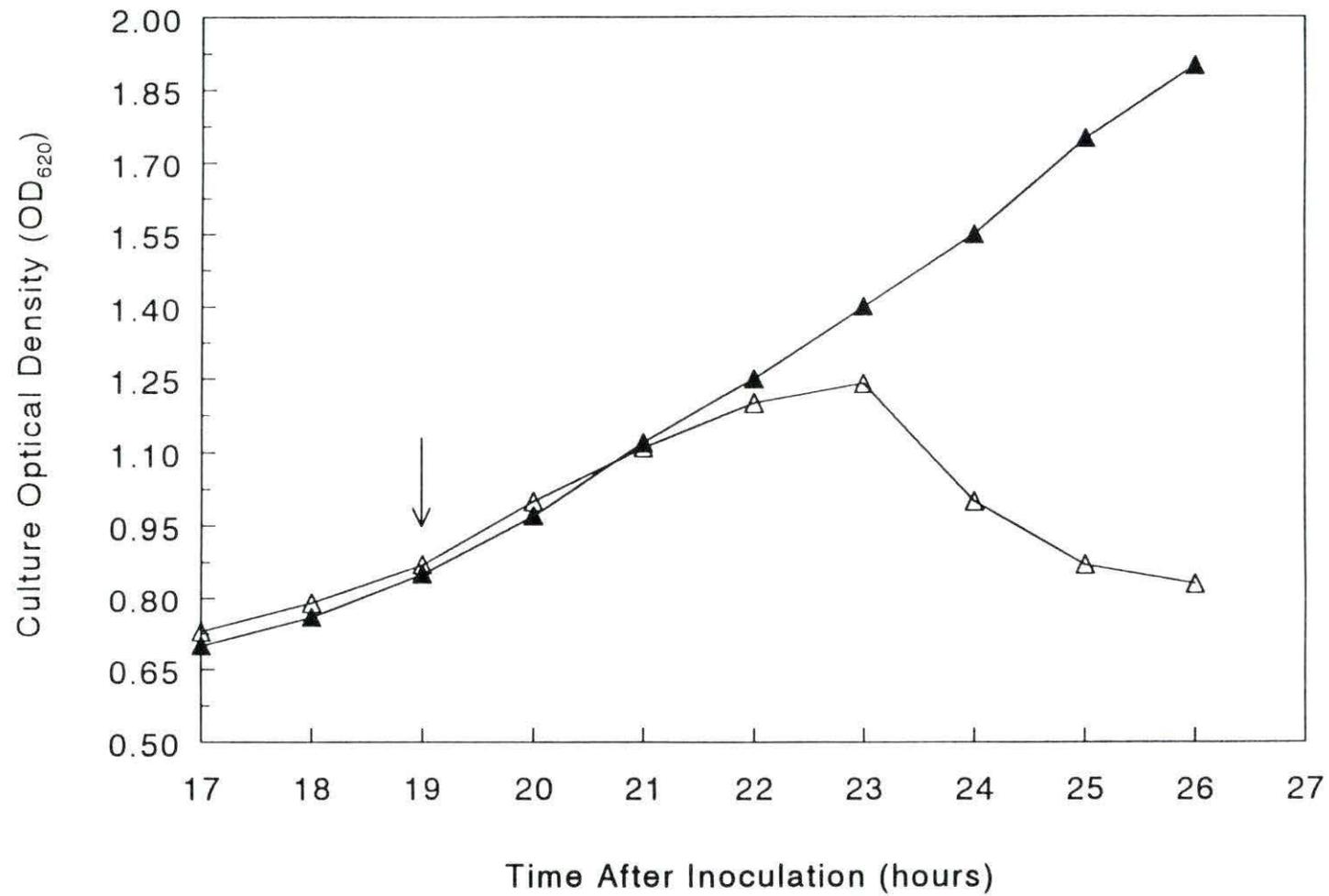
Electron microscopy observations of *S. hyodysenteriae* B204 cells treated with mitomycin C revealed numerous virions attached to whole cells and cell debris (Fig. 2). In control cultures (not treated with mitomycin C) no phages were detected. Phage particles were of uniform size and comprised of a head (45 nm diameter) and a tail (64 nm x 9 nm). Measurements were obtained from fifty particles from two different preparations. Only one morphologic type was seen suggesting only one prophage was induced. Phage tail size was uniform for each attached particle suggesting that it was noncontractile. In parallel experiments, λ phage from *Escherichia coli* were examined by electron microscopy . Size measurements of the λ head (56 nm diameter) and tail (150 nm x 14 nm) were consistent with published values [18]. Thus the *S. hyodysenteriae* B204 bacteriophage is smaller in size, with apparently less head volume than λ.

Examination of nucleic acid extracted from cells from mitomycin C treated cultures revealed an extrachromosomal band of nucleic acid not present in cells from noninduced cultures (Fig. 3). The extrachromosomal band of nucleic acid migrated on an electrophoresis gel at a position between the 7 and 8 kb linear double-stranded DNA markers. Genomic DNA was visible at or above the 23 kb marker. The extrachromosomal nucleic acid was sensitive to DNase but resistant to RNase and is therefore DNA. The staining intensity of the extrachromosomal DNA band increased from 3 to 5 hours after the addition of mitomycin C indicating an increase in cellular production of this DNA. The appearance of this extrachromosomal DNA correlated well with the appearance of bacteriophage in mitomycin C treated cultures, consequently it seems highly likely that the extrachromosomal DNA is phage DNA.

Figure 2. Negatively stained *S. hyodysenteriae* B204 cells from mitomycin C treated cultures. A - Cell with phage particles attached to outer membrane and cellular debris (46,355X). Bar = 0.4µm. B - Inset, magnified portion of cell in A showing phage attached to cell membrane over periplasmic flagella (169,000X). Bar = 0.1µm.

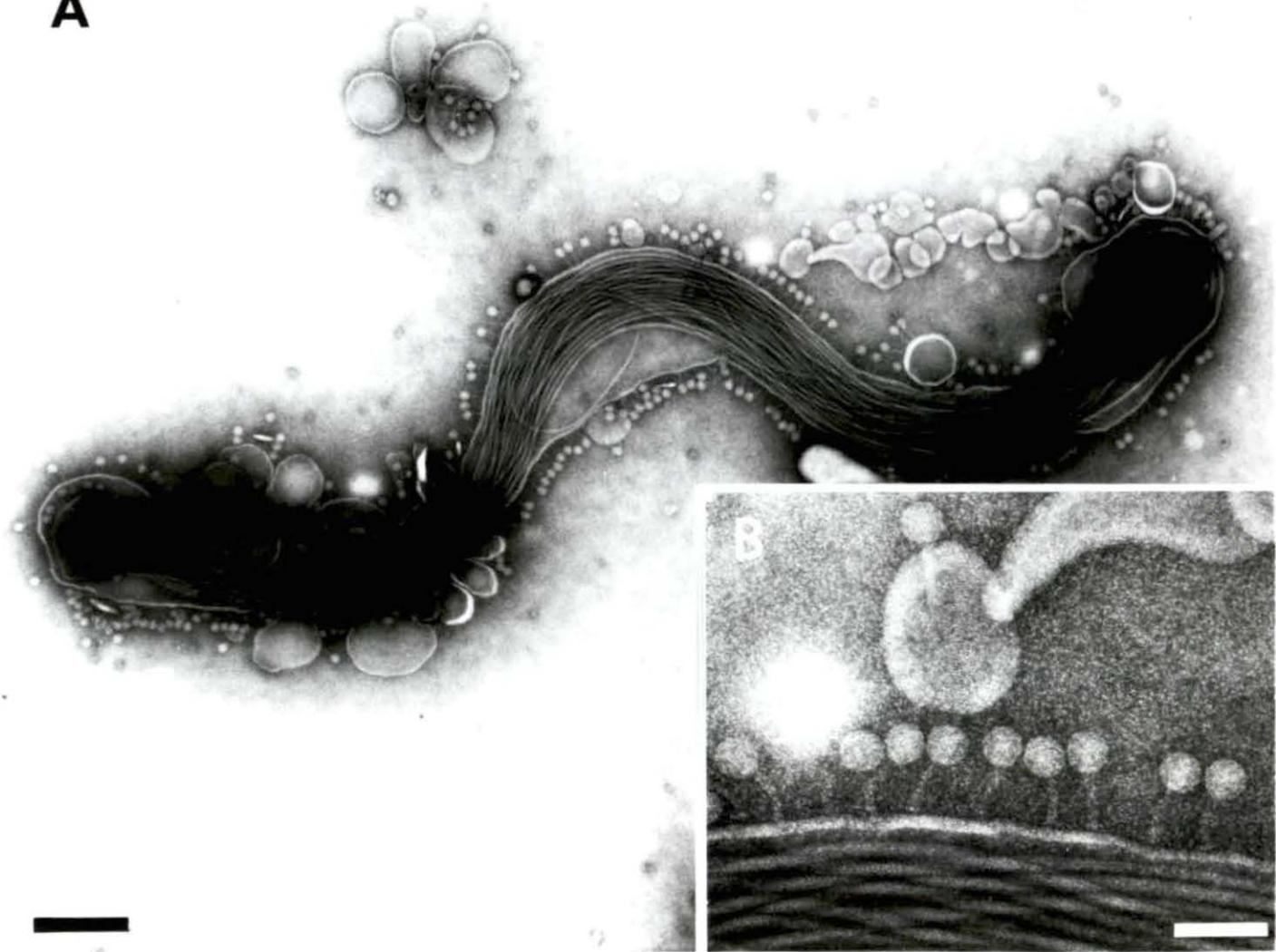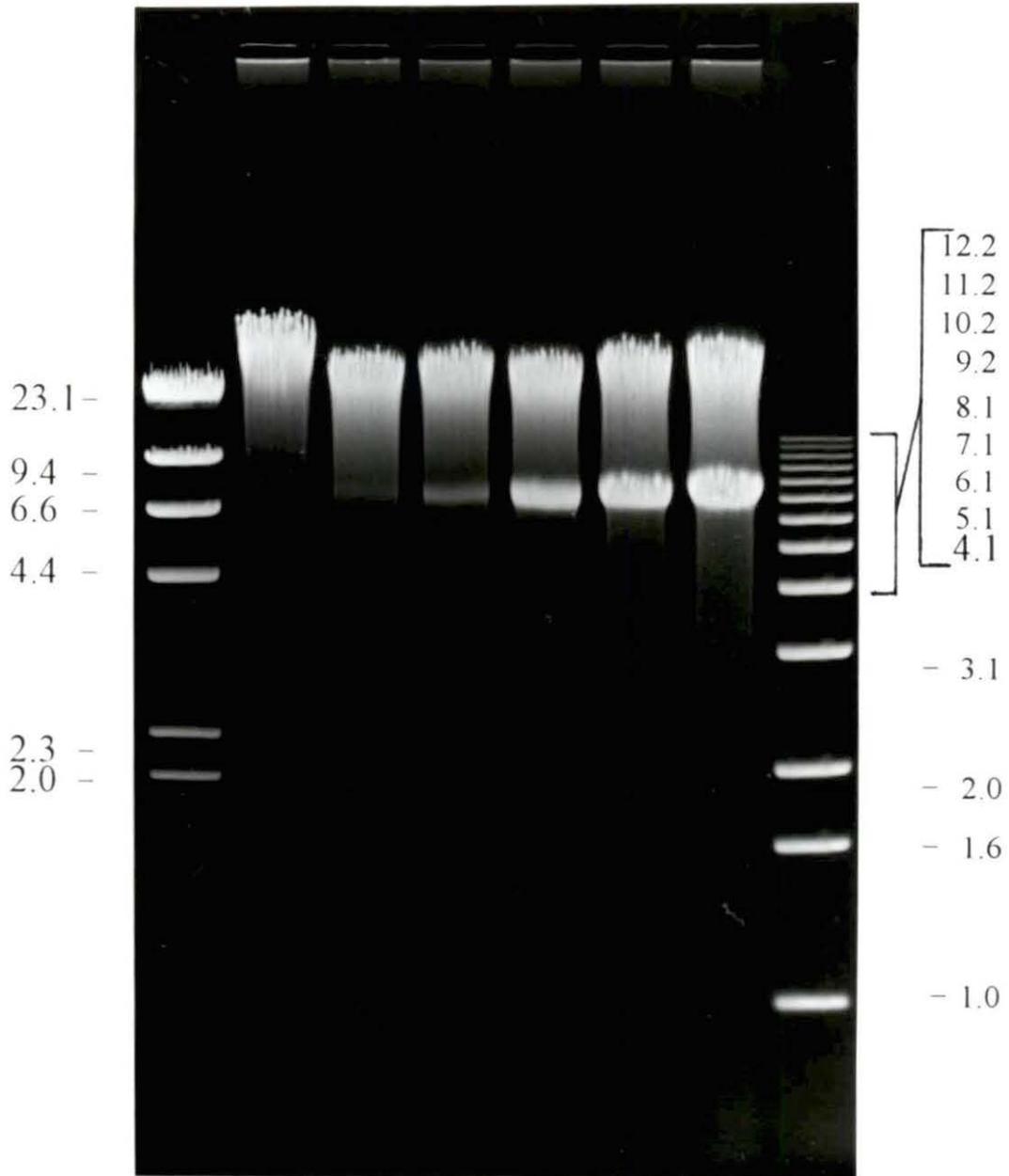**Figure 3. Appearance of extra-chromosomal DNA in *S. hyodysenteriae* B204 cultures at 3 to 5 hours after mitomycin C treatment.** A- λ Hind III fragments- 500 ng DNA; B- untreated control culture; C through G- cultures treated with mitomycin C for 3, 3.5, 4, 4.5, 5 hours, respectively; H- 1 kb ladder (Gibco-BRL, Inc.)- 400 ng DNA. 600 ng of total DNA was loaded in lanes B - G.

