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Vesicle-Mediated Transfer of Virulence Genes from *Escherichia coli* O157:H7 to Other Enteric Bacteria

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Membrane vesicles are released from the surfaces of many gram-negative bacteria during growth. Vesicles consist of proteins, lipopolysaccharide, phospholipids, RNA, and DNA. Results of the present study demonstrate that membrane vesicles isolated from the food-borne pathogen *Escherichia coli* O157:H7 facilitate the transfer of genes, which are then expressed by recipient *Salmonella enterica* serovar Enteritidis or *E. coli* JM109. Electron micrographs of purified DNA from *E. coli* O157:H7 vesicles showed large rosette-like structures, linear DNA fragments, and small open-circle plasmids. PCR analysis of vesicle DNA demonstrated the presence of specific genes from host and recombinant plasmids (*hly*, L7095, *mobA*, and *gfp*), chromosomal DNA (*uidA* and *eaeA*), and phage DNA (*stx1* and *stx2*). The results of PCR and the Vero cell assay demonstrate that genetic material, including virulence genes, is transferred to recipient bacteria and subsequently expressed. The cytotoxicity of the transformed enteric bacteria was sixfold higher than that of the parent isolate (*E. coli* JM109). Utilization of the nonhost plasmid (pGFP) permitted the evaluation of transformation efficiency (ca. 10^3 transformants μg of DNA^{-1}) and demonstrated that vesicles can deliver antibiotic resistance. Transformed *E. coli* JM109 cells were resistant to ampicillin and fluoresced a brilliant green. The role vesicles play in genetic exchange between different species in the environment or host has yet to be defined.

Many gram-negative bacteria produce membrane vesicles, suggesting that vesicle production is not purposeless; indeed, studies during the last two decades have presented strong evidence supporting the importance of vesicles. Typical vesicles released from the surfaces of gram-negative bacteria are 50 to 250 nm, spherical, and made up of outer membrane and encapsulated periplasmic components (4, 26). Vesicle components include outer membrane proteins, lipopolysaccharide, periplasmic proteins, phospholipids, DNA, and RNA (9, 12, 15, 22, 34, 40). Vesicles from gram-negative bacteria were reported to fuse to both gram-positive and gram-negative bacteria and in some instances to promote lysis of the target cell (28). Moreover, vesicles may function as an alternative secretory pathway (3, 23) and promote adherence of the parent cell to host cells (17, 32). By virtue of their small size, bilayer protecting envelope, and ability to integrate into the membranes of foreign bacteria and to adhere to or be engulfed by eukaryotic cells, a potential role of vesicles in delivery of virulence factors, including enzymes and toxins, is not unlikely (23). In fact, virulence factors associated with the parent strain, including proteases, phospholipases, autolysin, hemolysins, and Shiga toxins, have been isolated from vesicles (3, 22, 26, 28).

Aside from toxic compounds, DNA has also been isolated from vesicles. Vesicles produced by *Pseudomonas aeruginosa* were reported to contain DNA (22). Vesicles released by *Neisseria gonorrhoeae* harbor both linear and circular DNA, including 4.2- and 7.1-kb plasmids (12). Chromosomal and bacteriophage-associated virulence genes were detected in *Escherichia coli* O157:H7 vesicles (26). Moreover, this research demonstrated that DNA was protected from digestion by DNase, suggesting that DNA is packaged within vesicles (26).

Bacterial evolution often proceeds by horizontal gene transfer between different genera and species (1, 7). Antibiotic resistance genes and pathogenicity islands have been acquired by a variety of pathogens, including *E. coli*, *Salmonella enterica* serovar Typhimurium, *Yersinia pestis*, *Dichelobacter nodosis*, and *Helicobacter pylori* (19). Virulence factors contributing to the pathogenicity of *E. coli* O157:H7, including Shiga toxins (45, 46) and intimin (31, 44), are encoded on pathogenicity islands in the O157 chromosome and are thought to have been acquired by horizontal transfer. Results of previous studies suggest that vesicles may be involved in the transfer of genetic material among similar bacterial species (8, 12, 26). The hypothesis has been put forth that vesicles influence antibiotic resistance in other bacteria in two ways: by physical dissemination of preformed antibiotic-inactivating enzymes into the recipient periplasm and by delivery of antibiotic resistance plasmids (3, 12). Competent *Haemophilus influenzae* produces vesicles which are released into the medium when cells are returned to normal growth conditions or a noncompetent state (8). Specific DNA-binding peptides were reported to be present on the surfaces of *H. influenzae* vesicles (24, 25) and to be associated with vesicles from *N. gonorrhoeae* (11).

