Complete nucleotide sequence of the prophage VT2-Sakai carrying the verotoxin 2 genes of the enterohemorrhagic *Escherichia coli* O157:H7 derived from the Sakai outbreak

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The enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 strain RIMD 0509952, derived from an outbreak in Sakai city, Japan, in 1996, produces two kinds of verotoxins, VT1 and VT2, encoded by the *stx1* and *stx2* genes. In the EHEC strains, as well as in other VT-producing *E. coli* strains, the toxins are encoded by lysogenic bacteriophages. The EHEC O157:H7 strain RIMD 0509952 did not produce plaque-forming phage particles upon inducing treatments. We have determined the complete nucleotide sequence of a prophage, VT2-Sakai, carrying the *stx2A* and *stx2B* genes on the chromosome, and presumed the putative functions of the encoded proteins and the *cis*-acting DNA elements based on sequence homology data. To our surprise, the sequences in the regions of VT2-Sakai corresponding to the early gene regulators and replication proteins, and the DNA sequences recognized by the regulators share very limited homology to those of the VT2-encoding 933W phage carried by the EHEC O157:H7 strain EDL933 reported by Plunkett et al. (J. Bacteriol., p1767–1778, **181**, 1999), although the sequences corresponding to the structural components are almost identical. These data suggest that these two phages were derived from a common ancestral phage and that either or both of them underwent multiple genetic rearrangements. An *IS629* insertion was found downstream of the *stx2B* gene and upstream of the lysis gene *S*, and this might be responsible for the absence of plaque-forming activity in the lysate obtained after inducing treatments.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is defined

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as *E. coli* that produces one or more forms of Shiga toxin (Stx) and factors for attaching-and-effacing lesions on the host epithelial cells, and carries a characteristic large plasmid (Levine, 1987). EHEC causes not only hemorrhagic

colitis, but also serious complications such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura, sometimes resulting in death (Cohen and Giannella, 1991; Griffin and Tauxe, 1991). Among EHEC strains, the *E. coli* serotype O157:H7 is the most common one, and was first recognized in 1982 during outbreaks of bloody diarrhea in Oregon and Michigan, USA (Wells et al., 1983; Riley et al., 1983).

In Japan, several quite large outbreaks of EHEC O157:H7 occurred in 1996 (Watanabe et al., 1996). The biggest outbreak occurred in Sakai city, Osaka, where more than 6,000 patients suffered from diarrhea, hemorrhagic colitis and HUS. In this outbreak, approximately 1,000 patients were hospitalized for their severe gastrointestinal symptoms, and about 100 cases were complicated with HUS, resulting in 3 deaths.

We have previously determined the complete DNA sequences of the large virulence plasmid pO157 and the small plasmid pOSAK1 found in an EHEC O157 strain (RIMD 0509952) isolated from a typical case in the Sakai outbreak (Makino et al., 1998). pO157 possessed many pathogenic genes, such as *hlyA*, *katP*, *espP* and *toxB*, encoding EHEC hemolysin, catalase-peroxidase, serine protease, and a protein homologous to ToxinB of *Clostridium difficile*, respectively. It carried chimeric systems of replication and plasmid maintenance similar to those of R100 plasmid and F-factor. The strain RIMD 0509952 produces Shiga toxins, Stx1 and Stx2, encoded by two prophage-like regions, to which we named VT1-Sakai and VT2-Sakai, respectively (unpublished data). When repeated efforts to detect phage plaque formation with the mitomycinC-induced Sakai strain were carried out with an indicator *E. coli* K-12 strain such as C600, no plaques were detected, indicating that the prophages carrying the *stx1* and *stx2* genes of this strain are defective for forming plaques. On the other hand, Matsushiro et al. (1999) reported that another O157:H7 Sakai strain RIMD 0509894 produced plaque-forming phages VT1-Sa and VT2-Sa, carrying the *stx1* and *stx2* genes, respectively.

Here we report the complete DNA sequence of the prophage VT2-Sakai on the chromosome of the O157:H7 Sakai strain RIMD 0509952. This project is part of an ongoing effort to sequence the entire genome of the EHEC O157:H7 strain derived from the Sakai outbreak. Since Plunkett et al. (1999) published the complete DNA sequence of 933W phage harboring the *stx2* genes of EHEC O157:H7 strain EDL933 (during our preparation of this manuscript), we compared the sequence between VT2-Sakai and 933W and found substantial differences.

