Phage-Mediated Shiga Toxin 2 Gene Transfer in Food and Water[▽]

Lejla Imamovic,¹ Juan Jofre,¹ Herbert Schmidt,² Ruth Serra-Moreno,¹ and Maite Muniesa^{1*}

Department of Microbiology, Faculty of Biology, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain,¹ and Institute of Food Science and Biotechnology, Department of Food Microbiology, University of Hohenheim, Garbenstrasse 28, 70599 Stuttgart, Germany²

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Shiga toxin (*stx*) transduction in various food matrices has been evaluated with lysogens of Stx phages. *stx* transduction events were observed for many phages under appropriate conditions. Transduction did not occur at low pH and low temperatures. A total of 10^3 to 10^4 CFU ml⁻¹ was the minimal amount of donor and recipient strains necessary to generate transductants.

Shiga toxin-producing *E. coli* (STEC) strains are emergent food-borne pathogens (6, 11, 18, 27, 34). *E. coli* O157:H7 and other serotypes have been implicated in human diseases (18, 27, 34). The cardinal cause of virulence of STEC is the production of Shiga toxins (Stx) (6, 18). *stx* genes are carried in the genomes of temperate phages (15, 26), which are efficient vectors for the transfer of *stx* and play an important role in the evolution of new pathogens (31, 38). Stx phages can be induced from the lysogenic strain (19, 20, 41). As a result of prophage induction, bacterial host cells lyse and release free phage particles that can infect other bacteria and transduce the *stx* gene (2, 9, 17, 22, 28, 33, 35).

Although STEC strains are food-borne pathogens (8, 10, 11, 32, 36), there are no data on the potential transduction that could occur in food. Some food treatments can increase the rate of induction of prophages and the number of transduction events (3). In order to guarantee food quality, it is necessary to evaluate whether transduction by Stx phages can occur in food and the conditions that could favor *stx* transfer in food samples.

Sixty-two strains (Table 1) were tested for susceptibility to Stx phages. From this strain collection, tetracycline-resistant (Tc^r) and chloramphenicol-susceptible (Cm^s) recipients were selected. Two nalidixic acid-resistant (Nal^r) laboratory strains were also used as recipients: *E. coli* strain WG5 (ATCC 700078) (4) and strain C600^{Nal}, generated in our laboratory. The donor strains used (Table 1) were *E. coli* lysogens harboring Stx phages with a modified *stx::cat* gene (28, 29, 30).

Luria-Bertani (LB) agar or broth was used for routine bacterial propagation. Chromocult coliform agar (Merck, Darmstadt, Germany) was used for the evaluation of background flora and for the detection of transductants. When necessary, media were supplemented with Cm (25 µg ml⁻¹), Tc (25 µg ml⁻¹), and Nal (100 µg ml⁻¹) (Sigma-Aldrich, Steinheim, Germany).

Food samples were purchased from local retailers. Liquid samples were commercial bottled water and ultra-high-temperature-processed whole milk. Some additional experiments in-

cluded 100% pure orange juice (pH 3.7). Twenty-five milliliters of a liquid sample was used for the transduction experiments. Solid samples used were salad and ground beef. Salad was a commercially prepared mixture of frisée lettuce, corn salad, and radicchio (red chicory). The beef used was freshly minced on request at local supermarkets. Twenty-five grams of minced beef or salad was diluted 1:4 (wt/vol) in one-quarter-strength Ringer's solution. The mixture was placed in stomacher bags with filters (Afora, Barcelona, Spain) and then homogenated for 1 min in a stomacher (Masticator; IUL Instruments GmbH, Königswinter, Germany) for the transduction experiments.

To prevent the interference of the present background flora and phages infecting *E. coli* with the transduction experiments, 25 ml or g of a sample was tested for the presence of phages infecting *E. coli* strains WG5, C600^{Nal}, and DH5α (coliphages) by double-agar layer methods as described previously (4) and for antibiotic-resistant flora using Chromocult agar with antibiotics (Tc/Cm or Nal/Cm).

