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In Vivo Transduction with Shiga Toxin 1-Encoding Phage

DAVID W. K. ACHESON,[1] JOACHIM REIDL,[2] XIAOPING ZHANG,[1] GERALD T. KEUSCH,[1] JOHN J. MEKALANOS,[3] AND MATTHEW K. WALDOR[1]*

Division of Geographic Medicine and Infectious Diseases and Tupper Research Institute, Tufts University School of Medicine,[1] and Department of Microbiology and Molecular Genetics and the Shipley Institute of Medicine, Harvard Medical School,[3] Boston, Massachusetts, and Research Center for Infectious Disease, University of Wuerzburg, Wuerzburg, Germany[2]

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To facilitate the study of intestinal transmission of the Shiga toxin 1 (Stx1)-converting phage H-19B, Tn*10d-bla* mutagenesis of an *Escherichia coli* H-19B lysogen was undertaken. Two mutants containing insertions in the gene encoding the A subunit of Stx1 were isolated. The resultant ampicillin-resistant *E. coli* strains lysogenic for these phages produced infectious H-19B particles but not active toxin. These lysogens were capable of transducing an *E. coli* recipient strain in the murine gastrointestinal tract, thereby demonstrating that lysogens of Shiga toxin-converting phages give rise to infectious virions within the host gastrointestinal tract.

Bacteriophages have played a critical role in the evolution of many bacterial pathogens (reviewed in references 3 and 6). Integrating (temperate) phages often alter the properties of the host bacterial cell upon establishment of lysogeny, a process known as phage conversion. The toxins of a number of both gram-negative and gram-positive pathogens have been found to be encoded in the genomes of temperate bacteriophages. The presence of genes encoding virulence factors in phage genomes provides a means for the dissemination of these genes; however, the sites and conditions which favor bacteriophage conversion have not been studied.

The bacteriophage-encoded Shiga toxins (Stxs) are believed to play an important role in the pathogenesis of hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura that may result from human infection with lysogenic *Escherichia coli* (8). Stxs are A-B-type toxins which bind to the host glycolipid Gb3 (11). The enzymatically active A subunit of Stx acts as an rRNA *N*-glycosidase on the eukaryotic 60S ribosomal subunit and thereby inhibits protein synthesis (7). Stx1 and Stx2 are the two principal Stxs found in *E. coli*. Williams Smith and Linggood originally reported in 1971 that lysates of H19B, an *E. coli* O26:H11 strain isolated from an outbreak of infantile diarrhea, could transfer enterotoxinogenicity to *E. coli* K-12 in vitro (21). This phage, known as H-19B, was subsequently shown to encode Stx1 and to have DNA sequence homology to phage λ (10). Another lambdoid phage, designated 933W (isolated from an *E. coli* O157:H7 strain from a patient with hemorrhagic colitis), encodes Stx2 (15, 16). Although O157:H7 is the most commonly isolated *E. coli* serotype associated with Stx production and its related diseases in the United States, more than 30 other *E. coli* serotypes have been found to produce Stx and to be associated with disease (2). Transient Stx production has also been observed in other (non-*E. coli*) bacterial species, including *Enterobacter cloacae* (18) and *Citrobacter freundii* (20) isolated from patients with hemolytic-uremic syndrome. Dissemination of the lambdoid phages which encode Stx1 and Stx2 is the likely mechanism accounting for the spread of these toxins among diverse *E. coli* serotypes and into other bacterial species. In the present study, we sought to determine whether the Stx1-encoding phage H-19B can be transmitted to another *E. coli* strain within the murine intestine. An H-19B phage that was marked with an antibiotic resistance marker was constructed to allow detection of intraintestinal H-19B transmission by transduction.