MATERIALS AND METHODS

Construction of cosmid- and lambda-based libraries of O157 genomic DNA. The EHEC O157:H7 (RIMD 0509952) cells were grown as described previously

(Makino et al., 1998) and the whole genomic DNA was prepared by the standard method (Silhavy et al., 1984). The DNA was partially digested with *Sau3A* I restriction enzyme, treated with bacterial alkaline phosphatase to prevent multiple inserts, and ligated with SuperCos1 or lambda EMBL3 pre-digested with *Bam*H I (Stratagene). Gigapack III XL packaging extracts (Stratagene) were used to package both the recombinant cosmids and lambda phages following the protocols of the supplier.

Isolation of cosmid and lambda phage clones carrying VT2-Sakai DNA, and filling the gap between the contigs by PCR. The *stx2* DNA fragment (Fig. 1, probe A) used as a probe for colony and plaque hybridization was amplified by PCR using a TaKaRa Ex Taq kit (Takara Shuzo) with primers EVS1/2 (Takara Shuzo). The fragment was separated by conventional gel electrophoresis, and purified by using a QIAquick Gel Extraction kit (QIAGEN). An AlkPhos Direct kit (Amersham Life Science) was used to detect positive clones in hybridization experiments. To isolate the lambda clones which contain distant regions, such as the L31E09 clone (Fig. 1A), the appropriate primers were designed to make the probes B and C by PCR based on the sequence of the cosmid clone C49E05 (Fig. 1A), and then probes B and C were used for plaque hybridization. In the case of the lambda clones, the inserted DNA fragments in the recombinant phages were amplified by PCR using a TaKaRa LA Taq kit (Takara Shuzo) with two primers flanking the cloning site: primer EMBL-1, (5'-ACAGTCGGTGGTCCGGC-AGTACAATGGATTACC-3') and primer EMBL-2, (5'-GCAACCTGCAACGTATTGAGCGCAAGAATCAGC-3'). The PCR products were treated with exonuclease I and shrimp alkaline phosphatase to remove excess dNTPs and primers (Amersham). To obtain the DNA fragment that fills the gap (Fig. 1A), primers A and B were used for PCR, and the PCR products were similarly treated with exonuclease I and shrimp alkaline phosphatase. These DNA fragments were used for shotgun cloning as described below.

Preparation of DNA templates for sequencing.

Ten micrograms of the DNA fragments amplified by PCR or cosmid DNA were sonicated for 30 sec with a sonic disrupter. The sonicated DNA was blunt-ended using a DNA blunting kit (Takara Shuzo) and fractionated by 6% polyacrylamide gel electrophoresis. A gel slice containing DNA fragments of 1~2 kb in length was excised from the gel, and DNA was eluted by immersing the gel in the MG elution buffer [500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% (w/v) SDS] overnight at 37°C. The recovered DNA was ligated to the *Sma* I site of vector M13mp18 DNA (Yanisch-Perron et al., 1985), which had been pretreated with bacterial alkaline phosphatase, and introduced into *E. coli* DH11S com-

Fig. 1. Schematic illustration of the prophage VT2-Sakai. A) The location of VT2-Sakai on the chromosome is shown relative to the chromosome map of K-12 strain. The thick bars indicate the extents of the DNA fragments obtained from the lambda and cosmid clones, and by PCR amplification. To obtain clones L37B11 and C49E05, probe A was used for plaque or colony hybridization. To obtain L31E09, probes B and C were used for plaque hybridization. To fill the gap by PCR in the prophage region, two primers (A and B) were used. Open bars indicate regions corresponding to the K-12 chromosome. Black bars indicate regions corresponding to the prophage VT2-Sakai. Dotted bars indicate the *IS629* region. The nucleotide numbers are counted from the left end of the PCR product. B) The predicted ORFs of VT2-Sakai are illustrated. Hatched boxes indicate ORFs also found in the K-12 genome. White and shaded boxes are ORFs encoded by the prophage VT2-Sakai. Shaded boxes are ORFs which have no or only limited homology to 933W. ORFs encoded by *IS629* are also shaded. The boxes above and below the horizontal line are the ORFs with rightward and leftward transcription directions, respectively.

