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Characterization of a Shiga Toxin-Encoding Temperate Bacteriophage of *Shigella sonnei*

ECKHARD STRAUCH,[1] RUDI LURZ,[2] AND LOTHAR BEUTIN[1]*

Robert Koch-Institut, D-13353 Berlin,[1] and Max-Planck-Institut für Molekulare Genetik, D-14195 Berlin,[2] Germany

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A Shiga toxin (Stx)-encoding temperate bacteriophage of *Shigella sonnei* strain CB7888 was investigated for its morphology, DNA similarity, host range, and lysogenization in *Shigella* and *Escherichia coli* strains. Phage 7888 formed plaques on a broad spectrum of *Shigella* strains belonging to different species and serotypes, including Stx-producing *Shigella dysenteriae* type 1. With *E. coli*, only strains with rough lipopolysaccharide were sensitive to this phage. The phage integrated into the genome of nontoxigenic *S. sonnei* and laboratory *E. coli* K-12 strains, which became Stx positive upon lysogenization. Moreover, phage 7888 is capable of transducing chromosomal genes in *E. coli* K-12. The relationships of phage 7888 with the *E. coli* Stx1-producing phage H-19B and the *E. coli* Stx2-producing phage 933W were investigated by DNA cross-hybridization of phage genomes and by nucleotide sequencing of an 8,053-bp DNA region of the phage 7888 genome flanking the *stx* genes. By these methods, a high similarity was found between phages 7888 and 933W. Much less similarity was found between phages H-19B and 7888. As in the other Stx phages, a regulatory region involved in Q-dependent expression is found upstream of *stxA* and *stxB* (*stx* gene) in phage 7888. The morphology of phage 7888 was similar to that of phage 933W, which shows a hexagonal head and a short tail. Our findings demonstrate that *stx* genes are naturally transferable and are expressed in strains of *S. sonnei*, which points to the continuous evolution of human-pathogenic *Shigella* by horizontal gene transfer.

The production of Shiga toxins, also called verocytotoxins, is associated with strains of *Shigella dysenteriae* type 1 and with certain serotypes of *Escherichia coli*, such as enterohemorrhagic *E. coli* O157:H7 and others (22, 29). *S. dysenteriae* type 1 produces the prototypical Shiga toxin (Stx), whereas different variants of Stx, designated Stx1 and Stx2, are produced by Shiga toxin-producing *E. coli* (STEC) strains. In *E. coli*, the genes encoding the different Stx types are frequently found associated with temperate lambdoid bacteriophages, which are integrated in the chromosome of the host bacterium. Shiga toxin-converting bacteriophages can multiply and lyse their bacterial host upon induction and are able to infect Stx-negative *E. coli* strains, which can thus be converted into STEC. At present, more than 200 different serotypes of STEC have been described (22).

Bacteriophage-specific DNA sequences are also found in the neighborhood of the *stxA* and *stxB* genes (*stx* gene) in the chromosomes of *S. dysenteriae* type 1 strains. However, intact Stx-converting phages are not produced by *S. dysenteriae* type 1, which is probably due to the loss of essential phage genes caused by transposition and recombination events (17, 37).

Other enterobacteria, such as *Citrobacter freundii* and *Enterobacter cloacae*, were occasionally found to be Stx producers (25, 32, 36). However, the *stx* genes are not stably inherited in these bacteria, which is also the case with certain strains of *E. coli* (13, 25, 32).

A human isolate of *Shigella sonnei* (strain CB7888) which carries a bacteriophage bearing the gene for production of Shiga toxin was described recently (9). Nucleotide sequence analysis revealed a high similarity between the *stx* gene of the *S. sonnei* phage (EMBL accession no. AJ132761) and a published *stx* sequence of *S. dysenteriae* type 1 (EMBL accession no. M19437), suggesting that the *S. sonnei* phage might have acquired its *stx* gene from an *S. dysenteriae* type 1 strain. In order to gather more information about the origin of Shiga toxin-producing phage 7888 of *S. sonnei*, we examined its host range and integration in *Shigella* and *E. coli* and studied its relationship to the Stx1- and Stx2-encoding phages of *E. coli*.

MATERIALS AND METHODS

Bacteria. The bacterial strains which were used as sources of phages and for transduction experiments are listed in Table 1. The reference strains for the different subgroups and serotypes of *Shigella* and *E. coli* are described elsewhere (4, 24).

Detection of Shiga toxins and phages bearing *Q* and *Rz* genes. Production of Stx was investigated with the Vero cell test and the VTEC-RPLA test (10). stx_1- and stx_2-specific DNA sequences were detected by PCR as described previously (9). PCRs for the *Q* gene and the *Rz* gene were developed on the basis of published nucleotide sequences (GenBank accession no. AF125520, AP000363, AF034975, LO4539, and Y10775) using McVector software (Oxford Molecular Group). The oligonucleotides Q-up (5′ GTC CTG TGA CGA TGA TGC GAT C 3′) and Q-down (5′ ATG CCT TCA ACA ATC CCC TCC G 3′) were selected as common primers for the amplification of *Q* genes present on different Shiga toxin-encoding bacteriophages. The PCR was run for 30 cycles (1 min at 94°C, 1 min at 57°C, and 1 min at 72°C for each cycle) and yielded a 195-bp amplification product. Primers RZ-up (5′ GCT GAA AAT GAA ACT CTT CGC 3′) and RZ-down (5′ GCT CTC TGA GGG TGA AAT AAT CC 3′) were used for amplification of a 172-bp internal segment of the phage *Rz* gene. The PCR conditions were the same as for the *Q*-gene-specific PCR.