Extrachromosomal DNA was detected previously in *S. hyodysenteriae* and *S. innocens* strains by other investigators and considered to be a plasmid [19,20]. The DNA was approximately 6-8 kb in size and has not been characterized. In view of our results, this extrachromosomal DNA could have originated from phage present in their cultures.

Additional strains of *S. hyodysenteriae* and *S. innocens* 4/71 were tested for mitomycin C inducible prophages. All strains tested (B78, B169, A-1, B8044, B6933, Ack300/8, R-1, and *S. innocens* 4/71) yielded phages similar in appearance to the phage from strain B204. The effective concentration of mitomycin C used for induction with each strain varied (*S. hyodysenteriae*: B78, 8 μg/ml; B169, 8 μg/ml; A-1, 8 μg/ml; B8044, 12 μg/ml; B6933, 12 μg/ml; Ack300/8, 15 μg/ml; R-1, 8 μg/ml; and *S. innocens* 4/71, 8 μg/ml). The induced bacteriophage particles were of uniform size and resembled those observed in cultures of strain B204. In untreated control cultures of all strains, no phages were detected by electron microscopy.

The results of this study indicate that *S. hyodysenteriae* strains and *S. innocens* 4/71 contain prophages which are induced by mitomycin C treatment of cells. The lysogenic phage of *S. hyodysenteriae* strain B204 is similar in size and morphology to the phages reported to appear spontaneously in *S. hyodysenteriae* cultures by Ritchie and colleagues [6]. Although we did not detect spontaneously appearing phages (*S. hyodysenteriae* cultures not treated with mitomycin C), our culture conditions (both medium and atmosphere) were different from those used previously [6]. It is possible that other culture conditions favor spontaneous prophage induction leading to high numbers of phage particles present in culture.

Little is known about lysogenic phages of spirochaetes in general, and of *S.*

hyodysenteriae in particular. The ability to induce prophages with mitomycin C is an essential

first step towards purifying and characterizing the *S. hyodysenteriae* phages and investigating

possible roles for these phages in the ecology, pathogenesis, and gene transfer mechanisms of

S. hyodysenteriae cells.

Acknowledgements

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VSH-1, A BACTERIOPHAGE INDUCIBLE FROM *Serpulina hyodysenteriae* AND CAPABLE OF GENE TRANSFER.

A paper to be submitted to the Journal of Bacteriology

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Abstract

When *Serpulina hyodysenteriae* B204 cultures are treated with mitomycin C (20µg/ml broth), the cells lyse and release bacteriophages, designated "VSH-1" (**V**irus of **S**. **h**yodysenteriae). VSH-1 particles, precipitated by using PEG and purified by CsCl density gradient ultra-centrifugation, had a buoyant density of 1.375 g/cm^3 and consisted of a head (45nm diameter) and a noncontractile tail (64nm x 9nm). The bacteriophage was incapable of lytic growth on any of five intestinal spirochete strains, representing three *Serpulina* species. VSH-1 nucleic acid was determined to be 7.5 kB, linear, double-stranded DNA based on differential staining with acridine orange, DNase I sensitivity, electrophoretic mobility, and contour length measured by electron microscopy. Phage DNA digested by the restriction enzymes *Ssp* I, *Ase* I, *Eco*R V, and *Afl* II gave electrophoretic banding patterns nearly identical to those of digested *S. hyodysenteriae* chromosomal DNA. Additionally, VSH-1

DNA hybridized with probes complementary to *S. hyodysenteriae* chromosomal genes (*nox* and *fla*A1). Thus, restriction endonuclease analysis and Southern hybridization findings indicated that VSH-1 particles package host DNA and suggested the phage might be capable of transferring genes between *S. hyodysenteriae* cells. To evaluate whether or not VSH-1 is a transducing virus, purified bacteriophages induced from cultures of *S. hyodysenteriae* strain A203 (*flaA1::cat*) were added to growing cells of recipient strain A216 (*nox::kan*) for 8 hours and plated onto Trypticase soy blood agar containing both chloramphenicol (10 μg/ml) and kanamycin (200 μg/ml). Transductants (*flaA1::cat, nox::kan*) were obtained at a frequency of 1.5 x 10^{-6} per phage particle. Homologous recombination incorporating the *flaA1::cat* locus from donor strain A203 was confirmed by Southern blot analysis and PCR. These results suggest that VSH-1, a phage apparently incapable of lytic growth, packages the DNA of its bacterial host, *S. hyodysenteriae*, and is capable of transferring genes between cells of that spirochete.

Introduction

The spirochete *Serpulina hyodysenteriae* is a gram negative, anaerobic bacterium which can be isolated from the cecum, colon and feces of dysenteric swine. In the early 1970's it was established that *S. hyodysenteriae* is the causative agent of swine dysentery, an enteric disease producing a severe mucohemorrhagic diarrhea in infected swine (7).

Spirochetes represent a diverse group of organisms with widely varying genetic composition. *Borrelia burgdorferi* has a linear chromosome of approximately 1,000 kb and several extrachromosomal elements varying in size and conformation; *Leptospira interrogans* has a genome comprised of a 4,400 kb circular chromosome and a 350 kb circular chromosome; *S. hyodysenteriae* has a single circular chromosome of 3,200 kb; and *Treponema pallidum* possesses a single circular chromosome of approximately 900 kb (23,32,34,35). In addition, an extrachromosomal plasmid of 2.6 kb has been isolated and characterized from *Treponema denticola* (9). Natural gene transfer systems for spirochetes have neither been identified nor characterized. However, the existence of DNA filled membrane vesicles on the surface of *B. burgdorferi* cells has been reported and indirect evidence of lateral gene transfer has been obtained for *Borrelia* (6,15). Bacteriophages have been observed in cultures of spirochetes free, attached to cells or within cells (8). To the best of our knowledge three lytic phages of *Leptospira biflexa* have been the only spirochete phages isolated and characterized (22). Unfortunately, no evidence that these three phages play a role in gene transfer was obtained.

We previously reported an inducible prophage in *S. hyodysenteriae* and *S. innocens* cells (8). The research described in this article extends our previous findings and describes the isolation and characterization of a mitomycin C inducible phage, designated VSH-1, from *S. hyodysenteriae* B204 cells. VSH-1 bacteriophages package 7.5 kb fragments of host chromosomal DNA. Furthermore, virions were determined to transduce either *flaA1* or *nox* genes between *S. hyodysenteriae* strains indicating this phage, VSH-1, behaves like a generalized transducing phage.

Materials and Methods

Bacterial strains and culture conditions

Cells of *S. hyodysenteriae* were routinely cultured in BHIS as described previously (25). *S. hyodysenteriae* strain B204 used in these studies has been described (11). The *S. hyodysenteriae* strain A203 (21), a *FlaA1* mutant (*flaA1::cat*), was used in transduction studies along with an NADH oxidase (*nox*) mutant A216 (*nox::kan*). Both strains A203 and A216 were constructed from strain B204 by electroporation-mediated allelic exchange. Strain A203 contains a 852 bp chloramphenicol resistance gene from pER919a inserted into a 169 bp deletion site between nucleotide position #593 and #762 in the *flaA1* coding region. Strain A216 contains a 1252 bp kanamycin resistance gene from pUC4K inserted into a 322 bp deletion site between nucleotide position #438 and #760 in the NADH oxidase coding region.

In order to purify phage particles *S. hyodysenteriae* cells had to be cultured in NT broth, a low protein and serum-free medium. To prepare NT broth, brain heart infusion broth was ultrafiltered (Ultrafiltration cell; 10,000 MW cutoff filter- YM10; Amicon, Inc.) to remove proteins with high molecular mass. The filtrate was used to make sterile anaerobic medium as previously described (25). This basal medium was supplemented with cholesterol (25 µg/ml, final conc.) and phosphatidylcholine (50 µg/ml, final conc.) dissolved in ethanol. Cultures in NT broth (400 ml) were grown under an initial $N_2:O_2$ (99:1) atmosphere at 37°C. When growing strain A203, both liquid and solid media contained chloramphenicol (10 µg/ml) and when A216 was grown media contained kanamycin (200 µg/ml).

Induction and purification of VSH-1 particles

Mitomycin C was added to cultures of spirochete cells in exponential growth phase

(OD_{620} = 0.9; 8 x 10^7 cells/ml). A mitomycin C concentration (20 µg/ml, final conc.)

was used that resulted in a gradual decrease (0.1 - 0.2 units) in culture optical density

within 5-8 hours after addition.

