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Comparative Survival of Free Shiga Toxin 2-Encoding Phages and *Escherichia coli* Strains outside the Gut

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The behavior outside the gut of seeded *Escherichia coli* O157:H7, naturally occurring *E. coli*, somatic coliphages, bacteriophages infecting O157:H7, and Shiga toxin 2 (Stx2)-encoding bacteriophages was studied to determine whether the last persist in the environment more successfully than their host bacteria. The ratios between the numbers of *E. coli* and those of the different bacteriophages were clearly lower in river water than in sewage of the area, whereas the ratios between the numbers of the different phages were similar. In addition, the numbers of bacteria decreased between 2 and 3 log units in in situ survival experiments performed in river water, whereas the numbers of phages decreased between 1 and 2 log units. Chlorination and pasteurization treatments that reduced by approximately 4 log units the numbers of bacteria reduced by less than 1 log unit the numbers of bacteriophages. Thus, it can be concluded that Stx2-encoding phages persist longer than their host bacteria in the water environment and are more resistant than their host bacteria to chlorination and heat treatment.

Shiga-toxin producing *Escherichia coli* (STEC) strains are associated with diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome. Serotype O157:H7 constitutes the main STEC causative serotype, although others, such as O18, O26, O111, O128, and O138, are also involved (20). STEC isolated from patients with hemorrhagic colitis produces two immunologically distinct toxins known as Shiga toxin 1 (Stx1) and Stx2.

Several authors have reported that capability for the production of Stx1 and Stx2 in *E. coli* is conferred by toxin-converting bacteriophages (21, 27). A wide range of Shiga-toxin-converting bacteriophages has been described (25, 28). Dissemination of the lambdoid phages that encode Stx1 and Stx2 may account for the spread of these toxins to several *E. coli* serotypes and other bacterial species. Recently, it has been shown that an Stx1-encoding phage can be transmitted from a lysogenic strain to another strain within the murine intestine and that lysogens of Shiga toxin-converting phages give rise to infectious virions within the host gastrointestinal tract (1).

Significant numbers of bacteriophages infecting *E. coli* O157:H7 and carrying the Stx2 gene are found in sewage in different geographical areas (18, 19). The role of these phages, which are excreted by humans and animals, in the dissemination of Stx1 and Stx2 genes and the sites and conditions that favor bacteriophage conversion remain to be elucidated before we can understand the epidemiology and pathogenesis of *E. coli* O157 infections. The role of these phages in the dissemination of Stx1 and Stx2 genes may depend on their persistence outside the human and animal gut.

Experiments presented herein were envisaged to assess whether Stx2-encoding bacteriophages persist in the environment more successfully than their host bacteria, as many other phages infecting enteric bacteria do (10), and consequently whether the Stx2 gene incorporated in free bacteriophages persists outside the gut more successfully than the genes incorporated in the bacterial genome. Thus, the numbers in river water, the survival rates in river water, and the resistance to chlorination and pasteurization of phages and their bacterial hosts were compared. The protocol used in this study allows detection of all naturally occurring bacteriophages infecting *E. coli* O157:H7 and encoding Stx2 and, with the suitable primers, is applicable to any gene or phage involved in phage conversion, as for example, Stx1-encoding phages.

The presence of *E. coli* was evaluated by passage of water samples through 0.44-μm-pore-diameter membrane filters according to standard methods (2). Filters were then placed on plates of Fluorocult (Merck GgaA, Darmstast, Germany). This medium allows the detection of *E. coli* strains other than O157:H7, which is 4-methylumbelliferyl-β-D-glucuronide negative (15). To study the inactivation of *E. coli* O157:H7, counting in seeded samples was performed by using M-FC agar according to standard methods (2) and incubation at 37°C. Samples were always seeded with numbers of *E. coli* O157:H7 at least 1.5 log units greater than the numbers of naturally occurring fecal coliform bacteria. Somatic coliphages, defined as those infecting strain *E. coli* WG5 (11), and bacteriophages infecting strain ATCC 43888 of *E. coli* O157:H7 were counted by the double agar layer method on modified Scholten's agar according to standard methods (11).