Isolation of Stx-encoding phages and preparation of phage lysates. A single colony of *S. sonnei* strain CB7888 was grown in L broth to the exponential growth phase. For induction of lysogenic phages, mitomycin C was added to a final concentration of 0.5 μg/ml and the culture was further incubated for 3 h. The bacteria were then harvested by centrifugation, and the culture fluid was filtered through 0.22-μm-pore-size membranes (Schleicher and Schüll, Dassel, Germany). Dilutions of the supernatant were titrated on *E. coli* K-12 strain C600 and on the Stx-negative *S. sonnei* strain 737/85. Single plaques grown on either strain

* Corresponding author. Mailing address: (P13) Division of Emerging Bacterial Pathogens, Robert Koch-Institut, Nordufer 20, D-13353 Berlin, Germany. Phone: 49 (30) 45472484. Fax: 49 (30) 45472673. E-mail: BeutinL@rki.de.

TABLE 1. Bacterial strains

Strain	Species	Relevant characteristic(s)	Reference or source
CB7888	*S. sonnei*	Lysogenic for Stx phage 7888	9
C600	*E. coli* K-12	Stx negative, *Q* gene negative, O rough; Lac^- Trp^+ His^+	30
HB101	*E. coli* K-12	O rough, Stx negative, *Q* gene negative	30
P400	*E. coli* K-12	O rough, Stx negative, *Q* gene negative	P. Manning[a]
JC3272	*E. coli* K-12	O rough, Stx negative, *Q* gene negative; Lac^- Lys^- Trp^- His^-	6
C600(H19)	*E. coli* K-12	C600 lysogenic for Stx1 phage H-19B	8
C600(933W)	*E. coli* K-12	C600 lysogenic for Stx2 phage 933W	8
737/85	*S. sonnei*	Stx negative, *Q* gene negative	Human isolate[b]
508/75	*S. sonnei*	Stx negative, *Q* gene negative	Human isolate[b]

[a] University of Adelaide, Adelaide, Australia.
[b] Collection of the Robert Koch-Institut, Berlin, Germany.

were used for preparation of high-titer phage stocks. High-titer lysates (10^{10} PFU/ml) of phage 7888 were grown on *E. coli* K-12 strain C600 on agarose plates as described previously (30). Stx1 (H-19B)- and Stx2 (933W)-encoding *E. coli* phages were isolated by mitomycin C induction from the lysogenic host strains C600(H19) and C600(933W), respectively (7). Phage H-19B and 933W lysates were propagated on *E. coli* C600 as described above.

Electron microscopy. Phages were isolated in CsCl step gradients according to standard protocols (30). Phage suspensions were prepared by negative staining with 1% uranyl acetate on carbon films according to the method of Steven et al. (34) and examined using a Philips CM100 transmission electron microscope. Phage DNA was prepared by the microdiffusion technique for size determination as previously described (15).

Isolation of phage DNA. Phage lysates were incubated with 1 μg of DNase (Qiagen, Hilden, Germany) per ml and 1 μl of RNase C (Qiagen) per ml for 30 min at 37°C. Phage DNA was prepared with a Qiagen lambda kit according to the instructions of the manufacturer.

Phage 7888 sensitivity test. Cultures of *E. coli* and *Shigella* strains were grown overnight in L broth at 37°C. The next day, a 100-μl overnight culture of a bacterial test strain was added to 3 ml of molten LC-top agar (6) and the mixture was poured on Luria-Bertani agar plates. Sensitivity to phage 7888 was tested by spotting 10 μl of phage 7888 lysate (10^8 PFU/ml) onto the inoculated top agar overlay. The plates were then incubated for 22 h at 37°C. Lysis of bacteria was recorded after 5 and 22 h of incubation time.

Lysogenization of *E. coli* and *S. sonnei* with phage 7888. Ten-microliter portions of diluted phage 7888 lysates were spotted on Luria-Bertani plates covered with 3 ml of LC-top agar containing 100 μl of an overnight culture of nonverotoxigenic *Shigella* or *E. coli* strains, which were negative for *stx*- and *Q*-gene-specific DNA sequences. The plates were incubated for 22 h at 37°C, and bacteria which had grown within the lysis zones were further subcultured to single colonies. The bacterial isolates were examined for verocytotoxicity and for *stx*-specific DNA sequences as described previously (9). *stx*-positive colonies were confirmed for the specific phenotype of the recipient host strain and for the presence of temperate phage 7888 by *Q* gene PCR and by induction of infectious phages as described above.