petent cells (Raleigh et al., 1988; Lin et al., 1992) with an electroporator. The cells were plated with LB soft agar containing X-gal (100 µg/ml), isopropyl β-D-thiogalactopyranoside (1 mM) and indicator cells to screen the transfectants. The isolated plaques were inoculated into 100-fold diluted suspensions of overnight cell cultures and grown for 3h at 37°C, and the cultures were used as templates for PCR. The DNA inserts in the phage clones were amplified by PCR using a TaKaRa Ex Taq kit (Takara Shuzo) with two primers flanking the cloning site: primer 1, (5'-TCCGGCTCGTATGTTGTGTGGA-3') and primer 2, (5'-GTGCTGCAAGGCGATTAAGTTGG-3'). The PCR products were treated with a PCR product pre-sequencing kit containing exonuclease I and shrimp alkaline phosphatase (Amersham).

DNA sequencing, sequence assembly and data analysis. The sequencing reactions were performed by using a Dye Primer Cycle Sequencing FS Ready Reaction Kit and a thermal cycler, GENEAMP PCR SYSTEM 9600 (Perkin-Elmer). DNA sequences were analyzed with an ABI PRISM 373XL DNA sequencer (Perkin-Elmer). Sequences were assembled by using the Factura 2.0

Feature Identification Software (Perkin-Elmer) and Autoassembler DNA Sequence Assembly Software Version 2.0 (Perkin-Elmer). To complete the sequencing of the entire prophage, 493 shotgun clones for the cosmid clone C49E05, 556 for the lambda clones L37B11 and L31E09, and 205 for the gap-filling PCR product were sequenced by using -21M13 or/and M13Rev primers (Perkin-Elmer)(Fig. 1A).

The ORF Finder (from Other NCBI Resources) was used to identify potential open reading frames (ORFs). Each ORF was searched for in the sequence database using WWW BLASTP 2.0.8 (Jan/05/1999 version) (Altschul et al., 1997).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AP000422.

RESULTS

Sequence of VT2-Sakai and its surrounding regions. To analyze the unique DNA sequence to the O157 strain

RIMD 0509952, we determined the entire sequence of the prophage VT2-Sakai carrying the *stx2A* and *stx2B* genes after making cosmid and lambda libraries with the genomic DNA. We screened the libraries by colony or plaque hybridization using *astx2* DNA fragment as a probe, and isolated clones that contained the *stx2* genes. One of them derived from the lambda library and another from the cosmid library were designated L37B11 and C49E05, respectively (Fig. 1A). The complete nucleotide sequences of the L37B11 and C49E05 clones were determined by the shotgun method as described in Materials and Methods. To clone lambda phages carrying the remaining regions, we screened the lambda library again by hybridization, using the probes B and C (Fig. 1A). The phages which hybridized with the two probes were partially sequenced, and one of the phages carried a sequence identical to that of a region of the K-12 genome, and was named L31E09 (Fig. 1A). Comparison of the complete nucleotide sequence of the insert of the L31E09 clone and that of the overlapping region of the K-12 genome revealed that the lambda clone carried the O157-specific region and the region corresponding to that of K-12 genome at 23 min (Fig. 1A) (Blattner et al., 1997). The end of the homologous region to the K-12 genome was located within the *wrbA* gene, which encodes the Trp repressor binding protein, WrbA. It has been proposed that the WrbA protein functions as an accessory protein in blocking TrpR-specific transcriptional repression (Yang et al., 1993). To fill the gap, we constructed primers that were specific for *wrbA* (Blattner et al., 1997) and the end of L37B11 based on its sequence information (Fig. 1A, primers A and B). The DNA fragment containing the gap region was amplified by PCR with the genomic DNA extracted from the O157:H7 cells, and its sequence was determined by the shotgun method (Fig. 1A). The combination of the sequence of the PCR product and all the sequence data described above indicated that the O157:H7-specific region was about 62.7-kb long and was integrated into the *wrbA* gene of the K-12 genome (Fig. 1).

Identification of the attachment site of VT2-Sakai.