Construction of H-19B derivatives which encode ampicillin resistance. An antibiotic resistance marker was introduced into the genome of the Stx1-encoding phage H-19B to facilitate the study of the intestinal transmissibility of this lambdoid phage. Tn*10d-bla*, an 861-bp minitransposon which was constructed to allow identification of phage-encoded exported proteins (19), was used for this purpose. This small element generates translational fusions between the mature portion of β-lactamase and the amino-terminal portion of the target gene product (19). Plasmid pJR207 (19), which harbors Tn*10d-bla*, was introduced into *E. coli* C600 H-19B in seven independent transformations, and ampicillin-resistant (Ap^r) colonies were selected as described previously (19). Approximately 10,000 Ap^r colonies were then pooled, washed, and UV induced to produce seven independent H-19B phage pool lysates. The streptomycin-resistant (Sm^r) *E. coli* strain MC4100 (5) was then used as a recipient strain for transduction with these lysates. Ampicillin-resistant colonies were purified and tested for lysogeny by cross-streaking. Lysogens were then used to produce new phage stocks which were subsequently used to lysogenize MC4100. All lysogens were resistant to ampicillin, thereby confirming the linkage of the Ap^r gene to the H-19B phage genome.

Subsequently one representative H-19B Tn*10d-bla* insertion from each of the seven independent phage pool lysates was characterized by restriction enzyme and Southern blot analysis. All Tn*10d-bla* insertions were located within the same 8.1-kb *Eco*RI fragment known to contain the *stxAB* operon (9) (Fig. 1). Two of the Tn*10d-bla* fusions were further characterized by subcloning the fusions from the corresponding phage DNA and determining the precise location of the 5′ fusion junctions by using an oligonucleotide corresponding to the 5′ end of the *bla* gene as a sequencing primer. We found that the Tn*10d-bla* insertion in H-19B-Ap1 was fused to the coding sequence

* Corresponding author. Mailing address: Division of Geographic Medicine and Infectious Diseases, NEMC 041, 750 Washington St., Boston, MA 02111. Phone: (617) 636-7618. Fax: (617) 636-5292. E-mail: matthew.waldor@es.nemc.org.

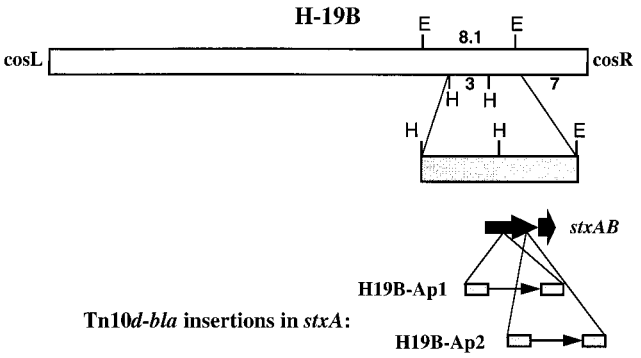

FIG. 1. Tn*10d-bla* insertions in the H-19B genome. The 3′ end of the *stxAB* operon is encoded on a 2.8-kb *Hin*dIII (H)-*Eco*RI (E) fragment (10). The orientation of the *stxAB* operon along with the locations of the Tn*10d-bla* insertions in H-19B-Ap1 and H-19B-Ap2 is shown.

corresponding to amino acid 70 of the A subunit of Stx1, while the Tn*10d-bla* insertion in H-19B-Ap2 was fused to the sequence corresponding to amino acid 167 of the A subunit of Stx1 (Fig. 1). As anticipated, both of these Tn*10d-bla* insertions were fused to the correct reading frame of *stxA*. These results reconfirm the utility of Tn*10d-bla* insertion mutagenesis for the identification of phage-encoded exported proteins.

The properties of MC4100 H-19B, MC4100 H-19B-Ap1, and MC4100 H-19B-Ap2 were compared. All three lysogens spontaneously produced approximately the same number of virions as measured by PFU per cell (Table 1). Also, virion production from all three lysogens was induced more than 4 orders of magnitude with mitomycin C (Table 1). The Tn*10d-bla* insertion in MC4100 H-19B-Ap1 and MC4100 H-19B-Ap2 resulted in the complete abrogation of detectable Vero cell cytotoxicity in cell-free supernatants of mitomycin C-induced cultures (data not shown). However, these insertions did not prevent production of the B subunit of Stx1 from MC4100 H-19B-Ap1 or MC4100 H-19B-Ap2 as detected by an enzyme-linked immunosorbent assay (1). Thus, MC4100 H-19B-Ap1 and MC4100 H-19B-Ap2 are Sm^r *E. coli* strains harboring Ap^r gene-marked detoxified H-19B prophages which, in vitro, produce numbers of infectious H-19B particles similar to those produced by MC4100 H-19B. We used these two Ap^r gene-marked H-19B lysogens to study the intraintestinal transmission of H-19B.