Previously, it was reported that vesicles released by *E. coli* O157:H7 into culture medium contain virulence genes and Shiga toxin (26). In the present study, we demonstrate that *E. coli* O157:H7 vesicles mediate the transfer of virulence genes, which are subsequently expressed by recipient enteric bacteria. Moreover, the origin of the DNA in *E. coli* O157:H7 vesicles is elucidated. Observations show that in addition to bacteriophage-associated genes, *E. coli* O157:H7 vesicles contain plasmids and fragments of chromosomal DNA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* O157:H7 (ATCC 43895), *E. coli* JM109 (Promega, Madison, Wis.), and *Salmonella enterica* serovar Enteritidis (ATCC 13076) were grown in Luria-Bertani (LB) broth (Difco, Detroit, Mich.) at 37°C with shaking (200 rpm). Transformation of pGFP (Clontech, Palo Alto, Calif.), which encodes green fluorescent protein (GFP), was performed using the calcium chloride method (43). Following transformation, *E. coli* O157:H7(pGFP) cells were grown on LB agar containing 100 μg of ampicillin ml^{-1}.

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TABLE 1. DNA primers used for amplification of specific genes

Gene	Primer name	Primer sequence (5′→3′)	Gene source (size [kb])	Product size (bp)	Reference or source
mobA	n-mobA	CGCGCAGGCGGGCTATCAGTC	p4821 (3.3)	576	18
	c-mobA	CCAAAGCCACATCATTTGC			
cdaA	n-cdaA	CTCGATGGTCGTGTGGCCG	pColD157 (6.7)	632	20
	c-cdaA	GGAGTATTCATCTGACCCACAC			
L7095	n-L7095	CAGTTCGCTCGTAAAGCAG	pO157 (92)	668	6, 29
	c-L7095	GTATAAATGGCTCTATGTCTAC			
hlyCA[a]	n-hlyCA	CTTTTGACGTCATGGGGAAGG	pO157 (92)	792	6, 29
	c-hlyCA	CGAATATTGCAACACCACGTTCAG			
stx1	stx-1F	ACACTGGATGATCTCAGTGG	Phage 933J	614	16
	stx-1R	CTGAATCCCCCTCCATTATG			
stx2	stx-2F	CCATGACAACGGACAGCAGT	Temperate phage 933W	779	16
	stx-2R	CCTGTCAACTGAGCACTTTG			
eaeA	n-eae	CCCGAATTCGGCACAAGCATAAGC	Chromosomal DNA	863	44
	c-eae	CCCGAATCCGTCTCGCCAGTATTCG			
uidA	n-uidA	CTCTACACCACGCCGAACAC	Chromosomal DNA	922	26
	c-uidA	CCTTCTCTGCCGTTTCCAAAT			
933W	W61311F	GTCAGTATGCGATAGCTCTG	DNA endpoints of 933W	660	42
	W300R	GCGACATGGGATTCATTCAGC			
gfp	n-gfp	ATGAGTAAAGGAGAAGAACTTTTC	pGFP	800	Clontech
	c-gfp	CGAAAGGGCCCGTACGGCCG			

[a] The primer set n-hlyCA and c-hlyCA was designed to amplify the C- and N-terminus-encoding portions of *hlyC* and *hlyA*, respectively, from the pO157 hemolysin operon, *hlyCABD*.

Vesicle isolation. Vesicles were isolated from early-stationary-phase (14-h) cultures of *E. coli* O157:H7 and *E. coli* O157:H7(pGFP) according to the method of Kadurugamuwa and Beveridge (22). In brief, tryptic soy broth (150 ml) was inoculated with 10^6 *E. coli* cells and incubated at 37°C for 15 h with shaking (150 rpm). Following incubation, cells were pelleted by centrifugation (10,000 × *g*, 10 min, 4°C), and the supernatant was decanted and passed through a 0.22-μm-pore-size filter (Micron Separations Inc., Westboro, Mass.) to remove remaining cells and cellular debris. Vesicles were collected by centrifugation (150,000 × *g*, 3 h, 4°C) (Ti 45 rotor; Beckman Instruments, Inc., Fullerton, Calif.), washed, resuspended in 50 mM HEPES (Fisher Scientific, Pittsburgh, Pa.) supplemented with 0.5 mM dithiothreitol (Sigma Chemical Co., St. Louis, Mo.), and stored at −20°C until needed. Vesicle preparations were checked for the presence of *E. coli* by surface plating of the vesicle suspension on Trypticase soy agar and by electron microscopy. Vesicle preparations were treated with DNase (DNase I from bovine pancreas; Sigma Chemical Co.), as described by Kolling and Matthews (26).

Electron microscopy. DNA associated with *E. coli* O157:H7 vesicles was visualized using DNA shadowing as described by Inman and Schnos (21). Briefly, DNA isolated from vesicles was adsorbed onto collodion-coated copper grids (400 mesh) and stained. Rotary shadowing was performed using platinum-palladium. For negative staining, 5 μl of vesicle suspension was layered onto a Formvar-coated copper grid (300 mesh) and wicked off after 10 s of contact. A 5-μl aliquot of 2% uranyl acetate was used to stain vesicles. Samples were visualized using a JEM-1230 electron microscope (JEOL USA, Inc., Peabody, Mass.).