The sequence of the VT2-Sakai prophage is flanked by two copies of a 7-bp direct repeat sequence (GTTTCAA) present only once in the *wrbA* gene of K-12 (Fig. 1B). Like lambda phage, many temperate bacteriophages integrate into their host genomes by a site-specific recombination between the phage and bacterial attachment sites, *attP* and *attB*. As a result, the integration generates two copies of a direct repeat (*attL* and *attR*) flanking the integrated linear prophage genome. From these reasons, we concluded that the core *attL* and *attR* sequences were located at positions 555 to 561 (*attL*) and positions 63258 to 63263 (*attR*), respectively (Fig. 1B). The total length of the VT2-Sakai prophage with the two attachment sites was 62708 bp. The G + C content of the VT2-Sakai

prophage was 49.9%, which is slightly lower than that of the K-12 genome (50.8%).

IS element. The *wrbA* gene, whose sequence was identical to that of the gene from the K-12 strain was split by integration of VT2-Sakai, and the junctions were identified at two regions (1–554 and 63264–65550) (Fig. 1A). We found an IS element at positions 24899 to 26209 that was very similar to the *IS629* derived from *Shigella sonnei* (96.0% identity) (Fig. 1) (Matsutani and Ohtsubo, 1990). A 4-base direct repeat at the insertion site of *IS629* was identified and it split an ORF(H0115; see below) whose function could not be identified from the homology search. The *IS629* insertion is likely to be responsible for the deficiency in plaque-forming ability, although we do not know whether the deficiency is due to the insertion inactivation of an essential gene or the reduction of the transcription of downstream genes, such as the *S* and *R* genes, required for the lysis of the host cells.

Comparison between the DNA sequences of VT2-Sakai and 933W.

Plunkett et al. (1999) have recently determined the complete sequence the *stx2*-encoding phage 933W derived from the O157:H7 strain EDL933. We compared the DNA sequences and ORFs of the two phages, both of which carry the *stx2* genes. The integration site of VT2-Sakai on the chromosome is exactly the same as that of 933W. VT2-Sakai and 933W share very similar DNA sequences in most areas of the genomes, with only minor sequence differences and small gaps. Notable differences were found in eight blocks where there existed little sequence similarity, including the one created by the *IS629* insertion (Fig. 2). These regions included the regulator genes for the early gene expression, the genes for replication initiation, and the recognition sequences for the regulators (see below). Except for *IS629*, they were located between nucleotides 9137 and 18975, where six small nonidentical blocks were flanked by highly conserved sequences. Homology search analysis of the DNA sequence from nucleotide 8000 to 19500 revealed that this region is highly chimeric (Fig. 3). The DNA sequences from 8000 to 9137, from 10015 to 10084, and from 11459 to 11865 are very similar to those of lambda phage (Sanger et al., 1982). The DNA sequence from 9838 to 9888 is homologous to that of ϕ 21 phage (Franklin, 1985). The DNA sequences from 12577 to 15178 and from 18774 to 18844 are almost identical to those of HK022 phage (Oberto et al., 1989; Oberto et al., 1994; Clerget and Boccard, 1996). The DNA sequences corresponding to those from 17505 to 17700, from 18384 to 18740, and from 18962 to 19100 are very similar to those of ϕ 21 (Kroeger, 1999), P22 (Sauer et al., 1983), and H-19B phages (Neely and Friedman, 1998a; Neely and Friedman, 1998b), respectively. These results suggest that many rounds of recombination with these and other unknown phages gen-

Fig. 2. Schematic drawing of the regions nearly identical in VT2-Sakai (A) and 933W (B). Homologous regions are boxed. Numbers of identical bases per total number of bases compared are shown. The numbers with minus and plus signs in parentheses indicate the numbers of bases missing or added in the VT2-Sakai sequence as compared with the 933W, respectively.

Fig. 3. DNA sequence in the region of VT2-Sakai from nucleotide 8000 to 19500, which contains several blocks of sequences different from those of 933W. Black bars indicate the regions homologous to those of 933W. The regions homologous to other phages are shown by the boxes filled with various patterns specific to phages whose names are shown above. The numbers of identical bases per total number of bases compared are shown. The numbers in parentheses indicate the numbers of bases missing (-) or added (+) in the VT2-Sakai sequence as compared with those of other phages.

erated VT2-Sakai. It should be noted that the regions described above encode the immunity proteins, including CI and Cro proteins, and their recognition sequences and therefore VT2-Sakai is likely to have immunity specificity different from that of 933W.