Intraintestinal transduction of phage H-19B. Oral administration of streptomycin to adult mice has been shown to facilitate the colonization of the murine large intestine with laboratory strains of *E. coli* (4). We used this model of intestinal colonization in streptomycin-treated mice to investigate whether MC4100 H-19B-Ap1 and MC4100 H-19B-Ap2 produce infectious virions within the intestine. Recovery of intestinally derived transductants of a recipient strain was used as a means to detect intraintestinal virion production.

TABLE 1. Virion production by Ap^r gene-marked H-19B lysogens

Strain	Phage titer (PFU/10^4 CFU)[a]		Fold induction[c]
	Before mitomycin treatment	After mitomycin treatment[b]	
MC4100 H-19B	4	25×10^4	6.3×10^4
MC4100 H-19B-Ap1	11	64×10^4	5.8×10^4
MC4100 H-19B-Ap2	7	18×10^4	2.6×10^4

[a] Serial dilutions of chloroform-treated supernatants were plated on *E. coli* C600.
[b] Cultures with an optical density at 600 nm of 0.5 were treated with mitomycin C at 250 ng/ml for 4 h.
[c] Calculated by dividing the number of PFU after mitomycin C treatment by the number of PFU prior to mitomycin C treatment.

TABLE 2. Detection of intestinally derived transductants at 24 h

Donor strain[a]	Mouse	No. of cells in stool (CFU/ml)[b]			Transductant ratio[f]
		Recipient[c]	Donor[d]	Transductants[e]	
MC4100 H-19B-Ap1	1	6.2×10^5	2.1×10^6	2.0×10^2	9.5×10^{-5}
	2	8.8×10^6	1.1×10^7	4.2×10^3	3.8×10^{-4}
	3	4.4×10^6	4.8×10^6	8.0×10^2	1.7×10^{-4}
	4	2.2×10^6	9.1×10^5	3.8×10^1	4.2×10^{-5}
MC4100 H-19B-Ap2	5	7.6×10^6	5.8×10^4	4.0×10^1	6.8×10^{-4}
	6	6.8×10^6	8.9×10^4	2.1×10^2	2.3×10^{-3}

[a] Approximately 1×10^9 MC4100 H-19B-Ap1 cells or 4×10^6 MC4100 H-19B-Ap2 cells were inoculated intragastrically as donors. Approximately 10^9 MC4100 *srl*::Tn*5* (Kn^r) cells were intragastrically inoculated 45 min later as potential recipients.
[b] Individual pellets were resuspended in 1 ml of Luria broth.
[c] The recipient cell number was determined by counting the number of Sm^r Kn^r LacZ$^-$ CFU.
[d] The donor cell number was determined by counting the number of Sm^r Ap^r LacZ$^-$ CFU.
[e] The number of transductants was determined by counting the number of Sm^r Kn^r Ap^r LacZ$^-$ CFU. The assay detection limit is 10 CFU/ml.
[f] Calculated by dividing the number of transductants by the number of donor cells.