DNA isolation. Total cellular DNA was isolated from *E. coli* and *Salmonella* serovar Enteritidis cells using guanidium thiocyanate according to the method of Pitcher et al. (41). Prior to DNA isolation from vesicle preparations, samples (200 μl) were treated with DNase to hydrolyze surface-associated DNA and free DNA in the suspension. Reactions were stopped by heat treatment at 80°C for 10 min. DNase-treated vesicles were lysed with 0.125% Triton X-100 solution (30 min at 37°C), and DNA was purified using a glass fiber matrix binding and elution technique according to the manufacturer's directions (Amersham Pharmacia Biotech, Piscataway, N.J.). DNA was resuspended in 20 μl of TE (10 mM Tris, 1 mM EDTA; pH 8.0).

PCR. All PCRs were performed in a total volume of 50 μl containing 4 μg of each primer ml^{-1}, a 0.2 mM concentration of each deoxynucleoside triphosphate (New England Biolabs, Inc., Beverly, Mass.), 10× PCR buffer (Sigma), nucleotide-free water (Promega), and 2.5 U of *Taq* polymerase (Sigma). DNA primers used for amplification of genes are listed in Table 1. Primers were not designed to have a common melting temperature. The DNA template was either whole transformed *E. coli* JM109 cells, DNase-treated vesicles (~4 ng of DNA), or purified vesicle DNA (~20 ng). An *E. coli* O157:H7 whole-cell suspension or cellular DNA was used as a positive control, and *E. coli* JM109 was used as a negative control. Cell pellets were resuspended in TE to a concentration of 10^8 cells ml^{-1}, and 4 μl was used for PCR. PCRs were carried out in a GeneAmp PCR system 2400 thermocycler (Perkin-Elmer Corp., Foster City, Calif.). Reactions were started with 1 cycle of 5 min at 94°C, 2 min at 50°C, and 3 min at 72°C and continued with 25 cycles of 45 s at 94°C, 90 s at 50°C, and 120 s at 72°C. The reaction was completed with an extension step of 10 min at 72°C. PCR products were separated on agarose gels by electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

Vesicle-mediated transformation. *E. coli* JM109 and *Salmonella* serovar Enteritidis were cultured in LB broth at 37°C with shaking (200 rpm) for 3 to 4 h to an optical density at 600 nm (OD_{600}) of 0.6. Cells were pelleted and resuspended in cold SOC medium (Bio 101, Vista, Calif.) to a concentration of approximately 10^7 cells ml^{-1} (for GFP transformation, cells were concentrated to approximately 10^{11} cells ml^{-1}). The cell suspension (100 μl) was mixed with 800 μl of SOC medium and 100 μl of vesicle suspension (approximately 1.65 ng of DNA ml^{-1}) and DNase (final concentration, 1 ng ml^{-1}). The suspension was incubated statically at 37°C for 1 h and then for an additional 2 h with shaking (150 rpm). Ten milliliters of LB broth was added to the suspension, and incubation in LB broth continued at 37°C for 20 h with shaking (200 rpm). Samples (2 ml) for the Vero cell assay (as described below) were collected after 5 and 20 h of incubation after the addition of LB broth. Samples were centrifuged, and the supernatant was collected and passed through a 0.22-μm-pore-size filter (Micron Separations Inc.) to remove remaining cells. Samples were either used immediately or stored (at −20°C) for later use. Controls included *E. coli* O157:H7, *E. coli* O157:H7 vesicles alone (no cells), *Salmonella* serovar Enteritidis, and *E. coli* JM109 either alone or with total DNA isolated from *E. coli* O157:H7. For PCR experiments, cells were harvested, pelleted, washed twice, and resuspended in TE buffer. All experiments were done in duplicate and repeated twice. Bacteriophages, if present, may contaminate vesicle preparations during isolation. To ensure that vesicles were responsible for DNA transformation and not bacteriophages (2, 37), vesicle samples were treated with 50 μg of proteinase K (Sigma) ml^{-1} for 30 min at 37°C to hydrolyze phage coats and release phage contents. Prior to transformation, proteinase K was removed from treated samples using microconcentrators that exclude molecules with an M_r of <30,000 (Millipore Corp., Bedford, Mass.). DNase was added to treated vesicle preparations to degrade released DNA. To further demonstrate that DNA transfer was vesicle mediated, separate experiments were conducted using vesicles that were hydrolyzed with 50 mg of lysozyme ml^{-1} at 37°C for 30 min. A control experiment was conducted using 20 ng of purified total *E. coli* O157:H7 DNA.