Descriptions of selected ORFs based on homology search. We listed ORFs longer than 100 amino acids (aa) in Table 1 and used them for homology search analysis against the NCBI's non-redundant amino acid sequence database, nr (Jan/05/99 version). When ORFs shorter than 100 aa were found to be homologous to any

known gene product, they were also noted and listed in Table 1. Computational analyses of the entire nucleotide sequence identified 93 ORFs and three putative tRNA genes (Table 1), which are illustrated in Fig. 1B. ORFs in the VT2-Sakai prophage are labeled H0071 to H0160, from left to right in Fig. 1B. Genes with functional assignments are named, and the names of the equivalent lambda genes or *E. coli* K-12 genes were employed when possible.

The VT2-Sakai prophage is very similar to the bacteriophage 933W (Plunkett et al., 1999) carrying VT2 (*stx2*) genes at the nucleotide sequence and gene organization

Table 1. Genes of VT2-Sakai

Label	Gene	From	To	Strand ^a	Length of product (amino acids)	Homologous ORFs in 933W	Note
	wbrA	8	<561	–			truncated wbrA of E.coli K-12
	attL	555	561				attL core
H0071	int	583	1917	–	444	L0061	integrase
H0072	xis	1946	2245	–	99	L0062	excisionase
H0073		2316	2627	–	103	L0063	
H0074		2687	3052	–	121	L0064	
H0075		2964	3587	–	207	L0065	
H0076		3591	3878	–	95	L0066	
H0077		3880	4098	–	72	L0067	
H0078		4100	4387	–	93	L0068	
H0079		4657	5430	–	257	L0069	λ Ea22 homolog
H0080		5427	5648	–	73	L0141	
H0081		5747	6028	–	93	L0070	λ Orf-61 homolog
H0082		6039	6230	–	63	L0071	λ Orf-63 homolog
H0083		6203	6391	–	62	L0072	λ Orf-60a homolog
H0084	exo	6382	7062	–	226	L0073	λ Exo homolog; exonuclease
H0085	bet	7059	7844	–	261	L0074	λ Bet homolog; DNA annealing
H0086	gam	7850	8146	–	98	L0075	λ Gam homolog; inhibitor of RecBC
H0087	kil	8221	8490	–	89	L0076	λ Kil homolog; host killing
H0088	c III	8333	8497	–	54	L0077	λ CIII homolog; regulator for lysogenization
H0089	ea10 (ssb)	8570	8938	–	122	L0078	λ Ea10 homolog; ssDNA binding protein
H0090		9121	9372	–	83		
H0091	N	9431	9703	–	90	L0080	phage H-19B N homolog
H0092		10136	10258	–	40		
H0093		10432	10953	–	173		
H0094	c I	11455	12150	–	231		λ CI homolog; repressor for lysogeny
H0095	cro	12226	12441	+	71		phage L C2 homolog; repressor for lytic growth
H0096	c II	12583	12879	+	98	L0087	phage HK022 CII homolog; activator for c I
H0097		12912	13058	+	48		phage HK022 Orf48
H0098	O	13051	13950	+	299		phage HK022 O homolog
H0099	P	13940	15376	+	478		phage HK022 P homolog
H0100		15376	15645	+	89		
H0101		15716	15994	+	92		phage 933W
H0102		16127	16342	+	71		phage 933W
H0103		16353	16589	+	78		phage 933W
H0104		16546	16992	+	148	L0093	λ and P22 NinB Orf-146 homolog
H0105	dam	16989	17516	+	175	L0094	phage HP1 and T1 Dam homolog; DNA adenine methylase
H0106	ninE	17513	17695	+	60		phage 21 ninE homolog
H0107		17970	18485	+	171		phage TP901-1
H0108	roi	18768	19496	+	242	L0096	phage H-19B Roi homolog
H0109		19496	20101	+	201	L0097	λ NinG Orf-204 homolog
H0110		20098	20292	+	64	L0098	λ NinH Orf-68 homolog
H0111	Q	20285	20719	+	157	L0099	phage H-19B Q homolog
		20707	20734	+			pR'; putative late promoter
		20748	20755	+			qut; putative Q-utilization
		20767	20789	+			tR'1; possible rho-independent terminator
		20970	20998	+			tR'2; possible rho-independent terminator
		21123	21151	+			pS; putative tRNA promoter
	ileZ	21161	21236	+		L0100	tRNA-Ile
	argN	21246	21322	+		L0101	tRNA-Arg
	argO	21336	21412	+		L0102	tRNA-Arg
		21426	21449	+			tS;possible rho-independent terminator
H0112	stx2A	21503	22462	+	319	L0103	VT2 subunit A
H0113	stx2B	22474	22743	+	89	L0104	VT2 subunit B
H0114		23230	23613	+	127	L0105	
H0115		23657	25135	+	492	L0105	
H0116		24942	25832	–	296		IS629; transposase
H0117		25829	26155	–	108		IS629