The presence of viable phages infecting *E. coli* O157:H7 and carrying the Stx2 gene in a given volume of water was determined as described previously (18). Briefly, phages in the water sample were partially purified and treated with 10 U of DNase ml^{-1} for 30 min (24) to remove free DNA. Then, liquid cultures containing approximately 2×10^8 cells per ml of *E. coli* O157:H7 ATCC 43888, which does not possess the gene encoding for Stx2, were inoculated with partially purified bacteriophages corresponding to the volume of sample to be tested. The enrichment was performed in modified Scholten's broth (11). After overnight incubation at 37°C, phages in the supernatants of the cultures were purified. The DNA of the purified phages was then extracted and subjected to a two-step PCR DNA amplification, as described previously, with (18) the following primers: upper primer 5′-GCGTTTTGACCATCTTC GT-3′ and lower primer 5′-ACAGGAGCAGTTTCAGACA G-3′ for the first-round PCR and upper primer 5′-TAATAC

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TABLE 1. Occurrence and levels of different microbial parameters in the Llobregat river[a]

Value	Concn of:			
	E. coli (CFU/ml)	Somatic coliphages (PFU/ml)	Phages infecting O157:H7 (PFU/ml)	Phages infecting O157:H7 and carrying Stx2 gene (MPN of phages/ml)
Mean ± SD	59 ± 12	350 ± 254	10 ± 2	0.18 ± 0.12
Minimum	39	92	8	0.07
Maximum	73	540	15	0.43

[a] Values obtained from testing of eight different samples in duplicate.

GGCAACAAATACT-3′ and lower primer 5′-TGATGAAAC CAGTGAGTGA-3′ for the nested PCR. The conditions for amplification were 1 cycle at 94°C; 35 cycles at 94°C for 1.5 min, 55°C for 1 min, and 72°C for 1 min; and, finally, 1 cycle at 72°C for 4 min for the first-round PCR; for the nested PCR, the amplification program was as described above, but with an annealing temperature of 50°C. Five microliters of the amplified DNA mixture was analyzed for amplification products by gel electrophoresis on a 3% agarose gel and stained with ethidium bromide. Each amplification was performed in duplicate. *E. coli* O157:H7 ATCC 43889, which produces Stx2, and bacteriophage 933 W, obtained by mitomycin C lysogenic induction of *E. coli* strain C600(933W) (17), were used as positive controls. Negative controls with double-distilled water used as the template were processed in parallel with each sample (the number of controls was equal to the number of test samples). Triplicate analyses were run for various volumes of each water sample according to the protocol described above. Negative and positive signals observed on agarose gels were recorded, and the most probable numbers (MPN) (6) of bacteriophages infecting O157:H7 and carrying the Stx2 gene were calculated.

The presence of phages infecting *E. coli* O157:H7 and carrying the Stx2 gene was studied at one site of the Llobregat river, selected because the water there consistently contains numbers of fecal coliform bacteria and somatic coliphages approximately 3 orders of magnitude lower than the numbers in sewage. The numbers of phages infecting *E. coli* O157:H7 and carrying the Stx2 gene, determined by the MPN technique, averaged 0.18 bacteriophages per ml (Table 1). The values for *E. coli* and coliphages, both somatic coliphages and those infecting strain O157:H7, were consistent (Table 1) and indicate that fecal microorganisms at the studied site have suffered natural inactivation. It has been described that the ratio of *E. coli* cells to bacteriophages decreases as a consequence of natural inactivation (10), and in the samples from Llobregat river the *E. coli*-to-bacteriophage ratios were significantly lower than those in sewage from the area. Indeed, the ratios of *E. coli* to somatic coliphages and *E. coli* to phages infecting O157:H7 averaged 0.16 and 5.6 in the Llobregat river samples and were >1 and >100, respectively, in raw sewage from the area (18, 19). If we compare reported estimates (18) of the numbers of bacteriophages infecting *E. coli* O157:H7 and carrying the Stx2 gene in sewage of the area (3 to 5 bacteriophages per ml) with the numbers estimated to be present in the Llobregat river (0.18 bacteriophages per ml), we can conclude that this group of phages survive under natural conditions similarly to other phages and significantly longer than their host bacteria. Although data on the numbers of *E. coli* strains carrying the Stx2 gene in polluted waters are scarce (8, 14, 15, 29), they indicate that the numbers would range from 1/100 to <1/1000 of those of *E. coli* lacking the Stx2 gene. Consequently, in polluted surface waters, the number of Stx2 genes in bacteriophages may equal or surpass the number of Stx2 genes in their bacterial hosts.