The food samples were also analyzed for the presence of *stx*₂ before the experiments. To this end, 25 ml or g of sample was used for DNA extraction with the QIAamp DNA blood mini kit (Qiagen, Valencia, CA). The presence of the *stx*₂ gene was examined by PCR, as described below.

One milliliter of donor strain culture (lysogen carrying an *stx::cat* phage) (Table 1) and 1 ml of the recipient strain culture (Nal^r Cm^s or Tc^r Cm^s) (Table 1) were added to each sample and homogenized. The inoculated samples were then statically incubated at the corresponding temperature for 18 h. To detect transductants, the samples were 10-fold diluted and 0.1 ml was plated onto Chromocult agar plus antibiotics (Nal plus Cm or Tc plus Cm) and incubated overnight at 37°C. Those colonies grown on Chromocult agar plus antibiotics showing the same color as the recipient were considered Stx transductants. At least 10 colonies from each experiment were restreaked onto a new plate of Chromocult agar plus antibiotics and confirmed by PCR and sequencing.

Variations of this protocol, including different incubation temperatures (37, 30, 25, 22, 15, and 4°C) and lower pH, were assayed. The minimal amounts of the recipient and donor strains required to produce transductants were also evaluated.

PCRs were performed with primer pair S2Aup/3Cm3pKD3 and primer pair Cm5/Cm3 (29) to confirm the presence of recombinant phages in the suspected lysogens. Primer pair

* Corresponding author. Mailing address: Department of Microbiology, Faculty of Biology, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain. Phone: 34934039386. Fax: 34934039047. E-mail: mmuniesa@ub.edu.

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TABLE 1. Bacterial strains used in this study

Bacteria	Strain	No. of strains	Source	Genotype ^a	Antibiotic susceptibility	Reference(s) or source
Donor strains						
<i>E. coli</i> K-12 strain C600	LysφA9Cm	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	29
<i>E. coli</i> K-12 strain C600	Lys933WCm	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	29
<i>E. coli</i> K-12 strain DH5α	LysφA557Cm	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	29
<i>E. coli</i> K-12 strain DH5α	LysφVTB55Cm	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	29
<i>E. coli</i> K-12 strain DH5α	LysφA312Cm	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	29
<i>E. coli</i> K-12 strain DH5α	LysφA549Cm	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	29
<i>E. coli</i> K-12 strain DH5α	LysφA534Cm	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	29
<i>E. coli</i> K-12 strain C600	C600φ3538	1	Laboratory lysogen	Δstx ₂ ::cat	Cm ^r NaI ^s Tc ^s	28
Recipient strains						
<i>E. coli</i>	O157:H7	35	Cattle and environment	stx ₁ ⁻ , stx ₂ ⁺ , and variable (+ or -) for <i>eae</i> and EHEC <i>hly</i>	Tc ^r Cm ^s	14, 22
<i>E. coli</i>	Non-O157:H7	18	Cattle, sheep, and food	stx ₁ ⁻ , stx ₂ ⁺ , and variable (+ or -) for <i>eae</i> and EHEC <i>hly</i>	Tc ^r Cm ^s	14, 22
<i>E. coli</i>	Non-O157:H7	4	Human	stx ₁ ⁻ , stx ₂ ⁻ , <i>eae</i> ⁻ , EHEC <i>hly</i> ⁻	Tc ^r Cm ^s	This study
<i>Klebsiella pneumoniae</i>	CF55	1	Environment		Tc ^r Cm ^s	This study
<i>Shigella flexneri</i>		2	Human		Tc ^r Cm ^s	25
<i>Shigella sonnei</i>		2	Human		Tc ^r Cm ^s	25
<i>E. coli</i> K-12	C600 ^{NaI}	1	Laboratory		NaI ^r Cm ^s	This study
<i>E. coli</i> K-12	WG5	1	ATCC 700078		NaI ^r Cm ^s	4

^a EHEC, enterohemorrhagic *Escherichia coli*.