Label	Gene	From	To	Strand ^a	Length of product (amino acids)	Homologous ORFs in 933W	Note
H0118		26479	26604	+	42		phage 933W
H0119		26617	26796	+	59		phage 933W
H0120		26837	27109	+	90	L0106	
H0121	S	27186	27401	+	71	L0107	phage H-19B and Qin S homolog
H0122	R	27406	27939	+	177	L0108	phage Qin R homolog
H0123	ant	28210	28779	+	189	L0109	phage P22 Ant homolog
H0124	Rz	28933	29397	+	154	L0110	λ Rz homolog
H0125	Rz1	29153	29338	+	61	L0143	λ Rz1 homolog
H0126	bor	29429	29722	−	97	L0111	λ Bor homolog
H0127		30131	30937	+	268	L0112	
H0128		30918	32267	+	449	L0113	
H0129		32207	32623	+	138	L0113	
H0130		32623	34767	+	714	L0114	
H0131		34925	35932	+	335	L0115	
H0132		35956	37170	+	404	L0116	
H0133		37226	37615	+	129	L0117	
H0134		37638	38126	+	162	L0118	
H0135		38110	38673	+	187	L0119	
H0136		38673	39323	+	216	L0120	
H0137		39320	41257	+	645	L0121	similar to tail fiber protein
H0138		41259	41528	+	129	L0122	
H0139		41575	41856	+	93	L0123	
H0140		42073	43776	+	567	L0124	
H0141		43773	45041	+	422	L0125	
H0142		45107	45334	+	75	L0126	
H0143		45340	45957	+	205	L0127	
H0144	lom	46048	46782	+	244	L0128	λ Lom homolog
H0145		47212	47613	+	133	L0129	
H0146		47707	48363	+	218	L0130	
H0147		48366	48812	+	148	L0131	
H0148		48822	49073	+	83	L0132	
H0149		49084	50349	+	421	L0133	
H0150		50380	58800	+	2806	L0134	
H0151		59083	59271	+	62	L0135	
H0152		59351	59695	−	114	L0136	similar to YgiW and YdeI of <i>E. coli</i>
H0153	hokW	59815	59970	−	51	L0137	similar to Ref and Gef
H0154	mokW	59815	60027	−	70	L0144	similar to Gef leader protein
	sokW	60019	60089	+		L0138	antisense RNA
H0155		60270	60656	−	128	L0139	
H0156		60656	61315	−	219	L0140	
H0157		61336	61554	−	72	L0140	
H0158		61541	61825	−	94		
H0159		61822	62043	−	73	L0141	
H0160		62091	62720	−	209	L0142	
	attR	63258	63263	+			attR core
	wrbA'	<63258	63307	−			truncated wrbA of <i>E. coli</i> K-12
		63613	63786	+			Hypothetical protein of <i>E. coli</i> K-12
	pyrP	63869	65197	−			uracil transport protein of <i>E. coli</i> K-12

a. A plus sign indicates the forward orientation of the gene and a minus sign indicates the reverse orientation of the gene.

levels (Fig. 2 and Table 1).