Generalized transduction experiments. Bacteriophage P1*vir* was from the collection of the Robert Koch-Institut. Transduction of chromosomal DNA using phages P1*vir* and 7888 was performed as described previously (19). Phage 7888 lysates grown on *E. coli* strain C600 (Trp^+ His^+) were used to transduce *E. coli* K-12 strain JC3272, which carries mutations in the chromosomal genes involved in the synthesis of tryptophan (Trp) and histidine (His) (6). JC3272 requires exogenous lysine, histidine, and tryptophan for growth, and Trp^+ and His^+ transductants were selected on M9 medium containing glucose, vitamin B_1, and lysine but lacking tryptophan or histidine, respectively. His^+ and Trp^+ transductant colonies were confirmed as JC3272 derivatives by their phenotypical properties and were examined for cotransduction of the phage 7888-associated *stx* and *Q* genes by PCR as described above.

Hybridization of *Xba*I-digested total bacterial DNA separated by PFGE. Preparation of total DNA, *Xba*I digestion, and pulsed-field gel electrophoresis (PFGE) were performed as described before (8). Southern blots of *Xba*I-digested total bacterial DNA were hybridized with *Hin*cII-digested digoxigenin-11–dUTP-labeled phage 7888 DNA or with an *stx*- and stx_1-specific gene probe which was prepared from PCR-amplified DNA from strain C600(H19) as described previously (8).

Cross-hybridization of phage genomes. Restriction endonuclease-digested phage DNA was separated in 0.8% agarose gels and transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by capillary blotting. Hybridization was performed at 65°C with a mixture of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 0.5% blocking reagent, and 5% dextran sulfate. Washing steps were done at 65°C with 2× SSC–0.1% SDS and then with 0.2× SSC–0.1% SDS.

Nucleotide sequencing. Small PCR products of several hundred base pairs were purified with a QIAquick PCR purification kit (Qiagen) and used directly for sequencing. Larger PCR products were cloned using the pGEM-T Easy vector system (Promega, Madison, Wis.) and transformed into *E. coli* JM109. Plasmids were isolated using a NucleoSpin Plus plasmid miniprep kit (Clontech Laboratories, Inc., Palo Alto, Calif.). Nucleotide sequencing was carried out with universal primers for pBluescript SK+ and commercially synthesized primers. Sequencing reactions were carried out by the dideoxynucleotide termination cycle sequencing method using a Prism BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer/Applied Biosystems, Weiterstadt, Germany). Sequencing products were run on an automated DNA sequencer (ABI 377). The sequences were analyzed with Auto Assembler software (Perkin-Elmer/Applied Biosystems).

Nucleotide sequence accession number. The 8,053-bp nucleotide sequence of phage 7888 has been submitted to the EMBL data library under accession no. AJ279086 and includes the nucleotide sequences of the *stxA* and *stxB* genes, which were deposited previously under EMBL accession no. AJ132761.

RESULTS

Morphology of the phage 7888 virion and genome size. Phage 7888 was isolated from the Stx-producing *S. sonnei* strain CB7888, which was from feces from a human patient with diarrhea (9). Phages released from *S. sonnei* CB7888 after mitomycin C induction were purified in a CsCl step gradient and examined by transmission electron microscopy. Electron micrographs (Fig. 1) depicted phage particles with regular hexagonal heads about 65 nm wide. Phage 7888 particles showed short tails, approximately 26 nm long and 13 nm wide, which seemed to possess small base plates. As described for the *stx2*-carrying phage 933W, we also found aggregation of the phages due to interactions of the tail tips (26).

The size of the phage 7888 genome was calculated to be 63.4 ± 2.4 kb from the sum of the restriction fragments obtained by endonuclease digestion of the complete phage DNA. Additionally, a size determination deduced from 47 genomes based on electron micrographs suggested a length of 64.2 ± 2.0 kb for the virion DNA.

Analysis of the *stxA*- and *stxB* (*stx* gene)-flanking region of phage 7888. The *stx* gene of phage 7888 was described previously (EMBL accession number AJ132761) (9). In the present work, the region of phage 7888 flanking the *stx* coding region was analyzed by sequencing PCR products obtained by partial amplification of phage 7888 DNA as well as of genomic DNA

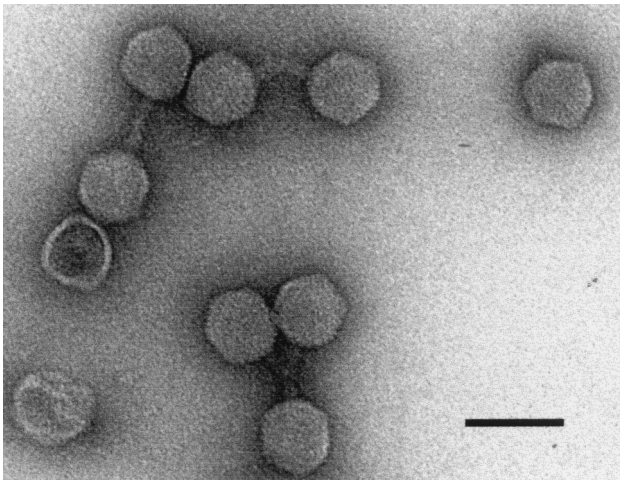

FIG. 1. Electron micrograph of CsCl-purified phage 7888 particles. Bar, 100 nm.

of strain CB7888. PCR products containing the 5′ upstream region of the *stx* gene were obtained by using primer Q-up and an internal primer located in the *stxA* gene. The region downstream of the *stx* gene was amplified using a primer binding in the *stxB* gene and the RZ-down primer. The physical map of the sequenced part of the phage genome with a length of 8,053 bp is shown in Fig. 2. Downstream of the *Q* gene of phage 7888, an open reading frame (ORF) which may encode a putative protein with a deduced size of 197 amino acids was identified. However, analysis of protein databases did not identify proteins with significant homology to the deduced gene product. The *stx* region is located downstream of this ORF and contains regulatory elements consisting of a promoter, $p_{R'}$, and a terminator, $t_{R'}$, involved in antitermination by the Q transcription activator, followed by the *stxA* and *stxB* genes. The region downstream of the *stx* gene contains a number of genes known from other Shiga toxin-producing phages in the order *yjhS, S, R, ant*, and *Rz*.