TABLE 2. Decimal reduction[a] of the numbers of different microbial parameters after 168 h in the in situ inactivation experiments

Experiment no.	Decimal reduction of the no. of:				
	E. coli[b] CFU	*E. coli* O157:H7[c] CFU	Somatic coliphages (PFU)	Phages infecting O157:H7 (PFU)	Phages infecting O157:H7 and carrying the Stx2 gene (MPN)
1	>2.5	2.9	1.6	1.7	>1
2	>2.5	2.4	0.9	1.2	>1
3	>3.0	>3.0	2.6	2.4	1.9
4	2.2	2.7	1.8	1.4	1.6
5	1.9	2.5	1.8	1.4	1.1
6	2.3	2.1	1.7	1.3	0.5

[a] Decimal reduction calculated as the decrease in log units.
[b] Naturally occurring *E. coli*.
[c] Seeded *E. coli* O157:H7.

Additionally, the survival of bacteriophages and their hosts in river water was studied through in situ survival experiments as follows. Raw urban sewage was diluted 1/50 in river-water samples (hardness, 380 mg of $CaCO_3$ per liter; alkalinity (complete alkalinity titer [CAT]), 181 mg per liter and pH 7.8) from the site described above. The mixtures were then placed in dialysis tubes, (each with a cutoff of 14,000 daltons), which were sealed and placed in the river at a depth of 20 to 25 cm at the same site where the river water was collected. In parallel, to study its inactivation, *E. coli* O157:H7 was inoculated to a final concentration of 10^5 CFU/ml, which surpassed the number of fecal coliforms by more than 1.5 log units, in another dialysis tube containing 100 ml of the above-mentioned mixture. Inactivation of O157:H7 was studied in parallel in order to avoid phage replication during the inactivation experiment. All the experiments were performed in the late summer, when the water temperature ranged from 14 to 25°C. Samples were taken 1 week later, and microbial parameters were counted as indicated above. Bacteriophages infecting *E. coli* O157:H7 and carrying the Stx2 gene survived similarly to somatic coliphages and all the bacteriophages infecting *E. coli* O157:H7, and more successfully than naturally occurring *E. coli* and seeded *E. coli* O157:H7 (Table 2). The survival rates of O157:H7 in river water reported here are very similar to those described elsewhere (16). Decimal reductions of phages were significantly (Mann-Whitney U test, $P < 0.01$) lower than decimal reductions of bacteria. The low decay in the number of bacteriophages can be attributed either to their resistance to inactivation or to replication, which, although very improbable (30), cannot be ruled out (13). The differences in the decimal reductions of the various groups of bacteriophages studied were not significant (Mann-Whitney U test, $P > 0.05$). These results are consistent with reports indicating that bacteriophages survive better than bacteria in natural water environments (10) and explain the ratios found in the river water.

Various treatments, such as chemical disinfection and pasteurization, are applied to eliminate microorganisms of fecal origin in water and food. For example, chlorination is applied to disinfect both secondary effluents and drinking waters, and

TABLE 3. Decimal reduction[a] of the numbers of different microbial parameters after chlorination of sewage

Experiment no.	Chlorination conditions		Decimal reduction of the no. of:				
	Total residual chlorine (mg/liter)	Time (min)	*E. coli*[b] CFU	*E. coli* O157:H7[c] CFU	Somatic coliphages (PFU)	Phages infecting O157:H7 (PFU)	Phages infecting O157:H7 and carrying the Stx2 gene (MPN)
1	10	20	3.9	4.0	0.5	0.3	>0.3, <0.8[d]
2	10	20	3.6	3.5	0.6	0.5	>0.4, <0.6
3	10	20	4.4	4.5	0.5	0.4	>0.3, <0.8
4	10	20	3.6	NT	0.8	0.7	0.4
5	10	30	4.4	NT	0.8	0.8	0.6
6	20	30	>4.8	NT	1.0	1.1	1.2

[a] Decimal reduction calculated as the decrease in log units.
[b] Naturally occurring *E. coli*.
[c] Seeded *E. coli* O157:H7. NT, not tested.
[d] Decimal reduction was between the indicated values. When the result of one of the triplicates of one of the volumes was missing, the inactivation was considered to be between the MPN values calculated considering the missing value as positive or negative.