DNA packaging. Vesicles were isolated from *E. coli* O157:H7(pGFP) to investigate packaging and transfer of nonhost (the term "nonhost" is used to underscore the idea that pGFP is not naturally associated with *E. coli* O157:H7) DNA. Transformation was completed as described above, and *E. coli* JM109 cells were plated onto LB agar supplemented with 100 μg of ampicillin ml^{-1} and incubated at 37°C for 24 h. Ampicillin-resistant colonies, which fluoresce a brilliant green upon exposure to UV light, were considered transformants.

Vero cell assay. The Vero cell assay was completed as described previously (26, 33). In short, Vero cells suspended in growth medium (Basal Medium Eagle, 15% fetal bovine serum; pH 7.1) (Sigma) were seeded into wells (2×10^4 cells/well) of a 96-well microtiter plate and incubated for 24 h at 37°C. Growth medium was removed by aspiration and replaced with fresh medium, and 100 μl of the filtered supernatants (obtained as described above) was added to the first row of wells. Serial dilutions (1:2) were made, and the plates were incubated for 48 h. After 48 h, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 5 mg ml^{-1}) was added to each well without removal of supernatants. Controls included *E. coli* O157:H7 (ATCC 43895), *E. coli* JM109, and

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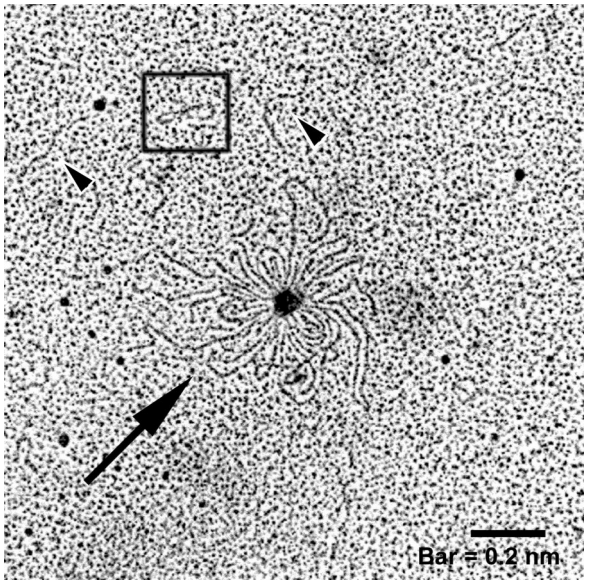

FIG. 1. Electron micrograph of DNA isolated from *E. coli* O157:H7 vesicles. Purified DNase-treated vesicles were lysed using 0.125% Triton X-100, and DNA was isolated and prepared for electron microscopy. Linear DNA (arrowheads), a plasmid (box), and rosette-like structures (arrow) were present.

Salmonella serovar Enteritidis (ATCC 13076) supernatants and vesicle preparations (treated like the transformed-cell supernatants), bacterial growth medium (LB) alone, and buffer (phosphate-buffered saline) alone. Results are based on cell survival using the following formula: percent live cells = ($OD_{treated\ cells}$/$OD_{control\ cells}$) × 100. The assay was performed in triplicate and repeated twice.

Plaque assay. The presence of phage in vesicle preparations was examined using a plaque assay. Samples (200 μl) of transformed *E. coli* JM109 cells, either immediately after vesicle transformation or after overnight growth, were diluted (1:10) in buffer (20 mM Tris, 10 mM NaCl, 10 mM $MgSO_4$; pH 7.4), mixed with 3 ml of molten (45°C) TB top agar (1% trypton, 0.5% NaCl, 10 mM $MgSO_4$, and 0.6% agarose), and poured onto the surfaces of LB plates. Plates were incubated at 37°C for 24 h and observed for plaque formation. In plaque assays, phage 933W is capable of forming plaques on *E. coli* O157:H7 (ATCC 43895) (also called EDL933).

RESULTS

Detection of genetic material within vesicles. Electron microscopy was performed on DNA isolated from vesicles to determine its origin, i.e., plasmid, chromosome, or phage. Electron photomicrographs show that various forms of DNA are associated with vesicles (Fig. 1). Large rosette-like structures, linear DNA fragments, and supercoiled plasmids were evident; small open-circle plasmids were also visualized (ca. 2 to 4 kb, based on comparisons to known plasmids). The rosette-like structures were frequently observed in vesicle samples.