Relationship of phage 7888 to Stx-converting coliphages H-19B and 933W. To assess the relationship of *S. sonnei* phage 7888 to other characterized Stx-converting phages, Stx2-converting phage 933W and Stx1-converting phage H-19B were chosen for comparison. All three phage genomes were subjected to restriction endonuclease digestions and to cross-hybridization experiments. The *Hin*cII and *Acc*I restriction patterns for all virion DNAs differed considerably (Fig. 3A and C).

The hybridization experiments were carried out under stringent conditions using the complete genome of phage 7888 as the probe. The hybridization patterns obtained from Southern blots of *Hin*cII and *Acc*I digests of the complete genomes of the Shiga toxin-converting phages are shown in Fig. 3B and D. Whereas hybridization signals between the DNAs of phages 7888 and H-19B were restricted to a few bands (Fig. 3B, lanes 1 and 3), the hybridization between the genomes of 933W and 7888 revealed a large pattern of hybridizing signals (Fig. 3D, lanes 2 and 4). The observed hybridization patterns clearly indicate a very close relationship between phages 7888 and 933W, while the relationship of phage 7888 to phage H-19B is far more distant.

Host range of phage 7888. Reference strains of *S. dysenteriae* ($n = 12$), *Shigella boydii* ($n = 19$), *Shigella flexneri* ($n = 31$), and *S. sonnei* ($n = 7$) belonging to different serotypes were tested for their sensitivities to phage 7888 (Table 2). The phage formed plaques on a wide range of *Shigella* strains belonging to different groups and serotypes. With *E. coli*, only O-rough strains (*E. coli* K-12 and O-rough wild-type strains) were sensitive to phage 7888. Wild-type strains of *E. coli* belonging to

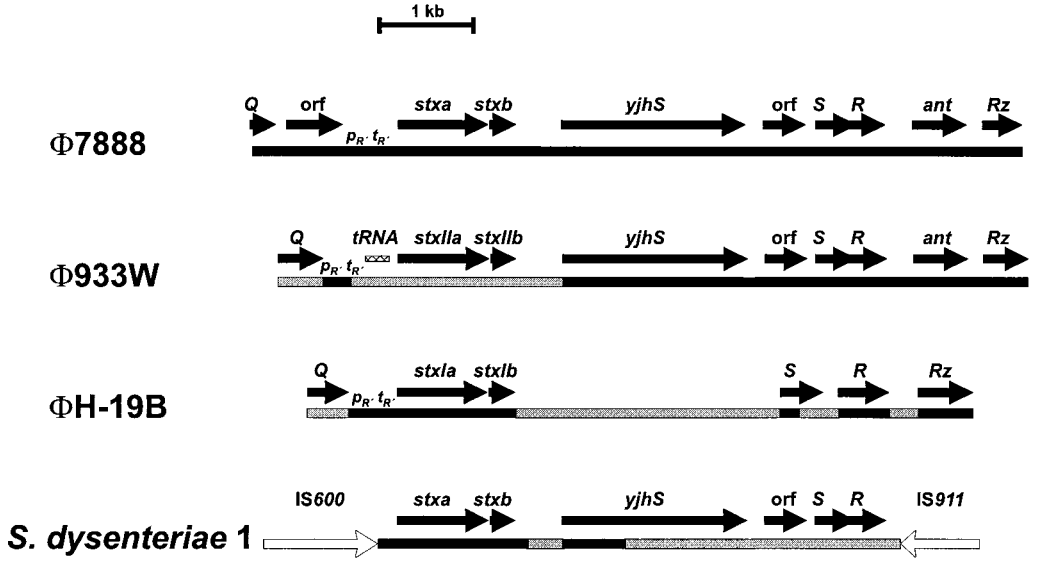

FIG. 2. Structures of Shiga toxin genes and flanking regions of phages 7888, 933W, and H-19B and of *S. dysenteriae* type 1. Black-shaded bars in the diagrams of 933W, H-19B, and *S. dysenteriae* indicate a DNA similarity of more than 95% to the corresponding region of phage 7888, while grey-shaded regions indicate a similarity of less than 95%. White arrows in the diagram of *S. dysenteriae* represent insertion elements IS*600* and IS*911*. $p_{R'}$ $t_{R'}$, regulatory region upstream of Shiga toxin genes.