VSH-1 was purified by PEG precipitation and CsCl density gradient

ultracentrifugation (Fig. 1b) based on methods for purification of λ phage (24). Culture

lysates were treated with DNase I (0.2 µg/ml, final conc.) and Stratagene RNace-it (10

µl/400 ml) and incubated at 37°C for 1 hour. NaCl (1M, final conc.) and chloroform

(1.5%, final conc.) were added and the lysate was placed on ice. After 1 hour the lysate

was centrifuged at 4,000xg for 5 min. at 4°C. The supernate was removed and centrifuged

again. The supernate was then harvested and polyethylene glycol (MW. 8,000) added

(10% final conc.). After 48 hours at 4°C the precipitated phage were pelleted by

centrifugation (4000 x g, 10 min., 4°C), resuspended in SM buffer (24), and treated with

DNase I (8 µg/ml, final conc.) and RNace-it (1 µl/ml) at 37°C for 1 hour. The phage

suspension was extracted with an equal volume of chloroform to remove polyethylene

glycol. Cesium chloride was added to the aqueous phase and the solution (1.32 g/cm^3, final

density) ultracentrifuged at 149,000 x g for 24 hours at 4°C in a Beckman SW55ti rotor. A

band of phage particles in the lower half of the ultracentrifuge tube (Fig. 1b) was collected

and ultrafiltered with a membrane (Microcon-100, MW. cutoff 100,000; Amicon, Inc.) to

remove CsCl. The phage particles were resuspended in SM buffer (0.5 ml), harvested by

ultrafiltration two more times, and then resuspended in SM buffer (200 µl).

VSH-1 virion characterization

Purified VSH-1 phage were negatively stained by mixing phage samples with phosphotungstic acid (2%, pH 7.0) at a ratio of 1:1. Samples were then deposited on parlodion-coated 200-mesh carbon-reinforced copper grids and viewed using a Philips model 410 electron microscope (80 kV).

To determine the size of VSH-1 virions measurements were made on viruses in electron micrographs from two separate preparations. A total of 100 particles were measured from 20 different fields. The buoyant density of VSH-1 particles in CsCl was estimated by measuring the refractive index of three separate samples taken from purified CsCl phage bands in a refractometer (ERMA Newt Peabbe's refractometer; Fischer, Inc.).

Purified VSH-1 virions were denatured by boiling for 10 min. in SDS sample buffer. Denatured proteins were then separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) using a 3.75% acrylamide stacking gel over a 12% acrylamide resolving gel (0.75mm thick). Electrophoresis was carried out at room temperature at 175V for 1 hour. A vertical gel electrophoresis apparatus (Mini-Protean II), power supply (Model 250/2.5), molecular mass standards (low range), and all electrophoresis reagents used were purchased from Bio-Rad Laboratories (Hercules, CA). Methods for buffer preparation and staining proteins with Coomassie blue were carried out according to the manufacturer's recommendations.

Protein concentrations were determined using the Lowry technique (19). Bovine

serum albumin (BSA; Sigma A-4378) was used as a standard. BSA at a concentration of 0.5

mg/ml in water had an OD_{280} of 0.33± 0.01 in a 1 cm pathlength cuvette.

VSH-1 nucleic acid analysis

Purified phage were treated with DNase I (16 µg/ml, final conc.) and RNase

(RNace-it 1 µl/ml; Stratagene), and incubated for 45 min. at 37°C. Proteinase K (50

µg/ml, final conc.), SDS (0.5% final conc.), and EDTA (20 mM, final conc.) were added

and the solution was incubated for 15 min. at 65°C. The solution was then extracted with

an equal volume of phenol:chloroform (1:1), followed by chloroform. To precipitate

nucleic acid in the aqueous phase 0.1X volume of sodium acetate (3 M, pH 7.0) and 2X

volumes of 100% ethanol were added to the solution which was then stored at -70°C for 1

hour before pelleting the precipitated DNA in a microcentrifuge.

To determine VSH-1 nucleic acid sensitivity to DNase I and RNase, VSH-1 nucleic

acid (equivalent to 100 ng of DNA) was incubated at 37°C for 1 hour with either DNase I (8

µg/ml) or RNace-it (1µl/ml; Stratagene). Samples were then analyzed for the presence of

nucleic acid by using agarose gel electrophoresis.

Acridine orange differential staining of nucleic acid in agarose gels was used to

determine whether VSH-1 nucleic acid was single or double-stranded (17). VSH-1 nucleic

acid (500 ng), 500 ng of a double-stranded control DNA (pBluescript SK⁻; Stratagene), and

500 ng of a single-stranded control DNA (M13mp18; United States Biochemical) were

stained after electrophoresis. The electrophoresis was carried out at 4°C in order to keep the

gel temperature down.

VSH-1 DNA and plasmid DNA (pBluescript II SK⁻; Stratagene) used as a size standard (2.96 kb) were prepared for electron microscopy by the aqueous monolayer spreading technique as described by Kleinschmidt (12). Nucleic acid/cytochrome C monolayers were absorbed onto parlodion-coated 200-mesh grids (carbon-reinforced on backsides only) and stained in uranyl acetate (10 µl of 0.05 M uranyl acetate in 0.05 M HCl mixed with 90% ethanol). The grids were then coated with platinum/palladium while rotating in a vacuum shadower.

Contour length of VSH-1 nucleic acid was determined by comparing the size of VSH-1 molecules to a size reference standard, pBluescript SK⁻ (2.96kb). A map measurer (Michelin) was used to obtain measurements of individual molecules in different electrom micrographs. The average size of 20 molecules of VSH-1 DNA and 20 molecules of pBluescript SK⁻ DNA were determined.

For VSH-1 nucleic acid DNase I sensitivity studies (Fig. 3) 500 ng of Bacteriophage λ DNA digested with *Hind* III and VSH-1 particles containing an estimated 100 ng of nucleic acid were used. In addition, 100 ng of VSH-1 nucleic acid was used for the sample in Figure 3, lane D. All samples were incubated at 37°C for 1 hour and put through the phage DNA extraction procedure (see above).

Restriction enzyme analysis and Southern hybridization

Genomic DNA was isolated from *S. hyodysenteriae* B204 cells in the exponential

phase of growth (OD_{620}= 1.5; 5 x 10^8 cells/ml) as previously described (27)

VSH-1 DNA (2 µg) and genomic DNA (2 µg) were digested overnight with various

restriction enzymes following manufacturer's instructions. The resulting DNA fragments were

separated by electrophoresis on a 1% agarose gel (100V; 3 hrs.) using 0.5X TBE buffer (24).

The DNA fragments were stained with ethidium bromide (0.5 µg/ml), illuminated with UV

light, and photographed. A downward blotting technique (4) was used to transfer the DNA

fragments onto nylon membranes (Hybond N; Amersham). The DNA was then crosslinked to

the membrane with UV light (Stratagene 2400 UV crosslinker; Stratagene) for use in

Southern hybridization experiments.

Southern hybridizations were carried out using either a radiolabeled *nox* gene probe

(5'-ATGAAAGTTATTGTAATAGG-3') corresponding to base positions #1-20 in the *nox*

coding region (26) or a radiolabeled probe from the coding region of the *flaA1* gene (5'-

GCGGTGCTGATGGTACTAA-3') corresponding to base positions #367-385 in the *flaA1*

gene (13). The probes were labeled at the 5' end with [γ^{32}P]-dATP (24). Pre-

hybridization/hybridization buffers consisted of 6 X SSC (24), 5 X Denhardt's solution, 0.1%

SDS, and 200 µg/ml denatured salmon sperm DNA. All solutions are final concentration

values. Pre-hybridization was for 2 hrs. at 50°C and hybridization overnight at 50°C with 4

µl/ml of radiolabeled probe (2 pmoles total; approx. 8 x 10^4 cpm) in the hybridization solution.

Hybridization was followed by three room temperature washes for 1 min. and then a final

wash at 50°C for 30 min. Wash buffer consisted of 5 X SSC and 0.5% SDS (final cons.).

Kodak X-Omat AR scientific imaging film was used for autoradiography.

Phage counts

VSH-1 bacteriophages were quantified by mixing phage preparations with dilutions of

latex beads (0.87 μm dia.) of known concentration (Earnst Fullam Inc.; #10530) at a 1:1

ratio. This suspension was then mixed with an equal volume of BSA (20 μg/ml; BSA fraction

V, Sigma #A-3059) and an equal volume of phosphotungstic acid (2%, pH 7.0). After 2

min. at room temperature this mix was loaded into a Pelco all glass nebulizer (Ted Pella,

Inc.; #14601) and sprayed onto parlodion-coated 200-mesh carbon-reinforced copper grids.

Photographs were taken of 20 different fields from each sample. VSH-1 particles and latex

beads in each photograph were counted. Based on the number of latex beads and their

known concentration, concentrations of virions in preparations were determined.

VSH-1 lytic growth

Five strains representing three *Serpulina* species were tested as hosts for VSH-1 lytic

growth. These were *S. hyodysenteriae* strains B204 and B78; *S. pilosicoli* strains P43/6/78

and Wes-B; and *S. innocens* strain B256 (28). Approximately 10^8 cells of each strain was

spotted onto a TSB agar plate and uniformly streaked across the plate with a sterile loop.

VSH-1 phage particles (10^{11}) were then spotted (3 μl) onto the streak and the plates incubated

at 39°C in an anaerobic chamber. After five days the plates were examined to determine

whether or not growth of the bacteria was inhibited by VSH-1.

Transduction studies

For transduction studies strains were propagated in 7 ml tubes of BHIS broth as previously described (25). In order to detect transductants and determine viable cell numbers liquid cultures were plated onto trypticase soy agar supplemented with 5% bovine blood (TSB) and incubated at $39°C$ in an anaerobic chamber. Transductants were selected by growing cells on selective media containing both chloramphenicol and kanamycin (CM^{10}; KM^{200}).

VSH-1 particles were induced and purified from strain A203 (*flaA1::cat*). These VSH-1 phage particles were then added to strain A216 (*nox::kan*) cells in the early exponential phase of growth ($OD_{620}= 0.9$; 8×10^7 cells/ml). A total of 8.2×10^{10} VSH-1 particles (46.8 μg protein) were added to 7 ml cultures. Culture samples were taken hourly and plated (200 μl) on TSB agar (CM^{10}; KM^{200}) to screen for transductants, cells capable of growth on selective media, and to obtain viable cell counts by plating (100 μl of a 10^{-5} or 10^{-6} dilution of the culture) on TSB agar.

To confirm that transductants contain both markers, *flaA1::cat* and *nox::kan*, PCR reactions were carried out with two primer sets specific for each locus. The first set contained a primer specific for the 5' end of the gene and a primer specific for the 5' end of the inserted antibiotic resistance gene. The second set of primers consisted of specific primers complementary to regions of the gene which flank the antibiotic resistance gene. For the *flaA1::cat* loci the *flaA1* 5' end primer ERL10 [5'-GGGGATCCT<u>ATG</u>AAAAAGTT-

ATTCGTAGTATTAACTTTCC-3', pos. #1-31 (21)] and the *cat* 5' end primer ERL16 [5'-

GATTAAAGATCTCTTTTCTCTTCC-3', pos. #55-32 (21)] were used for the first set.