UP378/LP378 (24) was used to detect the presence of the *stx*₂ gene in the samples. The identity of each PCR product was confirmed in duplicate by sequencing. Nucleotide sequence analyses were performed with the Wisconsin package version 10.2 (Genetics Computer Group [GCG], Madison, WI). BLAST analyses were done with the tools available on the NCBI webpage (<http://www.ncbi.nlm.nih.gov>).

The results indicate that the background flora present in the different food matrices did not generally interfere in the detection of the possible transductants because no growth was observed or because the background colonies (white) were distinguishable from the transductants (blue, pink, or violet). Coliphages and the *stx*₂ gene were not detected in any of the samples used. The controls performed with spiked samples indicated that no inhibitors potentially present in the food samples were responsible for the negative results. The detection limit of the PCR assay was 5.10¹ to 10² *stx*₂ copies ml⁻¹ of sample.

The protocol allowed the generation of transductants in milk, water, beef, and salad. The transductants were resistant to both antibiotics (NaI^r Cm^r or Tc^r Cm^r), had the same color as the recipient strain, and presented the *stx*::*cat* fragment, as confirmed by PCR and sequencing. The controls performed with only donor or with only recipient strains in the samples did not generate transductants.

Since mitomycin C (0.5 μg ml⁻¹) is commonly used to induce *stx*::*cat* phages from their lysogens (28, 29), it was used in the preliminary assays. Nevertheless, similar levels of transduction in samples treated with or without mitomycin C were observed (Fig. 1). Considering these results and since mitomycin C is not expected to be naturally present in food samples, the experiments presented were performed without mitomycin C induction.

Transductants were generated with several phages assayed and in various food samples. Since the generation of transductants occurred after 18 h of incubation without antibiotic selection, the results of the generation of transductants can only be considered qualitatively. In spite of this, some repetitive levels of transductants obtained in successive replicas allowed us to present some quantitative results, for example, the values obtained when phage 933WCm was transduced from its lysogen to three *E. coli* host strains (WG5, C600^{NaI}, and O157:H7 strain 278 [Tc^r]) (Fig. 2).

These first studies were performed under conditions that guarantee transduction, although such conditions may not be found outside the laboratory. These optimal conditions were later modified in an attempt to emulate the environments to which food samples may be exposed outside the laboratory. In some of these “nonoptimal” situations, transductants were also generated, and it is to these conditions that attention must be paid for the risk they may represent.

Transductants were not generated in orange juice at pH 3.7, which we attributed to the low pH. To evaluate this, the pH of the samples was increased to 6.0 by the addition of 0.1 M NaOH. In this case, transductants were generated using LysφA557Cm and C600φ3538 as donors and *E. coli* WG5 and C600^{NaI} as recipients. To confirm whether the survival of the recipient strain was the critical point, WG5 was then preconditioned at lower pH values in buffered LB medium. The lowest pH that allowed the growth of the recipient was 5.0. Transductants were then obtained. The survival of the donor strain does not appear to be relevant, probably because the phage particles, once induced from the donor, survive long enough at low pH to infect the recipient cells. The pH growth range of STEC is between 4.4 to 9.0, although some strains grow at lower values (<4.0) (12). The low number of trans-

FIG. 1. Comparison of the log CFU ml⁻¹ of transductants generated with or without mitomycin C (+Mit C and -Mit C, respectively) induction with strain Lys ϕ A557Cm as the donor strain and WG5 as the recipient strain. Results are the average of three independent experiments performed under identical conditions.

ductants generated at low pH, together with the range of pH survival of STEC and other *E. coli* at low pH, makes transduction unlikely in acidic environments. Low pH could therefore be considered a potential treatment to prevent transduction.

Assays at a range of temperatures, to which samples had been previously acclimated, showed the consistent generation of transductants at 37°C and most of the phages at 30°C and 25°C. At 15°C and 22°C, transductants were generated for some donor strains but not for others. Finally, transductants were not generated at 4°C. This could be due to the inhibition of DNA injection at low temperatures, as reported for phage T4 at 4°C (21) or for phage T5, for which the low temperatures rendered the recipient cell membranes rigid, thus preventing injection (13).