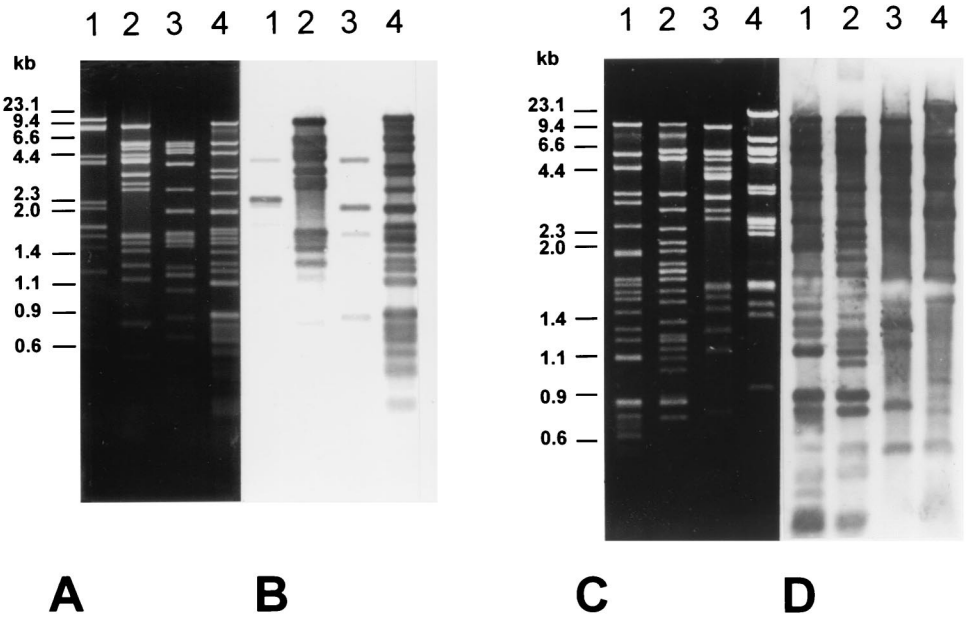

FIG. 3. Restriction patterns and Southern hybridization of DNA of phages H-19B, 933W, and 7888. (A) Agarose gel showing restriction enzyme-digested DNA of phages 7888 and H-19B. Lane 1, *Acc*I digest of H-19B; lane 2, *Acc*I digest of phage 7888; lane 3, *Hin*cII digest of phage H-19B; lane 4, *Hin*cII digest of phage 7888. (B) Southern blot of the gel shown in panel A after hybridization with labeled DNA of phage 7888. (C) Agarose gel showing restriction enzyme-digested DNAs of phages 7888 and 933W. Lane 1, *Hin*cII digest of phage 7888; lane 2, *Hin*cII digest of phage 933W; lane 3, *Acc*I digest of phage 7888; lane 4, *Acc*I digest of phage 933W. (D) Southern blot of the gel shown in panel C after hybridization with labeled DNA of phage 7888.

different O serogroups, including strains belonging to O groups O1, O53, O79, O87, O129, which are serologically related to certain O groups of *Shigella* (4), did not form plaques with the phage.

Lysogenization of *Shigella* with phage 7888. Lysogenization of *Shigella* with phage 7888 was performed as described in Materials and Methods. Stable lysogens were obtained with *S. sonnei* strains 737/85 and 508/75. Phage 7888 lysogeny was demonstrated by the production of Stx and by a positive *stx*- and *Q*-gene PCR result. Infectious phages could be induced by mitomycin C treatment of lysogenized *Shigella* strains.

The integration of phage 7888 in the genomes of Stx-negative *S. sonnei* strains 737/85 and 508/75 resulted in changes in their PFGE patterns, which led in both strains to the generation of an approximately 340-kb-size *Xba*I fragment which hybridized with digoxigenin-labeled *Hin*cII-digested phage 7888 DNA (Fig. 4A, lanes 2 and 3, and C, lane 2). This band was not present in the original *S. sonnei* host strains 737/85 and 508/75, which were also negative for phage 7888-specific DNA sequences (Fig. 4A and C, lanes 1). In addition, a second hybridizing *Xba*I fragment corresponding to the size of the phage 7888 genome (64 kb) was detected in each of the two *S. sonnei* strains which were lysogenized with phage 7888 (Fig. 4B, lanes 2 and 3, and D, lane 2).

TABLE 2. Host range of phage 7888

Species	Serotype(s) of strains	
	Sensitive to phage 7888	Resistant to phage 7888
S. dysenteriae	1, 4, 6, 7, 9	2, 3, 5, 8, 10, 11, 12
S. boydii	2, 3, 4, 5, 6, 7, 9, 17	1, 8, 10, 11, 12, 13, 14, 15, 16, 18
S. flexneri	1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5, 6	1a, 1b, 4a, 5[a]
S. sonnei	O rough and smooth	Strain CB7888[b]
E. coli	O rough[c]	O1, O53, O55, O79, O87, O129, O157

[a] Some *S. flexneri* 1a, 1b, 4a, and 5 strains were resistant to phage 7888.
[b] Source of phage 7888.
[c] *E. coli* K-12 strains C600 and P400, *E. coli* HB101, and *E. coli* O-rough wild-type strains.