FlaA1 primers 601 [5'-GCGGTGCTGATGGTACTAA-3', pos. #367-385 (13)] and 602 [5'-

TAGCAGCAGCACCTTGATC-3', pos. #915-897 (13)] were used for the second set. For

the *nox::kan* loci the nox 5 'end primer 1 [5'-ATGAAAGTTATTGTAATAGG-3', pos. #1-20

(26)]and the *kan* primer ERL12 [5'-CGCGGCCTCGAGCAAGACG-3', pos. #41-23 (21)]

were used for the first set. The *nox* 5' end primer 1 was used with the *nox* primer TS120 [5'-

AGCTTCCATTAAGATAACT-3', pos. #790-772 (unpublished)] for the second set.

Amplification of DNA was carried out using 100 ng of purified genomic DNA as a target.

Amplitaq DNA polymerase, ampliwax gems, and PCR core reagents (Roche Molecular

Systems, Inc.) were used in PCR DNA amplification according to manufacturers

recommendations. Samples were run on a thermocycler for 36 cycles of denaturation (95°C,

1min.), annealing (48°C, 1 min.), and extension (72°C, 2 min.) followed by a final extension at

72°C for 8 minutes. PCR products were separated by agarose gel electrophoresis, stained

with ethidium bromide, and detected by UV transillumination.

Results

VSH-1 structure

The amount of mitomycin C added and the time required to detect a decrease in culture optical density is different from previous values (8) because cultures used for purifying VSH-1 are grown in NT broth not BHIS. The use of NT broth is essential for culturing *S. hyodysenteriae* when the cultures will be used to induce and purify VSH-1 virions. CsCl density gradient formation necessary for VSH-1 purification is not easily accomplished when large amounts of protein and other serum components are present in the samples.

After ultracentrifugation phage particles formed a visible band in a CsCl density gradient (Fig. 1b). Based on the refractive index measurements of the CsCl solution at the position of the phage band the VSH-1 virions had a buoyant density of 1.375 g/cm³. Additional bands near the top of the CsCl gradient consisted primarily of flagella with some clumps of VSH-1 virions based on electron microscopy examination of material harvested from these bands. The VSH-1 particles (Fig. 1a) were of uniform size and consisted of a head (45 nm diameter) and a simple noncontractile tail (64 nm x 9nm). Based on morphology, VSH-1 is a member of the *Siphoviridae* family of tailed phages (1). Analysis of purified phage preparations by SDS-PAGE (Fig. 2) revealed thirteen Coomassie blue staining protein bands corresponding to proteins with a range of molecular weights between 13 and 101 kDa. Six of these bands (101, 53,45,38,19,and 13 kDa) stained more intensely than the others with

Figure 1. Negatively stained VSH-1 particles purified from mitomycin C treated cultures of *S. hyodysenteriae* B204 cells and the position of VSH-1 particles in a CsCl density gradient. A) Phage particles (169,000X). Bar = 0.1 μm. B) CsCl density gradient (phage band - buoyant density = 1.375 g/cm^3).
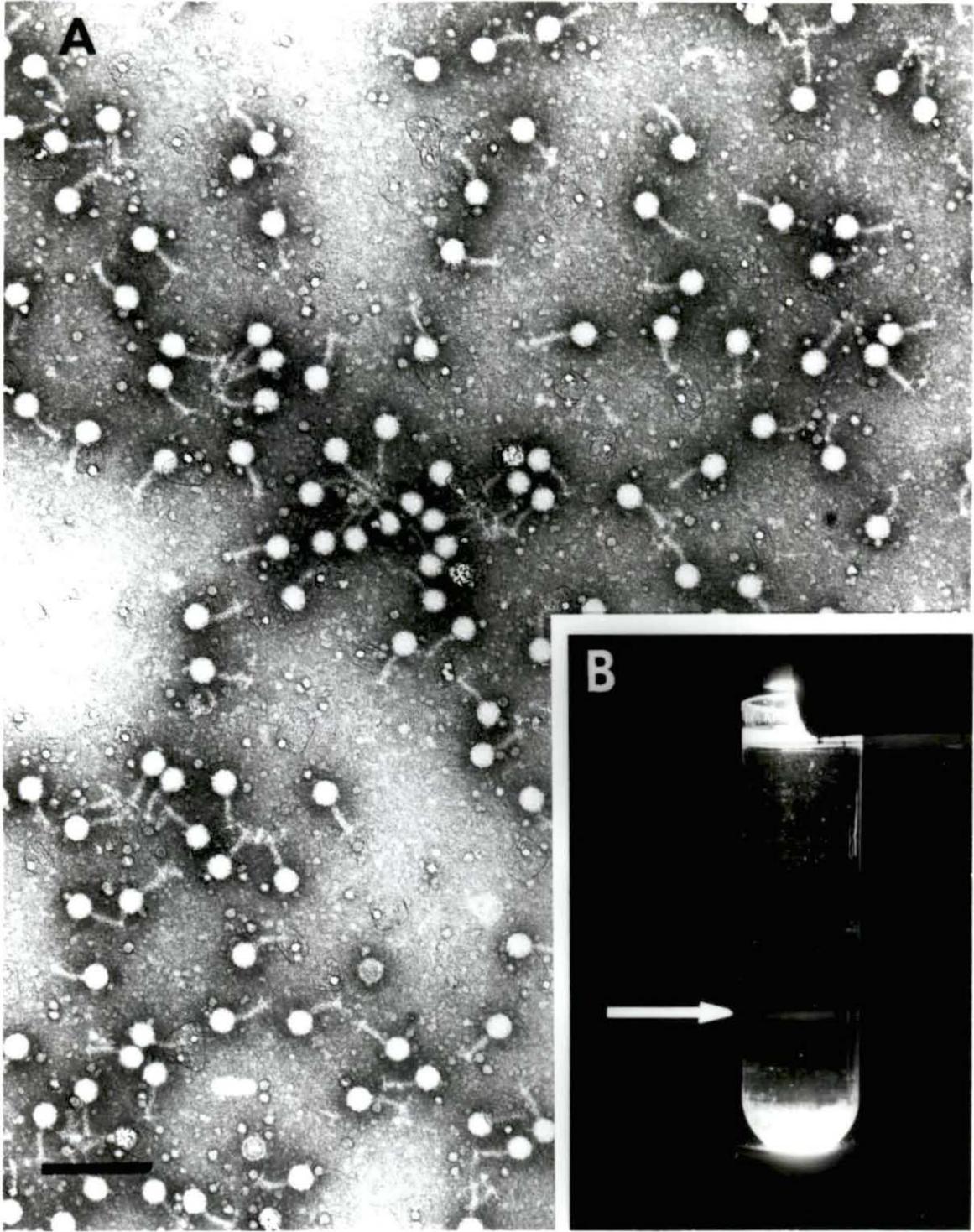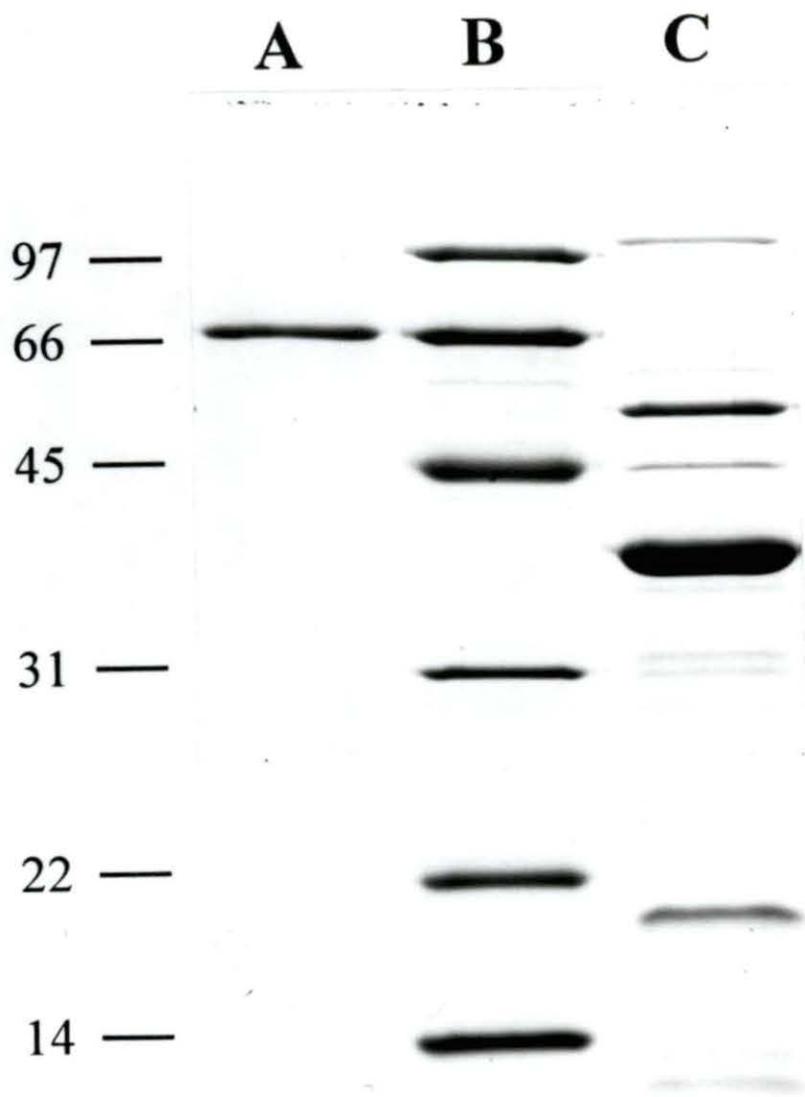
Figure 2. Protein electrophoresis of purified VSH-1 particles.

Lane: A. Bovine serum albumin control (0.7 µg).

B. Low molecular weight markers (Bio-Rad; 6.5 µg).

C. VSH-1 phage preparation (4 µg).

the 38 kDa band being dominant. Since this 38 kDa band is the most abundant it could be the major head protein of VSH-1.

VSH-1 Nucleic acid

VSH-1 nucleic acid was protected from DNase I treatment before extraction from purified virions (Fig. 3; lane C). After extraction VSH-1 nucleic acid was degraded by DNase I (Fig.3; lane D) but was found to be resistant to RNase treatment (not shown), indicating the nucleic acid packaged inside VSH-1 particles was DNA. In addition, VSH-1 nucleic acid in electrophoretic gels fluoresced green under UV light when stained with acridine orange (Fig. 4). The control DNA, M13mp18 and pBluescript SK⁻, Fluoresced orange and green respectively (Fig. 4). These results indicated VSH-1 DNA was double-stranded. This conclusion was further supported by the ability of the VSH-1 DNA to be digested by restriction enzymes (see below). Electron microscopy observations of VSH-1 nucleic acid revealed a linear molecule (Fig. 5) whose contour length corresponded to a 7.5 kb molecule of DNA. This size is similar to size estimates based on electrophoretic migration (Fig. 3). Thus VSH-1 particles contain 7.5 kb double-stranded linear DNA molecules.