The recipient strains of different serotypes that showed the required antibiotic susceptibility (Tc^r Cm^s) were selected (Table 1) to assay transduction in milk and beef. Sensitivities of 18.2% to the 933WCm phage for milk and 9% for beef were

observed in the selected strains (Table 2). Our results indicated a low range of potential recipients, although our studies were limited, due to the selection of the strains attending their antibiotic susceptibilities and to a reduced set of phages.

Strain WG5 was selected as the best recipient to evaluate the minimal amounts of the recipient and donor strains necessary to generate transductants at 37°C. Differences were observed between the three donor strains used. To calculate the threshold of each donor and recipient bacterium necessary for transduction to occur, the results (Table 3) should be interpreted as follows: if 10^X is the value of the donor strain while 10^Y is the value of WG5, then X + Y should be between 8 and 10 for C600 ϕ 3538, more than 10 for Lys ϕ 557Cm, and 14 for Lys933WCm to transduce WG5 in the solid samples. For the liquid samples, the threshold was generally lower. For phages C600 ϕ 3538 and Lys933WCm, X + Y was 8 and 13, respectively, and for Lys ϕ 557Cm, it was 6 to 7 in water and 10 in milk. Transductants were not obtained when the recipient strain

FIG. 2. Comparison of the log CFU ml⁻¹ of the transductants generated with Lys933WCm as the donor strain and three recipient strains (WG5, C600^{Nal}, and O157:H7-278) in the different food samples. Values were obtained after mitomycin C induction. Results are the average of three independent experiments performed under identical conditions.

TABLE 2. Transduction from lysogen Lys Φ 933WCm to different *E. coli* strains and other *Enterobacteriaceae* in milk and ground beef

Bacterium	Origin	No. of strains	Genotype	Milk	Beef
<i>E. coli</i> O111	Human	1	stx ₂ negative	—	—
<i>E. coli</i> O127	Human	1	stx ₂ negative	—	—
<i>E. coli</i> O26	Human	2	stx ₂ negative	—	—
<i>E. coli</i> O4:H4	Cattle	1	stx ₂	—	—
<i>E. coli</i> O6:H49	Beef meat	1	stx ₂	—	—
<i>E. coli</i> O8:H9	Sheep	1	stx ₂	—	—
<i>E. coli</i> O8:H21	Beef meat	1	stx ₂	—	—
<i>E. coli</i> O15:H16	Cattle	1	stx ₂	+	—
<i>E. coli</i> O21:H21	Beef meat	1	stx ₂	+	—
<i>E. coli</i> O64:H34	Cattle	2	stx ₂	—	—
<i>E. coli</i> O70:H-	Cattle	2	stx ₂	—	—
<i>E. coli</i> O91:H-	Sheep	2	stx ₂	—	—
<i>E. coli</i> O113:H21	Cattle	2	stx ₂ , EHEC hly ⁻	—	—
<i>E. coli</i> O113:H-	Cattle	1	stx ₂	—	—
<i>E. coli</i> O174:H-	Cattle	1	stx ₂	—	—
<i>E. coli</i> O162:H7	Cattle	1	stx ₂	+	+
<i>E. coli</i> O171:H25	Cattle	1	stx ₂	+	+
<i>E. coli</i> O157:H7	Cattle	30	stx ₂ , eae, EHEC hly	+	+
<i>E. coli</i> O157:H7	Environment	5	stx ₂ , eae, EHEC hly	—	—
<i>Shigella sonnei</i>	Human	2		+	+
<i>Shigella flexneri</i>	Human	2		—	—
<i>Klebsiella pneumoniae</i>	Environment	1		—	—
<i>E. coli</i> C600 ^{Nal}	Laboratory	1		+	+

value was lower than 10² CFU ml⁻¹. The controls were performed at different times to rule out the possibility that the donor and recipient strains may have grown during the process.