PCR amplification of specific genes provided additional information relating to the origin of vesicle-associated DNA. Ten primer pairs (Table 1) were used to amplify genes of chromosomal, phage, and plasmid origins. Genes of interest may not be associated with all *E. coli* O157:H7 isolates; therefore, cellular DNA isolated from strain ATCC 43895 was used as a template in PCRs to determine whether the strain harbors the selected genes (Fig. 2 and 3). The chromosomal genes *eaeA* (863 bp) and *uidA* (992 bp), the bacteriophage-related genes *stx1* (614 bp) and *stx2* (779 bp), and three of the four plasmid-related genes were amplified. Amplification of *hlyCA* (792 bp), L7095 (668 bp), and *mobA* (576 bp) suggests that strain ATCC 43895 harbors plasmids pO157 (92 kb) and p4821 (3.3 kb). The primer set n-hlyCA and c-hlyCA was designed to amplify the C- and N-terminus-encoding portions of *hlyC* and *hlyA*, respectively, from the pO157 hemolysin operon, *hlyCABD*. No PCR product was obtained using primers for *cdaA*, suggesting that the 6.7-kb plasmid pColD157, which is responsible for the colicinogenic phenotype of some *E. coli* O157 strains, is not carried by strain ATCC 43895 (5, 20). Two primers homologous to phage 933W DNA endpoints that correlate to circular phage DNA were used to amplify a 660-bp fragment from vesicle-associated DNA (42). Vesicles released by *E. coli* O157:H7 were screened for genes that gave positive results with whole-cell DNA analysis (Fig. 2 and 3). PCR products associated with *stx1*, *eaeA*, *uidA*, *mobA*, and L7095 were obtained using purified, concentrated (10-fold) vesicle DNA (Fig. 3). Other genes that were screened for were detected using nonconcentrated vesicle suspensions.

Vesicle-mediated transformation. Vesicle-mediated transfer of genetic material was determined qualitatively by PCR amplification of the selected *E. coli* O157:H7 genes using recipient *E. coli* JM109 cells (Fig. 3). Following transformation and overnight growth in LB broth, cells were concentrated (10,000 × *g*, 5 min) and washed, and PCR analysis was performed using primers specific for *eaeA*, *stx1*, *stx2*, *hlyCA*, L7095, *mobA*, and the endpoints of phage 933W. PCR products corresponding to *stx2* and *hlyCA* were visualized in ethidium bromide-stained agarose gels. The genes listed above were not amplified using nontransformed *E. coli* JM109. Two fragments of phage 933W were amplified using primers homologous to the phage endpoints, likely indicating the presence of the entire circular phage DNA.

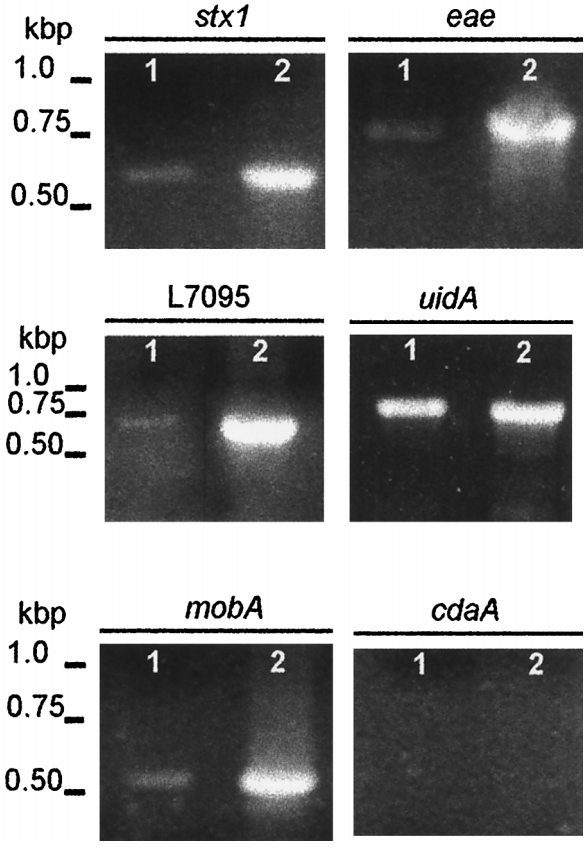

FIG. 2. PCR analysis of DNA isolated from *E. coli* O157:H7 vesicles. PCR amplification was performed with either DNA isolated from vesicles (lanes 1) or total cell DNA (lanes 2) using primers to specific regions of *stx1*, *eaeA*, L7095, *uidA*, *mobA*, and *cdaA* (Table 1).