Restriction enzyme digestion patterns for VSH-1 DNA and *S. hyodysenteriae* B204 chromosomal DNA were compared (Fig. 6A). The banding patterns for VSH-1 and *S. hyodysenteriae* were virtually identical to each other between 0.6 - 6 kb. For the *Eco*R V and *Afl* II digests of VSH-1 DNA no DNA fragments were observed above 7.5 kb, the size

Figure 3. Sensitivity of VSH-1 nucleic acid to DNase I.

Lane: A- Bacteriophage λ DNA digested by *Hind* III.

B- Bacteriophage λ DNA digested by *Hind* III and treated with DNase I before electrophoresis.

C- Bacteriophage λ DNA digested by *Hind* III mixed with VSH-1 virions. Mixture was treated with DNase I before VSH-1 nucleic acid was extracted and electrophoresed. Before extraction from virions, VSH-1 nucleic acid was resistant to DNase I.

D- VSH-1 nucleic acid extracted from virions and treated with DNase I before electrophoresis. Extracted nucleic acid was sensitive to DNase I.
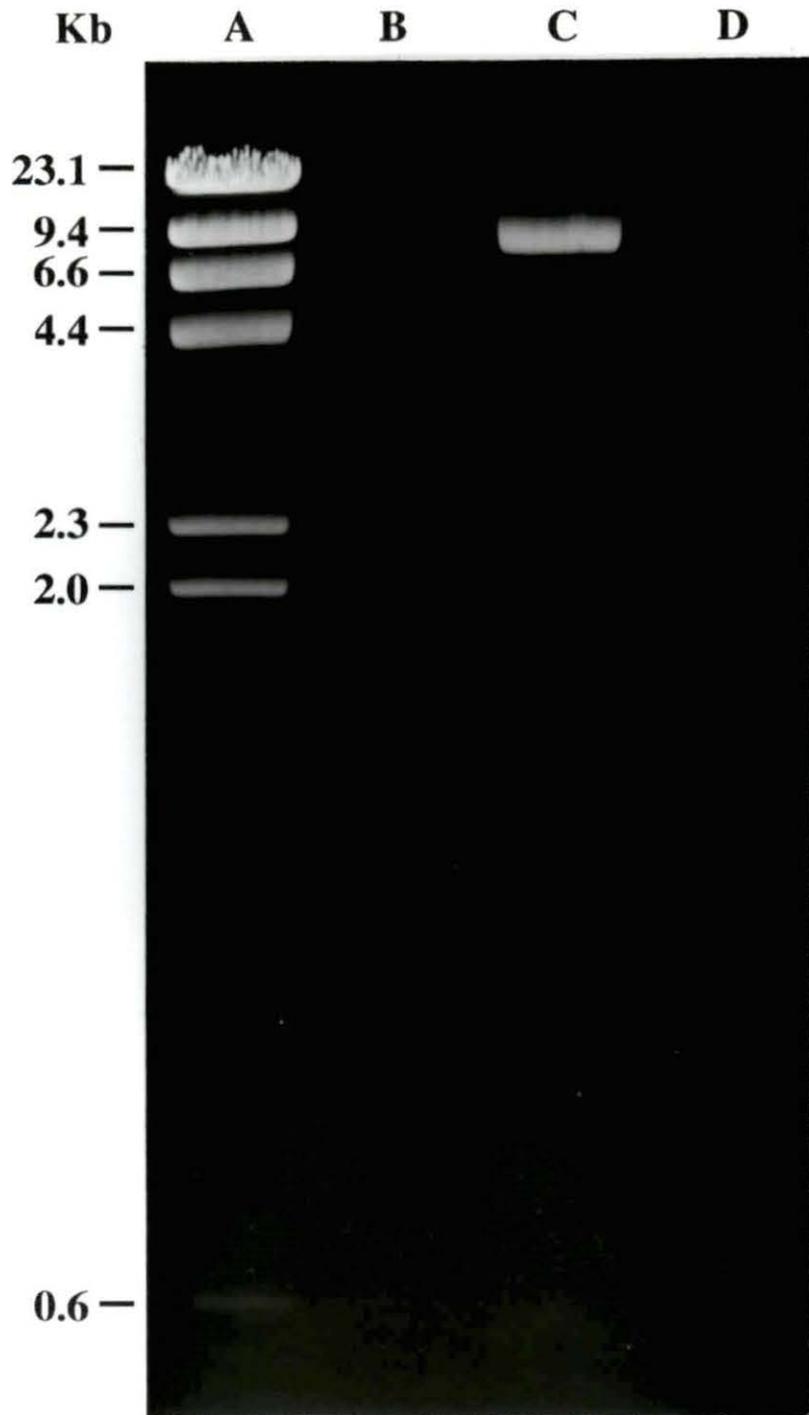
Figure 4. Acridine orange differential staining of VSH-1 DNA.

Lane   A- λ *Hind* III markers.

B- M13mp18 single-stranded control DNA.

C- pBluescript SK⁻ double-stranded control DNA.

D- VSH-1 DNA.
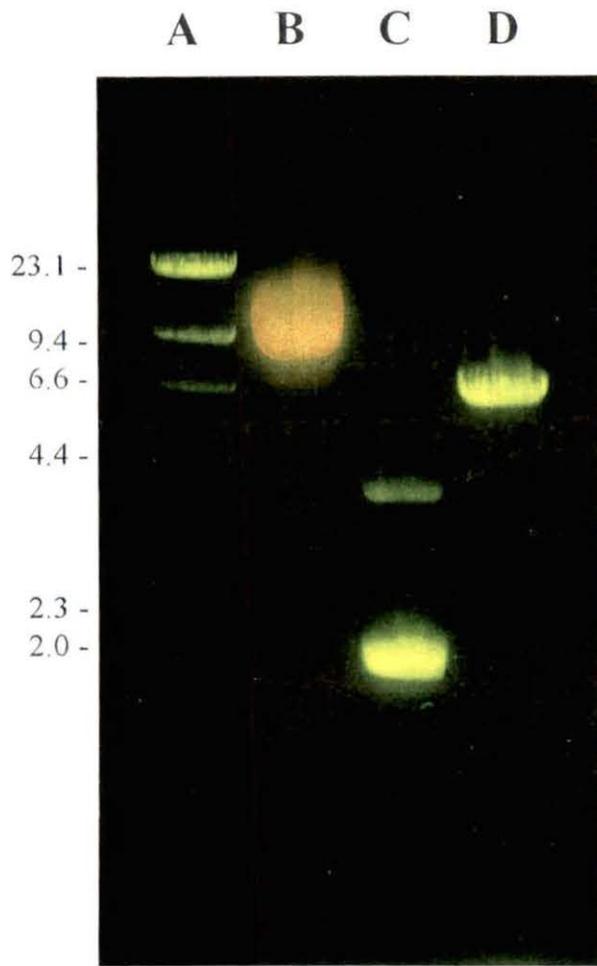
A total of 500 ng of DNA was loaded into each lane.

Figure 5. Electron microscopy of VSH-1 DNA. A - Linear double stranded VSH-1 DNA. Bar =0.2 μm. B - VSH-1

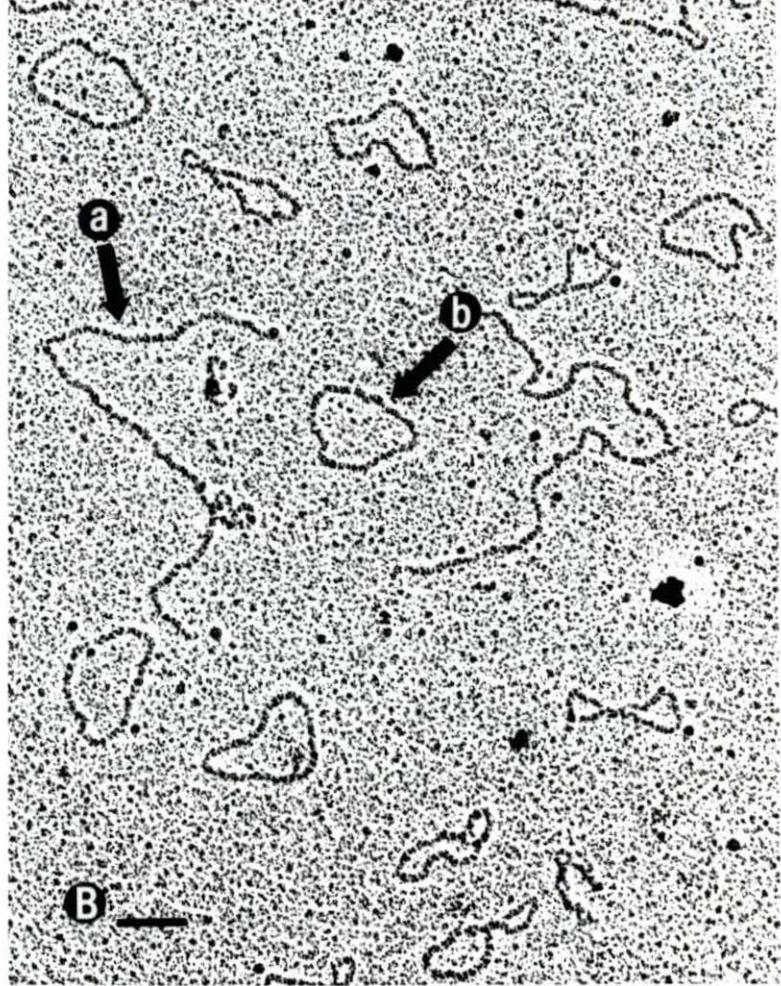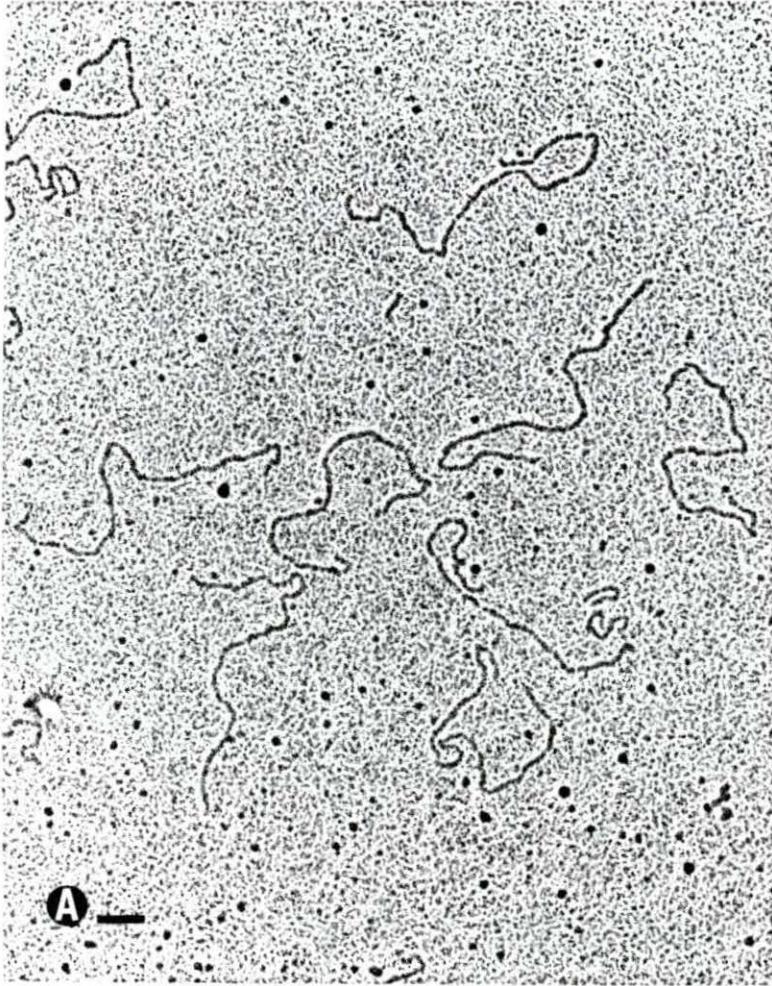DNA (a) mixed with pBluescript II SK⁻ (known size = 2.96 kb) (b). Bar =0.2μm.