Streptomycin (0.6 mg/ml) was added to water given to CD-1 mice for 48 h prior to the intragastric administration of MC4100 H-19B-Ap1 (Sm^r Ap^r LacZ$^-$), the phage donor strain. Stools collected from mice immediately prior to administration of the phage donor strain were found to have no detectable Sm^r LacZ$^-$ CFU. Forty-five minutes after the administration of the phage donor strain, the mice were intragastrically inoculated with a differentially marked potential phage recipient strain, AK16 (MC4100 *srl*::Tn*5*), which is identifiable as Sm^r kanamycin-resistant (Kn^r) LacZ$^-$ CFU. Approximately 1×10^9 cells of the MC4100 H-19B-Ap1 lysogen and 2×10^9 cells of AK16 were inoculated per mouse. Individual stool samples (pellets) were collected on each of the four days following inoculation, homogenized in 1 ml of Luria broth, and then plated on L agar containing antibiotics to allow the determination of the number of donor CFU (Sm^r Ap^r LacZ$^-$), recipient CFU (Sm^r Kn^r LacZ$^-$), and transductant CFU (Sm^r Kn^r Ap^r LacZ$^-$) in each pellet. Transductants (Ap^r colonies of MC4100 *srl*::Tn*5*) were recovered in stool samples on each of the four days following inoculation. Twenty-four hours postinoculation, there was approximately one transductant per 10^4 donor cells recovered in stool (Table 2). Each of 10 randomly picked Ap^r recipient colonies tested was found to produce StxB by an enzyme-linked immunosorbent assay (1). In addition, filtered supernatants from mitomycin C-induced cultures from each of these AK16 Ap^r transductant colonies was capable of transducing Ap^r to a new recipient strain. These results indicate that H-19B-Ap1 phage transduction accounted for the intestinal transmission of the Ap^r marker. There was a decrease in the frequency of transductants per recipients during the 4-day period when stools were tested (data not shown). At the end of this period the animals were sacrificed, and the frequency of transductants in cecal homogenates was found to be similar to the frequency of transductants in the stool homogenates.

A similarly designed experiment was carried out with MC4100 H-19B-Ap2 as a donor strain in two mice. In this experiment,

only 4×10^6 donor cells (more than 2 log units fewer donor cells than used in the previous experiment) were inoculated per mouse. Twenty-four hours later stool samples from both mice contained transductants at approximately the same frequency per donor cell as was observed when MC4100 H-19B-Ap1 was used as a donor (Table 2). Again all tested intestinally derived transductants produced StxB. The detection of H-19B-Ap^r transductants in stool samples and cecal homogenates demonstrates that infectious virions are produced within the host intestine and furthermore that these virions are capable of transducing other *E. coli* strains within the gastrointestinal tract.

Host environments may be the common site where new pathogenic strains evolve. Our demonstration that an *E. coli* H-19B lysogen produces infectious phage within the murine gastrointestinal tract suggests that the site of the emergence of the many Stx-producing *E. coli* serotypes and the Stx-producing non-*E. coli* genera may have been within the mammalian gastrointestinal tract. Similarly, a recent report by members of our group that *Vibrio cholerae* CTXϕ lysogens produce infectious virions within the gastrointestinal tract (12) may suggest that new toxinogenic *V. cholerae* serotypes, such as *V. cholerae* O139, may arise through phage-mediated transduction of nontoxinogenic strains within the host gastrointestinal tract. Similar phage conversion events may occur on other mucosal surfaces as well. Pappenheimer and Murphy reported a case of corynephage conversion of a nontoxinogenic *Corynebacterium diphtheriae* strain to toxinogenicity that probably occurred within a woman's upper respiratory tract (17). It has been proposed that phage conversion within a nonimmune host provides an efficient mechanism for the rapid dissemination of phage-encoded virulence genes (3). It is possible that specific mammalian host signals induce bacterial gene transfer events, as has been described for the plant pathogen *Agrobacterium tumefaciens* (13).

In the present study, the antibiotic resistance gene-marked H-19B facilitated our ability to monitor intraintestinal phage production by lysogens. The *bla*-marked H-19B phage will also facilitate the study of the bacterial host range for this virus and the study of intestinal factors, such as diet, and therapeutic interventions, such as antibiotics, that may influence the rate of intestinal H-19B transduction. Our previous work indicates that SOS induction of Stx-converting bacteriophages exerts a regulatory effect on Stx production both by increasing the number of toxin gene copies and by the increased expression of a phage-encoded regulatory molecule (14). Future studies will address the question of whether intraintestinal Stx production and Stx-related disease require the in vivo induction of Stx-converting bacteriophages from lysogens.

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