Lysogenization and transduction of *E. coli* chromosomal genes by phage 7888. Phage 7888 lysates grown on *E. coli* C600 (His^+ Trp^+) were used for generalized transduction with *E. coli* strain JC3272 (His^- and Trp^-) as the recipient. His^+ and Trp^+ recombinants of JC3272 were obtained according to the selection. The numbers of JC3272 His^+ and Trp^+ recombinants obtained were proportional to the amounts of phage lysates used for the transduction assays. We calculated that approximately 0.002% of the phage particles carried chromosomal DNA. Cotransduction of His^+ and Trp^+ was not observed with any of the His^+ ($n = 174$) or Trp^+ ($n = 231$) recombinants. However, 50 to 80% of the His^+ and Trp^+ transductional recombinants of JC3272 also became positive for production of Stx and were shown to carry *stx*- and *Q*-gene-specific DNA sequences. Intact phage 7888 could be isolated from these strains but not from Trp^+ or His^+ recombinants of strain JC3272, which were negative for Stx production and for the *Q* gene. In order to investigate whether the *stx* gene was genetically linked to the chromosomal genes encoding histidine and tryptophan, P1 lysates were made on Trp^+ Stx^+ and on His^+ Stx^+ JC3272 derivatives. The P1 lysates were used for

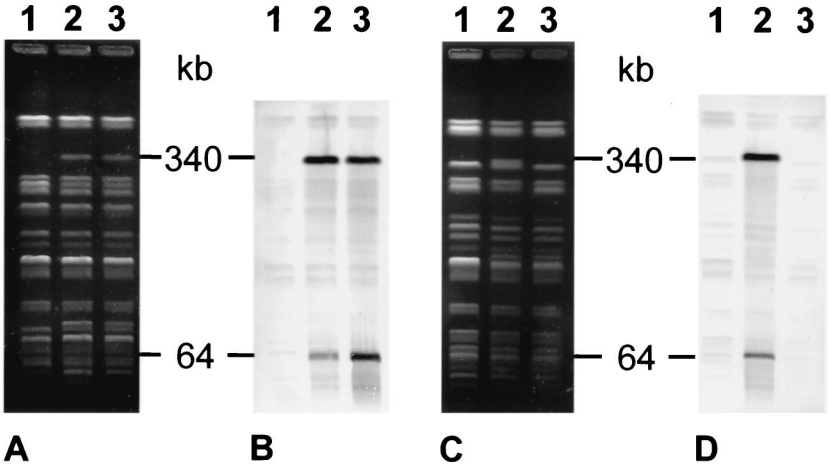

FIG. 4. Detection of prophage 7888 in the genomes of lysogenized strains of *S. sonnei*. (A) *Xba*I-digested total DNA of *S. sonnei* 737/85 and derivative strains. Lane 1, *S. sonnei* 737/85 (Stx negative); lanes 2 and 3, two derivatives of *S. sonnei* 737/85 lysogenized with phage 7888 (Stx positive). (B) Southern blot of the PFGE gel shown in panel A after hybridization with labeled DNA of phage 7888 as the DNA probe. The positions and sizes of the hybridizing fragments are indicated in kilobases. (C) *Xba*I-digested total DNA of *S. sonnei* 508/75 and derivative strains. Lane 1, *S. sonnei* 508/75 (Stx negative); lane 2, *S. sonnei* 508/75 carrying phage 7888 (Stx positive); lane 3, a derivative of an *S. sonnei* 508/75 transductant which has spontaneously lost phage 7888 (Stx negative). (D) Southern blot of the PFGE gel shown in panel C after hybridization with labeled DNA of phage 7888 as the DNA probe. The positions and sizes of the hybridizing fragments are indicated in kilobases.

transduction of JC3272 (His^- Trp^- to His^+ and to Trp^+ prototrophy, respectively). However, none of the resulting His^+ or Trp^+ recombinants were positive for *stx*- and *Q*-gene-specific DNA sequences, which indicates that the *stx* gene was not linked genetically to the chromosomal genes. This finding indicates that the Stx-positive JC3272 transductants were the result of double infections with wild-type and *E. coli* chromosomal markers transducing phage 7888 particles.

The integration of phage 7888 into the genome of *E. coli* JC3272 was investigated by PFGE and by DNA hybridization with an *stx*- and stx_1-specific gene probe. Nine independently isolated phage 7888 lysogens from transductional experiments with JC3272 were analyzed for their *Xba*I patterns by PFGE. The lysogenic JC3272 isolates were identical in their *Xba*I patterns but showed differences in two bands from the original host strain, JC32372 (Fig. 5A). A 243-kb *Xba*I fragment present in JC3272 was replaced by a 307-kb fragment, which was present in all nine JC3272 lysogens but absent in the parental strain JC3272. The 307-kb *Xba*I fragment hybridized with the *stx*- and stx_1-specific gene probe, which indicates that it was generated by integration of the phage 7888 genome (Fig. 5B). A second band 64 kb in size which hybridized with the *stx*- and stx_1-specific gene probe was found in all nine lysogenic JC3272 derivatives (Fig. 5B). The 64-kb fragment might represent free phage DNA, because it was overexpressed in two JC3272 lysogenic strains which were were growing poorly and which spontaneously liberated free plaque-forming phages during growth (Fig. 5, lanes 8 and 9).