Figure 6. Comparison of VSH-1 DNA to *S. hyodysenteriae* **B204 chromosomal DNA.** A. Restriction enzyme digestion patterns - Lanes: A) *Hind* III digested λ DNA; B) VSH-1 DNA, uncut; C) B204 DNA, uncut; D) *Ssp* I digested VSH-1 DNA; E) *Ssp* I digested B204 DNA; F) *Hin*f I digested VSH-1 DNA; H) *Hin*f I digested B204 DNA; I) *Eco*R V digested VSH-1 DNA; J) *Eco*R V digested B204 DNA; K) *Afl* II digested VSH-1 DNA; L) *Afl* II digested B204 DNA; M) *Bst*E II digested λ DNA. B. Southern blot analysis of DNA from *S. hyodysenteriae* B204 and VSH-1 (hybridization of membrane prepared from gel in A). DNA fragments were hybridized with ^{32}P-labeled *nox* probe.
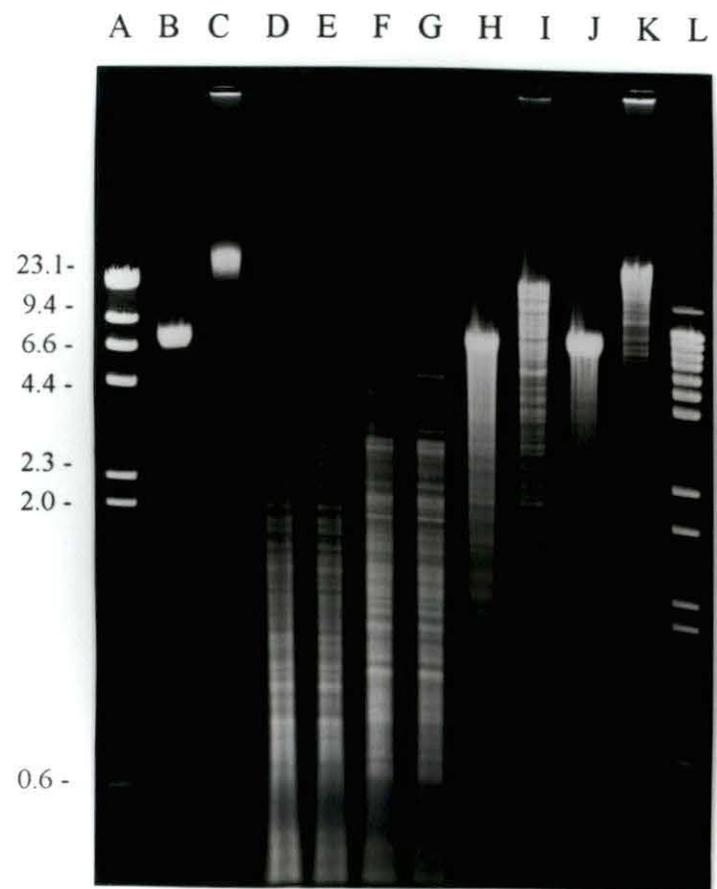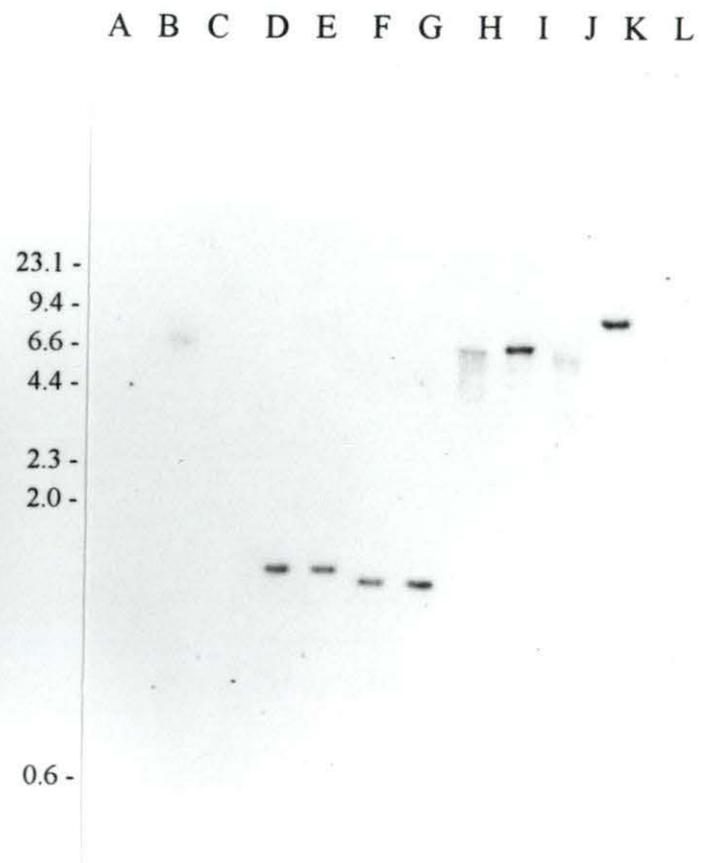
A   B

of VSH-1 DNA (Fig. 6A, lane B). A conclusion based on these results is that VSH-1 contains *S. hyodysenteriae* chromosomal DNA in random 7.5 kb fragments. In support of this conclusion, *S. hyodysenteriae* genes have been detected in purified VSH-1 DNA by Southern blot hybridization. When VSH-1 DNA restriction digests and *S. hyodysenteriae* B204 DNA restriction digests where transferred to a nylon membrane and hybridized to the NADH oxidase gene probe (*nox*) a positive probe reaction was obtained for both the VSH-1 digests and the B204 digests (Fig.6B). The *nox* probe hybridized to a DNA fragments of identical size (approx. 1.4 kb) for both VSH-1 and B204 in the *Ssp* I and *Hinf* I digest patterns (Fig. 6B, lanes D-G). However, the *nox* probe hybridized to a B204 DNA fragment of 7.5 kb in the *Eco*R V digest and a DNA fragment greater than 10 kb in the *Afl* II digest for B204 whereas a weak and diffuse hybridization reaction was obtained from VSH-1 DNA (*Eco*R V and *Afl* II restriction patterns) around the size range of 6-7.5kb. This result indicates that VSH-1 packages B204 DNA randomly in 7.5 kb fragments since larger restriction fragments carrying the *nox* gene become less defined. When the same membrane was reprobed with a *flaA*1 gene probe a similar result was obtained.

Growth inhibition studies

Of the five different strains tested for lytic growth of VSH-1 no growth inhibition was detectable. Therefore, it is not likely that VSH-1 lytic growth occurs on *S. hyodysenteriae* B204 and B78, *S. pilosicoli* P43/6/78 and Wes-B, or *S. innocens* B256.

Transduction

When purified VSH-1 particles induced from *S. hyodysenteriae* A203 (*flaA1::cat*) cells were added to growing cells of *S. hyodysenteriae* A216 (*nox::kan*) transductants, cells that were both resistant to kanamycin and chloramphenicol, were obtained at a maximum frequency of 1.5×10^{-6} transductants per phage particle.

To confirm that the transductants did contain both the *flaA1::cat* and *nox::kan* construct genomic DNA isolated from three of the transductants was digested with restriction endonucleases, the fragments separated by agarose gel electrophoresis and blotted onto a nylon membrane, and hybridized to both the *flaA1* gene probe (Fig. 7) and the *nox* gene probe (Fig 8). Strain B204, A203, and A216 DNAs were used as controls. The hybridization results confirmed that the transductants contained both constructs within their genomes. For the hybridization of the *flaA1* probe a shift of 700 bp, as predicted based on the sequence of the construct, was seen in the transductants for fragments produced from a *SspI* digest and a shift of 300 bp was seen for fragments produced from a *AseI* digest. For the hybridization of the *nox* probe a shift of 100 bp was seen for the *SspI* digest as predicted from the construct sequence. Therefore hybridization results for transductants resemble the results obtained from the donor strains, indicating homologous recombination had occurred.

For additional confirmation that the transductants did contain both of the *flaA1::cat* and the *nox::kan* markers PCR was performed on DNA extracted from transductants (Fig. 9). DNA from *S. hyodysenteriae* B204, and from strains A203 and

Figure 7. Restriction enzyme digestion patterns of transductants chromosomal DNA and Southern hybridization of resulting fragments to a radiolabelled *flaA*1 gene probe. A - Restriction digests separated by agarose gel electrophoresis. Lanes: A) *Bst*E II digested λ DNA; B) *Ase* I digested B204 DNA; C) *Ase* I digested A203 DNA; D) *Ase* I digested A216 DNA; E) *Ase* I digested transductant 1 DNA; F) *Ase* I digested transductant 2 DNA; G) *Ase* I digested transductant 3 DNA; H) *Ssp* I digested B204 DNA; I) *Ssp* I digested A203 DNA; J) *Ssp* I digested A216 DNA; K) *Ssp* I digested transductant 1 DNA; L) *Ssp* I digested transductant 2 DNA; M) *Ssp* I digested transductant 3 DNA; N) *Hind* III digested λ DNA. B - Southern hybridization of membrane prepared from gel in A. DNA fragments were hybridized to a ^{32}P- labelled *flaA*1 gene probe.
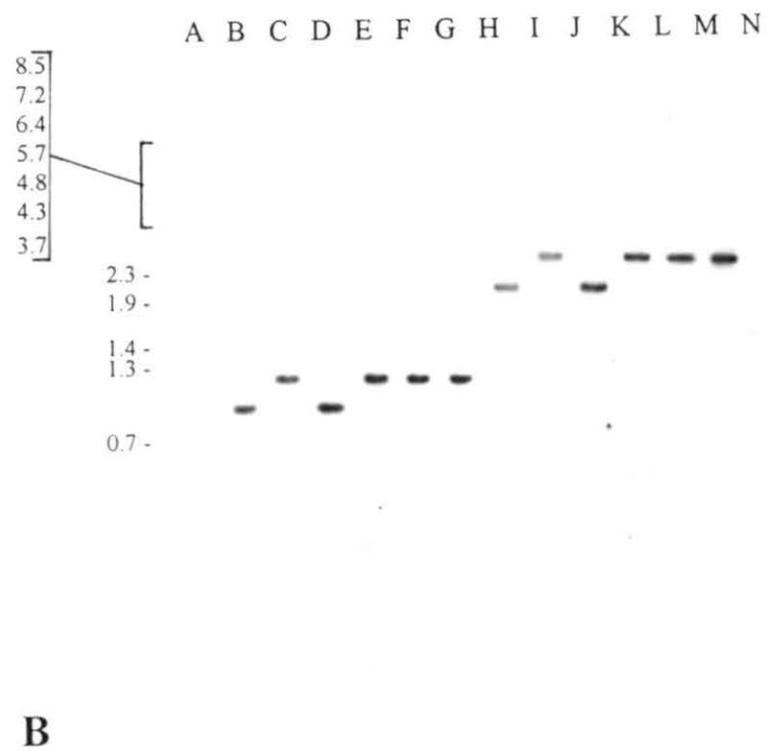
A