The higher threshold observed for the solid samples can be explained since a solid sample is a more complex and heterogeneous matrix, with an irregular distribution of the cells that reduces the phage-bacteria encounters necessary for infection to occur. The results for the liquid samples are similar to those reported elsewhere for replicating *E. coli* and a specific bacteriophage (39) and also for coliphages in water environments (23).

The levels of donor and recipient strains needed to generate transductants are not expected to be found in a food sample available to consumers, and so transduction is unlikely to occur in such samples. The low prevalence of O157 found in food samples (11, 12) indicates that transduction may be rare, although the prevalence of STEC in food (1) indicates that this possibility cannot be ruled out if some break in the control of the food chain happens (7, 16). The transduction of stx to recipient strains present in the background flora could be the main risk for the consumer.

Food products can be subjected to conditions that reduce the growth of some bacteria but prolong their survival. In these

TABLE 3. Generation of transductants using different values for the donor strains (lysogens of phages) and the recipient strain (*E. coli* WG5) in the food samples without mitomycin C induction^a

Donor strain and no. of CFU (10 ^x)	No. of recipient strain CFU (10 ^y)	Threshold (X + Y) ^b	Presence of transductants in ^d :				
			Water	Milk	Beef	Salad	LB medium
C600 Φ 3538 10 ⁸	10 ⁸	16	+	+	+	+	+
	10 ⁶	14	+	+	+	+	+
	10 ⁴	12	+	+	+	+/—	+
	10 ²	10	—	—	—	—	+
	10 ⁷	14	+	+	+	+	+
	10 ⁶	12	+	+	+	+	+
	10 ⁵	10	+	+	+	+	+
	10 ⁴	8	+	+	+/—	+/—	+
	10 ³	6–7 ^c	+/—	—	—	—	+/—
	10 ²	4–5 ^c	—	—	—	—	—
Lys Φ A557Cm 10 ⁷	10 ⁶	13	+	+	+	+	+
	10 ⁴	11	+	+	+	+	+
	10 ²	9	—	+/—	—	—	+
	10 ⁸	14	+	+	+	+	+
	10 ⁶	12	+	+	+	+	+
	10 ⁵	10	+	+	+/—	—	+
	10 ⁴	8	+	+/—	—	—	+
	10 ³	6–7 ^c	+	+/—	—	—	+/—
	10 ²	4–5 ^c	—	—	—	—	—
	10 ¹	2–3 ^c	—	—	—	—	—
Lys933WCm 10 ⁷	10 ⁷	14	+	+	+	+	+
	10 ⁶	13	+	+	—	—	+
	10 ⁵	12	—	—	—	—	—
	10 ⁷	13	+	+	+	+	+
	10 ⁶	12	—	—	—	—	—

^a Numbers of CFU ml⁻¹ or g⁻¹ of sample were measured for the donor and recipient strains.

^b The threshold necessary for the generation of transductants was estimated and is expressed as the sum of X + Y, where X is the exponential value for the donor strain and Y is the exponential value for the recipient strain.

^c When using lower values of donor and recipient cells, the X + Y calculation varies with the replicas and is expressed as a range between two log units.

^d +, transductants were generated, unless otherwise indicated, at >10³ CFU ml⁻¹ or g⁻¹ of sample; +/—, some replicas positive and some negative; —, no transductants.

conditions, bacterial populations suffer stress (37), which can increase the induction of prophages to their lytic cycle and therefore increase the number of free phage particles (5). Certain treatments used for the processing of food or water samples, such as chlorination (24), thermal treatment (24), radiation with ^{60}Co (40), or high hydrostatic pressure (3) fail to inactivate Stx phages. Indeed, some of these treatments may even increase the induction of the lytic cycle of certain Stx phages, which will persist after the application of the treatment (3). Further studies to evaluate the occurrence of free Stx phages in food matrices, especially in cattle products, are required. Attending to our results, transduction in food does not seem to be a high-risk way of *stx* transmission, although it can take place under the appropriate conditions and should not be discounted.

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