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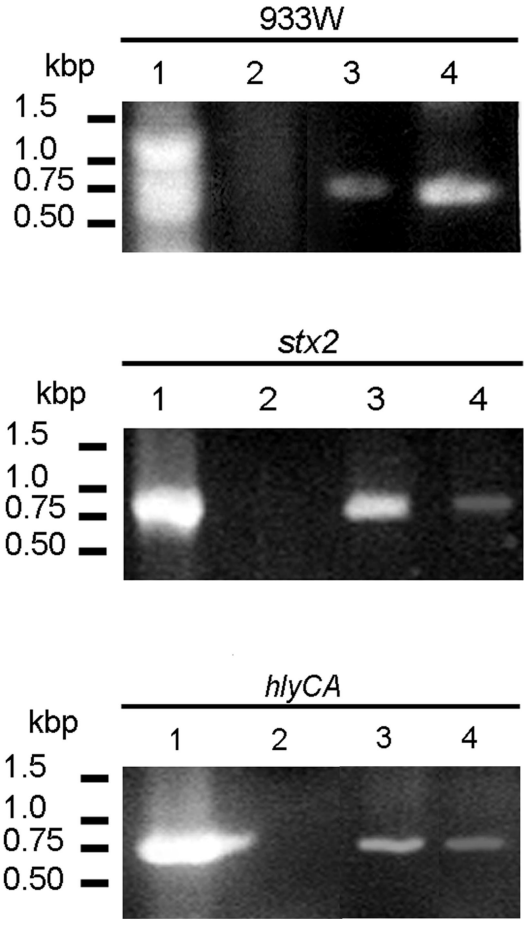

FIG. 3. PCR-detected genes transferred by *E. coli* O157:H7 vesicles to recipient JM109 cells. Lanes 1, *E. coli* O157:H7 cells; lanes 2, *E. coli* JM109 cells; lanes 3, intact vesicles; lanes 4, *E. coli* JM109, vesicle transformed. All genes listed in Table 1 were screened for, but only positive profiles are shown.

pGFP uptake, transfer, and expression in recipient cells. *E. coli* O157:H7 was transformed with pGFP, a derivative of the high-copy-number plasmid pUC18, to determine whether nonhost DNA would be entrapped in vesicles. Vesicles isolated from the transformed strain were analyzed by PCR for the presence of *gfp*. A single band of the appropriate size (800 bp) was amplified, indicating that plasmid DNA of foreign origin was packaged in the vesicles (Fig. 4). Vesicles (*gfp* positive) were used to transform *E. coli* JM109, which was plated onto LB agar alone or supplemented with ampicillin, and the transformation frequency was calculated as the number of transformants in the total cell count. The frequency of transformation of pGFP by vesicles was 3×10^{-10}, and transformation efficiency was 10^3 transformants per μg of DNA based on the assumption that 0.83 ng of DNA is associated with 10 μg of vesicle proteins (26). In fact, the efficiency is higher since pGFP is not the only DNA present in vesicles. No colonies formed in the following controls on LB agar supplemented with ampicillin: GFP-positive vesicles without cells, *E. coli* JM109 cells without vesicles, and *E. coli* JM109 cells incubated with 20 ng of purified plasmid ml^{-1}.

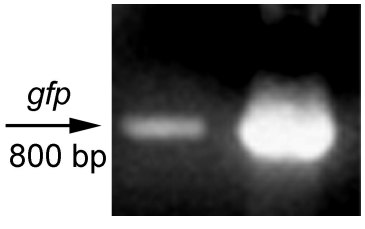

FIG. 4. PCR analysis of vesicle DNA for the presence of *gfp*. Vesicles isolated from *E. coli* O157:H7 harboring pGFP were treated with DNase and used as a template for PCR analysis with *gfp* primers. Left lane, vesicles containing *gfp*; right lane, purified pGFP (control). The molecular size of the expected fragment was 800 bp.

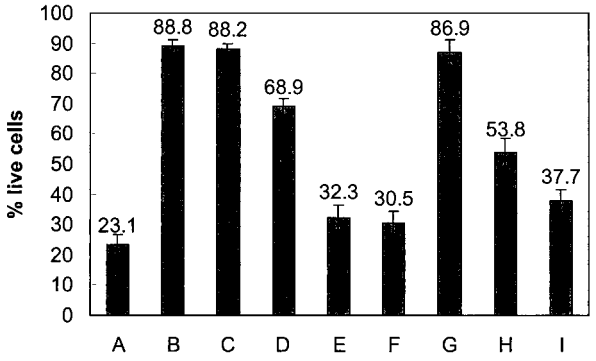

FIG. 5. Vero cell cytotoxicity of 20-h supernatants from transformed enteric bacteria. A, *E. coli* O157:H7 (positive control); B, *E. coli* JM109 (negative control); C, *Salmonella* serovar Enteritidis (negative control); D, *E. coli* O157:H7 vesicles; E, *E. coli* JM109, vesicle transformed; F, *Salmonella* serovar Enteritidis, vesicle transformed; G, *E. coli* JM109 transformed with total DNA purified from *E. coli* O157:H7; H, *E. coli* JM109 transformed with lysozyme-treated vesicles; I, *E. coli* JM109 transformed with proteinase K-treated vesicles. Values were determined as described in Materials and Methods. Error bars indicate the standard errors of the means. The assay was performed in triplicate and repeated twice. Values are from a representative experiment.