B
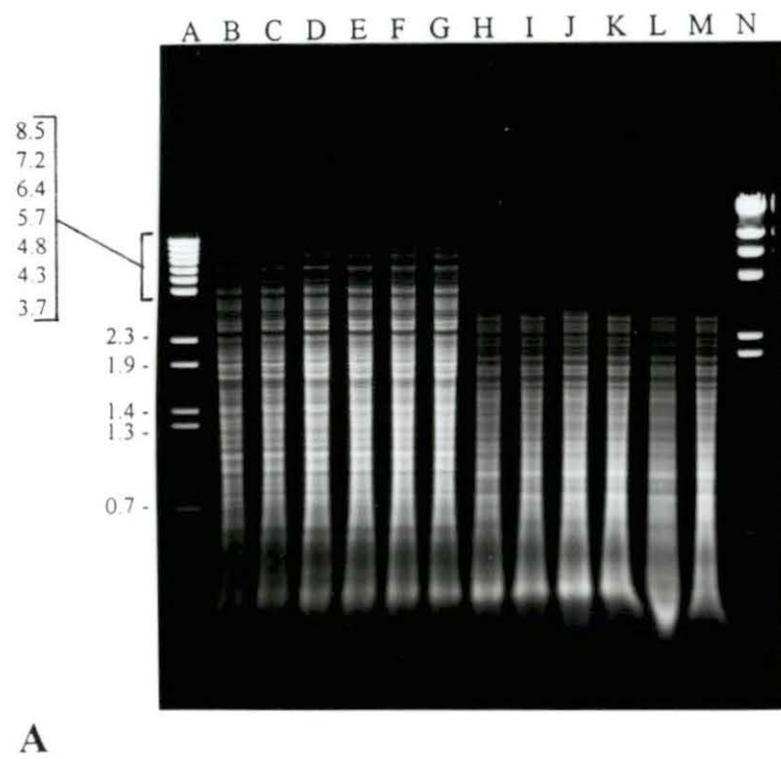
Figure 8. **Restriction enzyme digestion patterns of transductants chromosomal DNA and Southern hybridization of resulting fragments to a radiolabelled *nox* gene probe.** A - restriction digests separated by agarose gel electrophoresis. Lanes: A) *Hind* III digested λ DNA; B) *Ssp* I digested B204 DNA; C) *Ssp* I digested A203 DNA; D) *Ssp* I digested A216 DNA; E) *Ssp* I digested transductant 1 DNA; F) *Ssp* I digested transductant 2 DNA; G) *Ssp* I digested transductant 3 DNA. B - Southern hybridization of membrane prepared from gel in A. DNA fragments were hybridized to a ^{32}P-labelled *nox* gene probe.
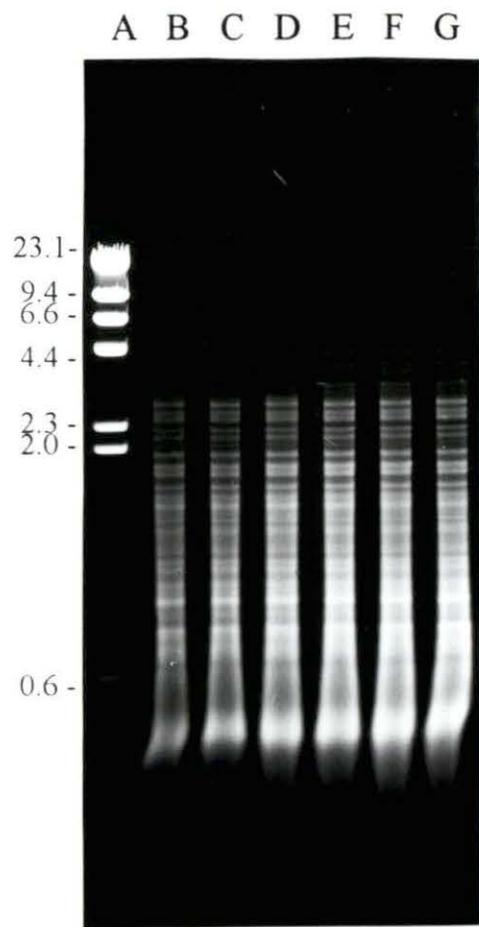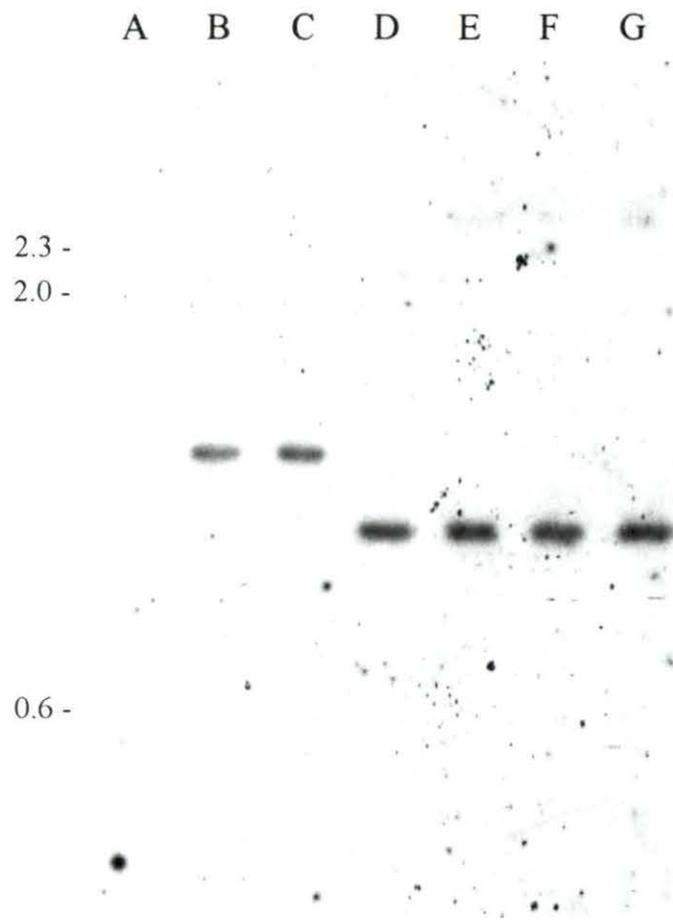
A

B

Figure 9. PCR analysis of transductant genes. **A.** PCR detection of the (*FlaA1::cat*) construct. Primers ERL-10 and ERL-16 used for amplification in lanes B-G and primers 601 and 602 were used for amplification in lanes H-M. Lanes: A) *Hind* III digested λ DNA, B) strain B204, C) strain A216, D) strain A203, E) transductant 1, F) transductant 2, G) transductant 3, H) strain B204, I) strain A216, J) strain A203, K) transductant 1, L) transductant 2, M) transductant 3, N) 100 bp markers (Pharmacia, Inc.). **B.** PCR detection of the (*nox::kan*) construct. Primers 1 and ERL-12 used for amplification in lanes B-G and primers 1 and 120 were used for amplification in lanes H-M. Lanes: A) *Hind* III digested λ DNA, B) strain B204, C) strain A203, D) strain A216, E) transductant 1, F) transductant 2, G) transductant 3, H) strain B204, I) strain A203, J) strain A216, K) transductant 1, L) transductant 2, M) transductant 3, N) 100 bp markers (Pharmacia, Inc.).

A216 were used as controls. When primer ERL-10 (*flaA1* gene) and primer ERL-16 (*cat* gene) were used to amplify DNA no product was obtained from B204 or A216 DNA (Fig. 9a, lanes B and C). This was to be expected since neither strain contains the *flaA1::cat* construct. However, when strain A203 and transductant DNA were used as target DNA, products of expected size (755 base pairs) were amplified (Fig.9A, lane D-G) indicating the presence of the *flaA1::cat* construct. When the primer 601 (*flaA1* gene) and the primer 602 (*flaA1* gene) were used to amplify DNA, products 550 base pairs in size were obtained when using B204 and A216 DNA as targets (Fig 9A, lanes H and I). Again these are products of expected size since the two strains do not contain the *cat* gene within the *flaA1* gene sequence. However, when these two primers, 601 and 602, were used to amplify the *flaA1* gene from strain A203 and transductants the product obtained (1250 base pairs) increased in size as predicted for the *flaA1::cat* construct (Fig. 9A, lanes J- M). This PCR evidence suggests that the transductants do carry the *flaA1::cat* construct at the *flaA1* locus. When primer 1 (*nox* gene) and primer ERL-12 (*kan* gene) were used to amplify DNA no product was obtained from B204 or A203 DNA (Fig. 9B, lanes B and C). This was to be expected since neither strain contains the *nox::kan* construct. However, when strain A216 and transductant DNA were used as target DNA, products of expected size (380 base pairs) were amplified (Fig.9B, lane D-G) indicating the presence of the *nox::kan* construct. When the primer 1 and the primer 120 (*nox* gene) were used to amplify DNA, products 564 base pairs in size were obtained when using B204 and A203 DNA as targets (Fig 9B, lanes H and I). Again these are products of expected size since the two strains do

not contain the *kan* gene within the *nox* gene sequence. However, when these two

primers, 1 and 120, were used to amplify the *nox* gene from strain A216 and transductants

the product obtained (1494 base pairs) increased in size as predicted for the *nox::kan*

construct (Fig. 9B, lanes J- M). This PCR evidence suggests that the transductants do

carry the *nox::kan* construct at the *nox* locus.

From these transduction studies we conclude that chromosomal DNA from strain

A203 was packaged into VSH-1 particles after induction with mitomycin C, the purified

particles then transferred this DNA into strain A216, and recombination at the *flaA1* loci

took place. When VSH-1 particles are purified from strain A216 (*nox::kan*) and added to

growing cells of strain A203 (*flaA1::cat*) transductants are obtained at the same maximum

frequency.

Optimization of transduction frequencies

In order to determine how much time was required for transduction to take place

and how much time was required for maximum transduction frequencies (transductants/

VSH-1 virions) to be obtained a time course experiment was set up (Table 1). Initially

VSH-1 virions were added to cultures and plated onto selective media at various time

points (Table 1; B); however, since the transduction frequencies increased as culture cell

density increased it became apparent that cell density was important in obtaining maximum

transduction frequencies. In order to increase initial cell densities VSH-1 particles were

added to 10 fold concentrated *S. hyodysenteriae* cells in early exponential growth phase

Table 1. Time course transduction studies for *S. hyodysenteriae* using VSH-1 particles purified from strain A203. Particles were added to strain A216 cultures. In sample A, VSH-1 particles were added to a 10 fold concentrated culture of the recipient strain A216. After 1 hour the culture was diluted back to the original concentration.