heat is applied to both sludges and food. Both chlorination and pasteurization inactivate bacteria more successfully than they do bacteriophages (10, 22). We tested the effect of chlorination and pasteurization on various naturally occurring bacteriophages, including those infecting *E. coli* O157:H7 and carrying the Stx2 gene, and on naturally occurring *E. coli* and seeded *E. coli* O157:H7. To assess the resistance of bacteriophages and their bacterial hosts to chemical disinfection, raw sewage samples (hardness, 368 mg of $CaCO_3$ per liter; alkalinity [CAT], 202 mg per liter and pH 7.9) were treated at 20 ± 2°C with various chlorine concentrations for the times indicated in Table 3. Chlorination was done by addition to the sewage samples of volumes of a hypochlorite solution calculated to provide the indicated amounts of total residual chlorine in the mixture. Pasteurization was performed by heating a sewage sample to 60°C for 30 min. In order to study the inactivation of *E. coli* O157:H7, in both types of inactivation experiments *E. coli* O157:H7 ATCC 43888 was seeded before the treatment of sewage to a final concentration of 10^6 CFU/ml, well over the concentration of naturally occurring fecal coliform bacteria in the sample. In both types of inactivation experiments, samples were analyzed before and after the treatment for all parameters described above. Data in Tables 3 and 4 show that phages, including those encoding Stx2, survive chlorination and pasteurization significantly (Mann-Whitney U test, $P < 0.01$) more successfully than *E. coli* strains. Thus, chlorine doses that reduced the numbers of naturally occurring *E. coli* and seeded *E. coli* O157:H7 by more than 3.5 log units inactivated bacteriophages, including those encoding Stx2, by less than 1 log unit (Table 3). Heat treatment that caused a reduction of more than 4 log units in the numbers of both naturally occurring *E. coli* and seeded *E. coli* O157:H7 reduced the numbers of the bacteriophages studied, including those encoding Stx2, by less than 1 log unit (Table 4).

TABLE 4. Decimal reduction[a] of the numbers of different microbial parameters after pasteurization of sewage

Experiment no.	Decimal reduction of the no. of:				
	E. coli[b] CFU	*E. coli* O157:H7[c] CFU	Somatic coliphages (PFU)	Phages infecting O157:H7 (PFU)	Phages infecting O157:H7 and carrying the Stx2 gene (MPN)
1	>4.2	>4.5	0.5	0.3	>0.4, <0.6[d]
2	>4.1	>4.6	0.7	0.6	>0.3, <0.8
3	>4.0	>4.4	0.6	0.4	>0.3, <0.6

[a] Reduction calculated as the decrease in log units.
[b] Naturally occurring *E. coli*.
[c] Seeded *E. coli* O157:H7.
[d] Decimal reduction was between the indicated values. When the result of one of the triplicates of one of the volumes was missing, the inactivation was considered to be between the MPN values calculated considering the missing value as positive or negative.

We conclude that naturally occurring bacteriophages that infect *E. coli* O157:H7 and carry the Stx2 gene persist in the water environment more successfully than their host bacteria and are more resistant than their host bacteria to chlorination and heat treatment. We also infer that the Stx2 gene incorporated in free bacteriophages persists outside the gut much more successfully than the genes incorporated in the bacterial genome. Bacteriophages may thus be the main reservoir of the Stx2 gene in nature, confirming the hypothesis framed some years ago by Reanney (23), which postulates that genes of a bacterial species may exist in ecologically dispersed form among the population of bacteriophages infecting the species. To ascertain whether conversion occurs in environments such as water and food in which high numbers of host bacteria and bacteriophages occur or in the gut of man and animals after ingestion of free bacteriophages requires further investigation. The extent of the role of free phages in the horizontal transmission of Stx2 genes requires further research as well, although existing data indicate that it may be very important. Indeed, STEC strains belong to different serotypes (20), and different molecular methods have shown a great heterogeneity within STEC serotypes (4, 7, 9, 12). Clonal analysis of *E. coli* of different serotypes has shown that these STEC serotypes are diverse mixtures of bacteria, often composed of a variety of clones that have acquired distinct combinations of virulence factors (7). Moreover, rapid changes, known as clonal turnover, had been shown to occur for different serotypes, for example, O157:H7 and O146:H2, within the population of individual patients or animals (4, 12). Existing evidence points to Shiga toxin (Stx)-encoding phages as a major cause of such genetic diversity (4, 5). Also, it has been recently reported that Stx-encoding phages can infect and convert different *E. coli* serotypes (26) and even *Shigella* (3). It seems clear that Stx-encoding phages may be the source of genetic diversity among STEC's and that phages outside the gut, because of their numbers and persistence, may be an important source of new toxigenic strains in natural environments.

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