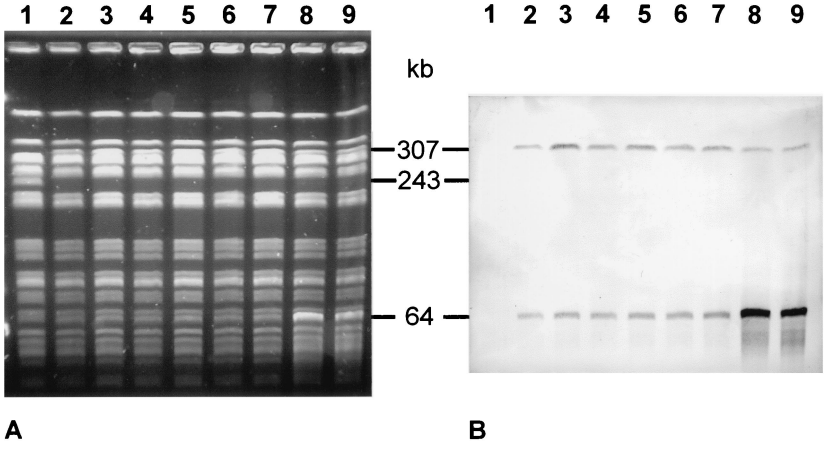

FIG. 5. Integration of phage 7888 in the genome of *E. coli* K-12 strain JC3272. (A) *Xba*I-digested total DNA of JC3272 and lysogenized derivatives separated by PFGE. Lane 1, JC3272; lanes 2 to 9, independent isolates of JC3272 carrying prophage 7888. (B) Southern blot of the PFGE gel shown in panel A after hybridization with an *stx*- and stx_1-specific DNA probe. The positions and sizes of the hybridizing fragments are indicated in kilobases.

Presence of *stx* genes in *Shigella* strains from human patients. Patient isolates of *S. sonnei* ($n = 149$), *S. flexneri* ($n = 118$), *S. boydii* ($n = 7$), and *S. dysenteriae* ($n = 8$), including two *S. dysenteriae* type 1 strains, were investigated for carriage of *stx*- and stx_1-specific DNA sequences by DNA hybridization. PCR-amplified DNA from strain C600(H19) was used as an *stx*- and stx_1-specific gene probe (8). Of the 282 strains, only the two *S. dysenteriae* type 1 strains and *S. sonnei* CB7888 were found to be positive for *stx*.

DISCUSSION

Bacteriophages are increasingly recognized as vectors of the horizontal transfer of virulence determinants in many bacterial species (for reviews, see references 11 and 18). In *Shigella*, some virulence functions are associated with bacteriophages, such as the genes for O-antigen modification of *S. flexneri* (3) and the aerobactin genes in *S. flexneri* and *S. boydii* strains (3, 28). Recent studies have shown that the *stx* gene of *S. dysenteriae* type 1 is associated with bacteriophage-related genes, which indicates that the *stx* gene was initially introduced by integration of an Stx-encoding phage (17). Stx1- and Stx2-encoding lambdoid phages are frequently associated with STEC strains, and in contrast to *S. dysenteriae* type 1, viable Stx phages can be isolated from serologically different STEC strains (21, 33, 35). These phages can spread their *stx* genes to Stx-negative *E. coli* strains by transduction and lysogenization (2, 20, 31). These findings therefore suggest that bacteriophages have contributed to the evolution and genetic diversity of the STEC group by the horizontal spread of *stx* determinants in *E. coli* (2, 31).

The Stx-encoding *S. sonnei* phage 7888 showed a broad host range for many *Shigella* strains and types, which indicates that it is well adapted to *Shigella* as a natural host and may have acquired its *stx* gene from *S. dysenteriae* type 1. On the other hand, the comparison of *S. sonnei* phage 7888 to the well-characterized *stx*-carrying *E. coli* phages H-19B and 933W suggested a close relationship of phage 7888 and 933W. Electron micrographs of 7888 virions revealed a morphology similar to that of the Stx2-converting phage 933W. Phage 933W shows a head 70 nm in diameter and a tail 27 nm long and 13 nm wide (26). In contrast, the stx_1-carrying H-19B virion has a considerably longer head and a flexible tail resembling those of phage lambda (39). The genome size of phage 7888 was found to be very similar to that of the 933W genome, i.e., 61,670 bp as determined by nucleotide sequence analysis (26).

The close genetic relationship between phages 933W and 7888 was demonstrated by DNA cross-hybridization analysis using the complete genome of phage 7888 as a gene probe (Fig. 3). In order to gain more information on the possible origin of Stx-encoding phage 7888 and on the genetic similarity between phages 7888 and 933W, we sequenced an 8,053-bp DNA region located between the *Q* and *Rz* genes of phage 7888. This part of the phage genome encompasses the *stx* gene and carries regulatory sites involved in the regulation of Shiga toxin expression (23, 26). By comparing this region to the corresponding regions of phages H-19B (AF034975) and 933W (AF125520), we found a regulatory site, $p_{R'}\ t_{R'}$, located upstream of the *stx* gene which is involved in expression of *stx* genes by Q antitermination and is highly conserved (more than 95% identity) in all three phage genomes (Fig. 2). The presence of the $P_{R'}\ t_{R'}$ site in the genome of phage 7888 suggests that the expression of the *stx* gene is also regulated by the action of the Q protein as a transcriptional activator, as was reported for phages H-19B and 933W (23, 38).