Sample	Hours after VSH-1(A203) addition	# of transductants /ml	Transduction frequency (# of transductants/ VSH-1 particle	Viable cell count (x 10^8)
Control- no VSH-1 added	8	0	0	6.0
A:VSH-1 (10 fold conc., 1 hr. incubation	2	120	3.2×10^{-7}	1
	4	305	1.3×10^{-7}	2.8
	6	1425	2.7×10^{-6}	4.1
	8	2565	1.5×10^{-6}	5.1
	10	1875	2.0×10^{-6}	3.8
B: VSH-1	2	5	1.0×10^{-8}	1.8
	4	65	5.9×10^{-7}	3.9
	6	405	9.5×10^{-6}	5.3
	8	2455	1.5×10^{-6}	5.8
	10	1475	2.6×10^{-6}	4.2

(Table 1; A).

Optimal transduction frequencies were reached 8 hours after the addition of the VSH-1 particles and occurred at a growth point which corresponded to the highest number of viable cells in cultures (maximum cell density). Transductants begin to appear 2 hours after the VSH-1 particles were added but at a lower frequency. If the VSH-1 particles were incubated for the first hour with 10 fold concentrated A216 cells and then diluted back to original concentration after 1 hour, transductants occurred at higher frequencies after two hours, but the maximum frequency (1.5×10^{-6} transductants per phage particle) was still obtained 8 hours after the addition of VSH-1 particles. When the number of VSH-1 phage particles added to cells was doubled the number of transductants doubled. We conclude from these observations that the maximum number of VSH-1 phage particles added to cells in culture to get the highest number of transductants was not reached and that cell density is important for maximum phage attachment and uptake of the DNA. This would explain why we saw higher transduction frequencies when cells were near their growth peak. Viable cell density was at a maximum. The addition of either $MgSO_4$ (50 mM) or $CaCl_2$ (10mM) to the BHIS growth media did not affect transduction frequencies and therefore may not be important for phage attachment.

Discussion

Bacteriophage particles have been observed attached to cells of *S. hyodysenteriae* in cultures (20) and the use of mitomycin C to induce bacteriophages from the spirochete *S. hyodysenteriae* has been described (8). The experiments in this paper describe the isolation and characterization of a bacteriophage, designated VSH-1, from *S. hyodysenteriae* B204 cells after treatment with mitomycin C. VSH-1 morphology and size is identical to those phages described in previous studies (8,20). The morphology of VSH-1 places it in the *Siphoviridae* family of tailed phages. The possibility that VSH-1 is the only *S. hyodysenteriae* bacteriophage does exist; however, there is little evidence to support that conclusion at present. The bacteriophage VSH-1 packages 7.5 kb fragments of *S. hyodysenteriae* chromosomal DNA when induced with mitomycin C. This type of a phenomenon is unusual but not unique. When *Bacillus subtilis* 168 cells are treated with mitomycin C the bacteriophage PBSX is induced and packages 13 kb fragments of host chromosomal DNA (2,18). Another inducible bacteriophage, PBND8, from a strain of *Bacillus natto* packages host chromosomal DNA in 8 kb fragments (31). PBSX packaging of host chromosomal DNA appears to be semi-random whereas PBND8 packaging of host DNA is random (2,31). Based on restriction digest comparisons of VSH-1 packaged DNA and *S. hyodysenteriae* B204 chromosomal DNA there is no enrichment for any particular DNA band. Therefore, this evidence suggests a random packaging of 7.5 kb B204 chromosomal fragments by VSH-1.

Many PBSX-like bacteriophages have been induced from strains of *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus* and *B. subtilis* with mitomycin C (29). These *Bacillus* phages are termed defective phages. They possess bacteriocin-like killing activities when mixed with susceptible strains, but are not capable of lytic infections or lysogeny (14,18,29). Most research work involving these defective *Bacillus* phages has focused on PBSX phage. PBSX particles are not capable of injecting the DNA they package into a cell (18). From this observation it is not surprising that there has been no evidence reported thus far to suggest that these defective *Bacillus* phages are generalized transducing particles. However, from our studies we have found that VSH-1 not only packages host chromosomal DNA but is also capable of generalized transduction.

Although we did not screen a large number of strains we found no evidence that VSH-1 is capable of lytic growth or possesses bacteriocin like killing activities. It is not surprising lytic growth is not observed since the DNA which VSH-1 delivers to *S. hyodysenteriae* cells would be mostly from the bacterial host. In addition, the small size of the DNA packaged within VSH-1 phage heads (7.5 kb) may be to small to code for all proteins necessary for VSH-1 replication. It is possible that VSH-1 is is capable of lytic growth; however, our findings suggest lytic growth would be a rare event if at all.

The frequency at which VSH-1 transduced the *flaA1::cat* marker and the *nox::kan* marker was 1.5×10^{-6} transductants per phage particle. It becomes difficult to compare the transducing frequency of VSH-1 to that of other phages since frequencies are generally expressed as number of transductants per infective phage particle or plaque forming unit.

VSH-1 does not form plaques and therefore any number of transductants must be related to the total number of phage particles obtained from direct counts. The frequencies of generalized transduction for the well studied P1 phage vary from 3×10^{-4} to 10^{-5} per infective phage particle depending upon the marker being transduced (16). For phage P22 frequencies vary from 10^{-6} to 10^{-9} transductants per infectious particle (33). Most generalized transducing phages package cellular DNA in a small fraction of the total number of particles produced during lysis whereas VSH-1, from our studies, essentially packages cellular DNA fragments in the majority of particles produced after induction. This should also be taken into account when comparing transducing frequencies.

Transferring genes into *S. hyodysenteriae* cells by electroporation to form stable recombinants has been accomplished (21,30). From these experimental results 500 ng of total DNA, consisting of plasmid and construct DNA, was electroporated into 1×10^{10} cells to obtain 5 recombinants. In our experiments approximately 640 ng of DNA, packaged inside VSH-1, was mixed with 5.6×10^8 cells and a total of 1.7×10^4 transductants were obtained. The estimate of the amount of DNA packaged inside VSH-1 particles is based on the assumption that one 7.5 kb molecule is packaged per particle. Taking into account that the electroporation studies used one type of molecule and that our transduction studies involved the introduction of markers by a heterogeneous population of cellular DNA fragments, packaged within VSH-1, transduction appears to be at least 10^6 times more efficient at moving DNA into cells to form stable recombinants than electroporation.

The conditions necessary for spontaneous induction of VSH-1 particles from *S.*

hyodysenteriae cells in nature are unknown as is the importance of VSH-1 to *S. hyodysenteriae* cell survival. Previous investigators have reported the spontaneous appearance of extrachromosomal DNA approximately 7.5 kb in size in DNA preparations made from *S. hyodysenteriae* cultures (5,10). The extrachromosomal DNA they have observed could have originated from VSH-1 virions or VSH-1 like virions. VSH-1 packages 7.5 kb linear fragments of *S. hyodysenteriae* chromosomal DNA after cultures are treated with mitomycin C and is capable of transferring this DNA to other *S. hyodysenteriae* cells. From this study evidence for the existence, within the VSH-1 head, of DNA encoding phage proteins does not exist. It is likely that VSH-1 plays an important role in natural gene transfer in populations of *S. hyodysenteriae* cells. More knowledge about the VSH-1 prophage and the induction of this prophage in nature would give insight into its importance to the ecology *of S. hyodysenteriae*. Additionally, isolation of the prophage DNA may lead to the isolation of autonomously replicating DNA molecules as was accomplished with the *Bacillus* phage PBSX (3). These could then lead to the development of shuttle vectors useful in studying the genetics and ecology of *S. hyodysenteriae* and related spirochetes.

Acknowledgements

We are grateful to Judi Stasko for her assistance with the electron microscopy and to Everett Rosey for providing us with the *flaA1* and *nox* mutants used in our transduction studies.

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SUMMARY AND DISCUSSION

The spirochete *Serpulina hyodysenteriae* is the causative agent of swine dysentery, a disease of economic importance in swine producing countries. In 1978, the existence of bacteriophages in pure cultures of this spirochete and a related spirochete, *Serpulina innocens*, was reported. The bacteriophages were all similar in morphology. Up until now, no other research on these bacteriophages has been reported.

In these studies we have demonstrated the induction of prophages from *S. hyodysenteriae* and *S. innocens* by using the DNA mutagen mitomycin C. Phage particles present after induction are morphologically identical to those reported to exist in pure cultures of *S. hyodysenteriae* and *S. innocens* in 1978. Whether these particles induced from different strains and species represent one prophage or a number of similar related prophages remains to be determined. We chose to focus on the isolation and characterization of the phage (designated VSH-1) induced from the *S. hyodysenteriae* strain B204.

After the induction and purification of VSH-1 particles from *S. hyodysenteriae* B204 it was determined that VSH-1 phage particles packaged 7.5 kb fragments of host chromosomal DNA. No evidence was obtained that indicated phage specific DNA was packaged. VSH-1 particles were found to be incapable of lytic growth on *S. hyodysenteriae* strains and strains of other related species. Bacteriocin-like killing activity which has been reported to be associated with phage particles that package host chromosomal DNA from *Bacillus* was not observed for VSH-1. Transduction studies revealed that VSH-1 particles can transfer the DNA they package between cells of *S. hyodysenteriae* and are therefore considered to be

generalized transducing particles.

Still to be answered are questions reguarding the source of VSH-1 and its functional significance. For instance, is VSH-1 a defective phage or is it a gene transfer system for *S. hyodysenteriae* cells? The evolutionary advantage that a gene transfer system like VSH-1 could give to *S. hyodysenteriae* cells could be very important to the survival of this organism. More knowledge about the prophage could help answer these questions.

Identifying where genes encoding VSH-1 proteins are located in the *S. hyodysenteriae* chromosome followed by the cloning of VSH-1 genes would be a useful step in understanding the nature of the VSH-1 *S. hyodysenteriae* relationship. VSH-1 specific genes may be useful in the development of *S. hyodysenteriae* specific PCR diagnostic tests. In addition, by cloning the prophage genes the isolation of a VSH-1 origin of replication may be obtained. The origin could then be used to construct vectors useful in the study of spirochete genetics. Other useful applications of VSH-1 may include the construction of strains containing multiple mutations or the use of VSH-1 in genetic mapping studies for *S. hyodysenteriae*.

Bacteriophages have been used to treat bacterial infections in animals. VSH-1 does not appear to be able to undergo lytic infections and therefore would not necessarily be useful in treating swine dysentery. However, inducing agents may be useful as therapeutic agents if they are capable of producing a significant decrease in the number of viable cells in active infections.

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ACKNOWLEDGEMENTS

I would first like to thank Dr. Thad Stanton for his patience and guidance on this project. All he has done for me has been greatly appreciated. Secondly, I would like to thank the other members of my graduate committee; Dr. Chris Minion, Dr. John Mayfield, and Dr. Richard Zuerner, for their time and input into the project. Lastly, I would like to thank my wife, Julia, and my son, Benton, for their support.