Cytotoxicity of supernatants from transformed enteric bacteria. The expression of virulence genes by recipient cells was analyzed using the Vero cell assay. Cytotoxic activity was sixfold greater for supernatants from transformed *E. coli* JM109 than for supernatants from parental *E. coli* JM109, indicating that Stx is expressed by the recipient cells (Fig. 5). Vesicles were also evaluated in the Vero cell assay to eliminate the possibility that vesicles were solely responsible for cytotoxicity. Cytotoxic activity was greater for supernatants from transformed cells than for supernatants from wild-type cells or vesicles alone. Transformation experiments were also conducted using total DNA from *E. coli* O157:H7 in place of vesicles; the cytotoxicities of supernatants from these experiments were similar to those for controls (Fig. 5). Treatment of vesicles with proteinase K did not affect the results; however, cytotoxicity decreased when lysozyme-treated vesicles were used, suggesting that vesicles and not phages are responsible for gene transfer (Fig. 5).

Vesicle-mediated transformation of enteric pathogens other than *E. coli* was determined using *Salmonella* serovar Enteritidis, a food-borne pathogen. Cell-free supernatants from transformants were used in the Vero cell assay. The cytotoxic activities of the supernatants from transformed *Salmonella* serovar Enteritidis and *E. coli* JM109 increased in 20-h supernatants compared to 5-h culture supernatants, suggesting continued expression of Stx by transformants (Table 2).

Plaque assay. *E. coli* O157:H7 strain ATCC 43895 (also called EDL933) is reported to spontaneously release phage 933W particles (42). The authors of the study indicate that propagation of the phage was unsuccessful in broth culture (as used in the present study); regardless, experiments were conducted to verify that the transfer of genetic material was in-

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TABLE 2. Effect of 5- and 20-h supernatants of transformants in Vero cell cytotoxicity assay

Incubation time (h)[a]	Vero cell survival (mean % live cells ± SEM)[b] with:		
	Transformed *E. coli* JM109[c]	Transformed *Salmonella* serovar Enteritidis[c]	Vesicles[d]
5	47.3 ± 2.8	46.0 ± 3.0	64.9 ± 3.2
20	36.4 ± 4.1	34.6 ± 3.9	68.9 ± 2.6

[a] Growth time in LB broth following transformation.
[b] Percent live cells was calculated as explained in Materials and Methods.
[c] Controls for the transformants were Vero cells treated with the appropriate bacteria (supernatant) and incubated for the same time under the same conditions.
[d] The control for vesicles was Vero cells treated with LB broth.

deed mediated by vesicles and not by lysogenic bacteriophages. Plunkett et al. (42) reported that phage 933W titers fell more than 20-fold after overnight storage at 4°C despite supplementation with $CaCl_2$, $MgCl_2$, or gelatin. In the present study, vesicle samples were stored for extended periods without supplementation of the above chemicals, and indeed, plaque assay results for putative transformants were negative.

DISCUSSION

In this study, the origin of DNA within membrane vesicles released by the food-borne pathogen *E. coli* O157:H7 was identified, and vesicle-mediated transformation of enteric pathogens other than *E. coli* was demonstrated. Vesicles harbored genes of different origins: chromosomal, phage, and plasmid. Electron micrographs of vesicle-associated DNA show the presence of plasmids and linear DNA. Transformation experiments demonstrated that vesicles can export and transfer genetic material to other enteric bacteria. The mechanism(s) by which DNA is entrapped or packaged into vesicles is not yet known. *E. coli* JM109 transformed with pGFP-positive vesicles also exhibited antibiotic resistance. The β-lactamase gene encoding resistance to ampicillin is carried on pGFP as a selectable marker.

Based on PCR, chromosomally integrated genes are associated with vesicles. The *uidA* gene is located in the chromosome, and its product, β-D-glucuronidase, is expressed in virtually all *E. coli* isolates (13, 14). The *eaeA* gene is located in a 35-kb chromosomal pathogenicity element and encodes intimin, a protein involved in the attachment of *E. coli* O157:H7 to epithelial cells (10, 30). The temperate bacteriophage 933W contains DNA encoding Stx2, while Stx1 is encoded by 933J, a putative cryptic prophage, and both genes are integrated into the chromosome (35, 36, 42). Analysis of the data set obtained during sequencing of phage 933W indicated that the assembled sequence is probably a circular permutation of prophage DNA (42). Amplification of the putative ends of phage 933W arms (*int* and *attR*) indicated that both arms are incorporated into vesicles and likely the circular form of the phage is present in vesicles (Fig. 2).