The largest stretch of DNA with sequence identity of >98% between phages 7888 and H-19B is a 1,895-bp region encompassing the *stx* and stx_1 genes and adjacent sequences. In both phages, the Q-dependent regulatory region $P_{R'}\ t_{R'}$ is located directly upstream of the *stx* or stx_1 gene, respectively. In phage 933W, the $P_{R'}\ t_{R'}$ region is separated by an approximately 300-bp stretch of tRNA genes from the adjacent stx_2 gene. The tRNA genes are not present in the corresponding regions of phages H-19B and 7888.

More than 95% similarity is found between the genomes of phages 7888 and 933W in an approximately 4,800-bp DNA stretch downstream of the *stx* gene. The region of high DNA similarity starts shortly upstream of a 1,938-bp ORF encoding an analogue of the *E. coli* K-12 YjhS protein, followed by an unknown ORF and the genes *S, R*, and *Rz*. In contrast, in the corresponding region on the genome of phage H-19B, small stretches of high sequence similarity to phage 933W and 7888 are found only in the coding regions of the *S, R*, and *Rz* genes. The complete genomic sequence of another Stx1-encoding prophage (VT1-Sakai) from an enterohemorrhagic *E. coli* O157 outbreak in Japan has been reported (GenBank accession no. AP000400) (40), though no Stx1 phage particles could be isolated from the strain. The *Q*-to-*Rz* region, which includes the stx_1 gene of this prophage, is very similar to that of phage 7888. As other parts of the genomic sequence of this prophage do not possess any DNA similarity to phage 933W, however, we suppose that the relationship to phage 7888 is minimal.

A specific feature of the *Q* to *Rz* region of phage 7888 is the presence of a 594-bp ORF of unknown function located downstream of the *Q* gene, which is absent in strains H-19B and 933W and in the VT1-Sakai prophage. Another difference concerns the 3′ coding region of the partially sequenced *Q* gene of phage 7888, which encodes a Q protein that is slightly different from the Q proteins of the H-19B and 933W phages; i.e., it has a carboxy-terminal region that is truncated by 18 amino acids.

A sequence comparison of the *stx* region of phage 7888 with the chromosomally located *stx* region of *S. dysenteriae* type 1 (AF153317), which are enclosed by IS*600* and IS*911* elements, revealed that the coding regions of the *stxA* and *stxB* genes as well as 187 bp 5′ upstream and 136 bp 3′ downstream of the *stx* gene are nearly identical (four nucleotide differences in a stretch of 1,550 bp). The *stx* gene of *S. dysenteriae* is likely to have been derived from an integrated prophage which became defective due to the integration of insertion sequence elements and rearrangements and thus resulted in a stable integration of the *stx* gene in the chromosome (17). The regulatory region for Q-dependent activation, however, is missing in *S. dysenteriae*. Downstream of the *stx* region, high sequence similarities (>98% identity) between phage 7888 and the *S. dysenteriae stx* region are found only in the 5′ coding region of *yhjS* genes.

The alignment of 22 published *stx* and stx_1 sequences (http://www.ncbi.nlm.nih.gov/BLASTN) originating from different *S. dysenteriae* type 1 strains, from *E. coli*, and from *S. sonnei* phage 7888 revealed only a few base exchanges in the *stxA* and

stxA$_1$ genes which cannot be related to the source of the toxin genes, *Shigella*, or *E. coli*. It is, therefore, not possible to determine whether the *stx* gene present on the genome of phage 7888 was originally acquired from *S. dysenteriae* type 1 or from an *E. coli* phage. However, our findings indicate that phage 7888 belongs to the family of lambdoid 933W-like phages, which are known to have a mosaic structure due to frequent gene exchange by recombination (12, 14, 37). It is possible that these lambdoid phages also differ in their host ranges and that similar phages might be more adapted to *Shigella*, which is highly related to *E. coli*.

Our finding that the lambdoid phage 7888 can mediate generalized transduction of chromosomal genes in *E. coli* points to its general role in the evolution of bacteria. It is known that bacteriophage lambda can behave as a generalized transducer in *E. coli* (16). The uniform hybridization patterns found with independent isolates of JC3272 phage 7888 lysogens indicate that phage 7888, like lambda, has a specific integration site in the bacterial chromosome. Further work is necessary to analyze the attachment site of phage 7888 in *E. coli* and *S. sonnei*.

The detection of an *stx* gene on a phage of *S. sonnei*, which can transfer the Stx-encoding characteristic to nontoxigenic *S. sonnei* strains, points to the continuous evolution of *Shigella* by horizontal gene transfer. In our study, we could not detect *stx* or *stx*$_1$ sequences in 280 *Shigella* strains other than *S. dysenteriae* type 1. However, older reports indicate that Stx production might occasionally occur in non-*S. dysenteriae* type 1 *Shigella* strains (5, 27). As *S. sonnei* isolates are not routinely examined for production of Shiga toxins in clinical laboratories, it is possible that Stx production in patient isolates of *S. sonnei* remains undetected in diagnosis. Among the different *Shigella* species, *S. sonnei* is the major isolate in the developed countries (1). In contrast to *S. dysenteriae* type 1, *S. sonnei* is distributed worldwide and the emergence of Stx-producing *S. sonnei* strains may contribute to an increase in the virulence of these widely occurring human pathogens.

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