ORFs H0071 (Int) to H0089 (Ea10) are identical or very similar to the corresponding ORFs of 933W (L0061 to L0078)(Fig. 1B and Table 1). This region includes the homologs encoding the integrase that mediates the site-specific recombination necessary for integration into the host genome and *xis*, encoding the excisionase that

together with integrase mediates excision of the phage genome from the host genome upon induction (Weisberg and Landy, 1983). The region from H0084 to H0086 contains the *exo*, *bet* and *gam* genes highly homologous to the homologous recombination system of lambda (Smith, 1983). Downstream of this region, the *kil* and *cIII* genes, which are involved in host cell killing and establishment

of lysogeny (Court and Oppenheim, 1983; Wulff and Rosenberg, 1983), respectively, are also very similar in sequence to the lambda homologs. ORFs H0090, 0092, 0093 and 0100 are not homologous to any known ORFs in the database. ORF H0091 is similar to the antitermination N proteins of bacteriophage H-19B and 933W, although the similarities are relatively low (42 and 41% identities, respectively) (Neely and Friedman, 1998a; Neely and Friedman, 1998b; Plunkett et al., 1999). ORF H0094 is the CI repressor of VT2-Sakai, which appears to be a hybrid of two species of repressors. The amino-terminal 96-amino acid sequence most closely resembles that of the repressor of bacteriophage L (56% similarity) (Schicklmaier and Schmieger, 1997), while the rest of the sequence is almost identical to that of lambda (99% identity) (Sanger et al., 1982). In the lambda repressor, the amino-terminal domain contains a helix-turn-helix motif for DNA binding that interacts with the operator sequences, while the carboxyl-terminal domain of the protein is involved in dimerization (Gussin et al., 1983). Although the CI of VT2-Sakai is not similar to that of 933W, the CI of 933W is a chimera of the repressors of two bacteriophages, HK022 and H-19B (Plunkett et al., 1999).

ORF H0095 is most similar to the Cro repressor of bacteriophage L (42% identity) (Schicklmaier and Schmieger, 1997). The similarities of the amino-terminal domain of CI described above and the Cro sequence to those of phage L are consistent with the facts that both CI and Cro recognize the same operator sequences, which has been shown in lambda (Gussin et al., 1983). ORF H0096 is similar to the CII proteins of HK022 (96% identity) (Oberto et al., 1989; Oberto et al., 1994), 933W (95% identity) (Plunkett et al., 1999), and H-19B (94% identity) (Neely and Friedman, 1998a; Neely and Friedman, 1998b), respectively. ORF H0097 is identical to the hypothetical protein 48 of phage HK022 (100% identity) (Oberto et al., 1994) and similar to ORF48 of phage P22 (85% identity) (Backhaus and Petri, 1984). This ORF is not present in 933W. ORFs H0098 and H0099 are nearly identical to the replication proteins O (98% identity) and P (98% identity) of phage HK022 (Oberto et al., 1994), respectively, but they show no homologies to those of 933W. In the well-characterized lambda replication system, the origin of replication is located in the coding region of the O protein, in which there are four 20-bp direct repeat sequences that are recognized by the O protein to initiate the replication (Furth and Wickner, 1983). Since the replication origin of HK022 is not known, we looked for such sequences based on the analogy to lambda. We found two 30-bp direct repeat sequences (5'-TGTACCAGCAGATTACCAGCAAATTACCAC) at positions 13443 to 13472 and 13509 to 13538 in the coding region for the O protein of VT2-Sakai. Consequently, the VPADYQQITT sequence appears twice in the O protein. This kind of repeat peptide is not present in the O protein of

lambda. Such repeat sequences of DNA and/or peptides may be related to the DNA-O protein interaction(s) required for the initiation of replication of VT2-Sakai. Although ORF H0100 is not similar to any known ORFs, it may correspond to the Ren protein involved in the reaction of Rex exclusion from the conserved relative position on the genome and its relative size. ORFs H0101 to H0103 were not noted in the 933W sequence reported by Plunkett et al. (1999), but Schmidt et al. (1997) reported their existence in 933W (GenBank; Y10775) (Schmidt et al., 1997). The DNA sequence of this region in VT2-Sakai is identical to that in 933W reported by Schmidt et al. (1997), and it differs from the one reported by Plunkett et al. (1999) by a 1-bp deletion in their sequence.

ORF H0104 and H0105 (Dam) are very similar to L0096 and L0097 of 933W (both 99% identities) (Plunkett et al., 1999). H0105 is similar to a hypothetical adenine-specific DNA methylase of bacteriophage HP1 (38% identity) (Esposito et al., 1996). It has been proposed that such a DNA modification is related to bacterial virulence (Heithoff et al., 1999).