E. coli O157:H7, like most gram-negative pathogens, contains plasmids, which generally carry genes encoding virulence determinants. Three known plasmids are associated with *E. coli* O157:H7; a large plasmid, of approximately 92 kb (pO157), is present in virtually all clinical isolates, and two small plasmids, 6.7 and 3.3 kb in size, are present less frequently (27, 38, 39). Based on the results of PCR analysis, *E. coli* O157:H7 (ATCC 43895) harbors both pO157 and the 3.3-kb plasmid but not the 6.7-kb plasmid. The complete DNA sequence of pO157 isolated from *E. coli* O157:H7 ATCC 43895 and RIMD 0509952 has recently been determined (6, 29). In this study, screening was done for the presence of three genes on pO157, *hlyC*, *hlyA*, and L7095. A protein encoded by L7095 has a putative cytotoxin active site and sequence homology to the large clostridial toxin family. The genes *hlyC* and *hlyA* are part of the *hlyCABD* operon, which encodes a pore-forming cytolysin and its secretion apparatus (6). All three genes were detected in vesicles (Fig. 2 and 3). The *hly* genes were amplified using template DNA from samples that were not concentrated, suggesting that pO157 is frequently contained in vesicles.

The 3.3-kb plasmid contains all the information necessary for its replication, stability, and mobilization (including origin of transfer and *mobA*, which encodes a mobility protein), but it lacks the *tra* genes, which mediate close physical contact of bacteria and are important for efficient conjugation. The lack of these genes is indicative of a nonconjugative but mobile plasmid (18). Nucleotide sequence analysis showed that the plasmid is extremely similar (>98%) to an antibiotic-resistant plasmid, NTP16, derived from *Salmonella* serovar Typhimurium, with the exception of antibiotic resistance transposons (18, 29). The presence of *mobA* (Fig. 3) indicates that the 3.3-kb plasmid is found within vesicles. Vesicles may play a role in the transfer of the nonconjugative 3.3-kb plasmid.

Based on the presence of chromosomal (derived from bacterial genomes and pathogenicity islands) and bacteriophage genes, large and small plasmids, and a foreign recombinant plasmid (pGFP), DNA entrapment likely occurs randomly. Although all genes that were screened for were detected within vesicles, results suggest that phage 933W and plasmid pO157 are more commonly associated with vesicles, since they were detected consistently without using concentrated vesicle DNA. Several ideas may explain these results, since the stability of genes is influenced by various factors. Assuming random DNA entrapment in vesicles, one would expect to find a similar distribution of genes in parental cells and vesicles, i.e., a higher frequency in cases of genes harbored by high-copy-number plasmids (like pGFP) or propagated phage. However, the mechanism by which DNA is entrapped in vesicles is not yet known. DNA-binding proteins were observed in vesicle lysates of *H. influenzae* (8) and *N. gonorrhoeae* (11). Vesicles released from *N. gonorrhoeae* sedimented into two fractions on a sucrose density gradient, termed BI and BII. Distinct profiles of DNA-binding proteins observed within the two fractions suggests that vesicles may play different roles and that specific mechanisms may exist for determining the association between DNA and vesicles (11).

The source of genetic material entrapped in vesicles was determined through PCR analysis. Electron microscopy was done to determine whether entire plasmids or intact phage was present in vesicles. Micrographs revealed that vesicles harbor intact plasmids; however, phage or phage components were not observed in micrographs of negative stained vesicle preparations. Large rosette structures observed in micrographs may be pO157 or the entire DNA of phage 933W. Although many small DNA fragments were evident, linear DNA fragments approximately a nanometer in length were also observed.

The intent of this study was also to investigate whether DNA contained within vesicles can be transferred to recipient bacteria and whether the gene products expressed will ultimately be active. A recent report indicates that vesicles derived from either *Shigella flexneri* or *P. aeruginosa* rapidly fused with the outer membrane of *Salmonella enterica* serovar Typhi, *Salmonella* serovar Typhimurium, or *E. coli* DH5α (22). The authors suggested that the integration of vesicles from a donor bacterium into the membrane of a recipient would introduce con-

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stituents from the donor directly into the recipient. In the present study, the results of PCR, antibiotic resistance selection, and the Vero cell assay demonstrate that genetic material is exported by *E. coli* O157:H7 vesicles and transferred to recipient enteric bacteria (*E. coli* JM109 and *Salmonella* serovar Enteritidis). Simply combining vesicles with noncompetent cells in vitro resulted in the transfer of genetic material and subsequent expression of active compounds (e.g., Shiga toxin). Although the experiments related to transformation and expression were restricted to replicons that do not require recombination, this does not preclude transformation and subsequent expression of chromosomal genes.

Three main mechanisms of gene transfer have been identified in bacteria: transformation, involving the uptake and incorporation of naked DNA; conjugation, a cell contact-dependent DNA transfer mechanism; and transduction, whereby host DNA is encapsidated into a bacteriophage which acts as the vector for its injection into a recipient cell. Perhaps vesicles constitute an alternative mode of gene transfer among bacteria. Based on the results of the present study, genes encoding virulence factors and antibiotic resistance can be transferred by vesicles. The role vesicles play in genetic exchange between different species and genera in the environment or host has yet to be defined.

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