ORF H0106 is almost identical to the to NinE proteins of bacteriophages 21 (98% identity) (Kroeger, 1999), P22 (98% identity) (Kroeger, 1994), H-19B (98% identity) (Neely and Friedman, 1998a; Neely and Friedman, 1998b) and lambda (93% identity) (Sanger et al., 1982), and very similar to that of 933W (85% identity) (Schmidt et al., 1997). ORF H0107 is not present in 933W and is most similar to a hypothetical protein of bacteriophage TP901-1 (37% identity) (Madsen and Hammer, 1998).

The ORFs from H0108 to H0160 are identical or very similar to the corresponding ORFs of 933W (L0096 to L0142) (Plunkett et al., 1999) except for the following: In VT2-Sakai, the ORF corresponding to L0105 of 933W is divided into two ORFs, H0114 and H0115, due to the addition of one G nucleotide at position 23589. Furthermore, the *H0115* gene is fused to the *IS629* sequence (Matsutani and Ohtsubo, 1990). ORFs H0116 and H0117 are the transposase and a hypothetical 12-kD protein encoded by *IS629*, respectively. ORFs H0118 and H0119 were not noted in the 933W sequence reported by Plunkett et al. (1999), but these ORFs were reported in the 933W sequence by Schmidt et al. (1997). L0140 of 933W is divided into ORFs H0156 and H0157 in VT2-Sakai due to a 1-bp deletion in the latter sequence. In VT2-Sakai, ORF H0158 is encoded by an additional 271-bp DNA sequence that is not present in 933W. Its sequence is not similar to any reported DNA sequence.

ORFs H0111, H0112, and H0113 correspond to Q, Stx2A and Stx2B. The existence of the transcriptional antiterminator Q and the related *cis*-acting DNA elements such as the late promoter pR', *qut*, terminators tR1' and tR2', suggest that the late gene transcription is activated by the antitermination function of Q, which has been well studied in the lambda system (Friedman and Court, 1995;

Ring and Roberts, 1994; Yarnell and Roberts, 1992). Therefore, the expression of the *stx2* genes is likely to be positively regulated by Q, like the expression of the downstream genes. This region is almost identical to that of 933W (Plunkett et al., 1999).

Promoters, operators, terminators and antiterminators in the immunity region. Between the *N* and *rexB* genes of lambda phage lie three tandemly repeated operator sites, *OL1*, *OL2* and *OL3*, each with inverted symmetry (Gussin et al., 1983). The operators recognized by CI and Cro proteins overlap and control the activities of the leftward promoter, pL (Gussin et al., 1983). The pL promoter is essential for the transcription of *N*, which is required for expression of the delayed early genes, such as *cIII*, *int*, *O*, *P*, and *Q* in lambda (Gussin et al., 1983). In lambda, the region between the *N* and *rexB* genes contains the *nutL* DNA sequence, whose transcript RNA signal is recognized by antiterminator protein N, resulting in readthrough of the terminators *tL1* and *tL2* (Friedman

and Gottesman, 1983). Moreover, this region contains the terminator *tRM*, which blocks the transcription initiated from the pRM promoter (Gussin et al., 1983). The nucleotide sequence of the corresponding region of VT2-Sakai is not similar to those of lambda, 933W, or other known bacteriophages, indicating that the recognition proteins N, CI and Cro are not similar to those of other known phages. As described in the previous section, the sequence similarities of N, CI and Cro to those of other phages are relatively low, suggesting that these proteins recognize sequences different from those of other phages. By analogy to lambda, we searched by eyes for nucleotide sequences that might serve such functions in the region between the *N* and *H0092* genes of VT2-Sakai. We found putative *nutL*, pL promoter, operators (*OL1*, *OL2* and *OL3*), and terminator (*tRM*) sequences, as shown in Fig. 4.

Like in lambda phage, we also found three tandemly repeated operators, *OR1*, *OR2* and *OR3*, each of which has an approximately inverted repeat structure, between the *cI* and *cro* genes of VT2-Sakai (Fig. 4). The sequence of

Fig. 4. Sequence of VT2-Sakai in the region between *H0091* (*N*) and *H0096* (*cII*). The nucleotide sequence is shown in the conventional orientation for lambdoid phages. The features described in the text are indicated. The two sets of the segments labeled "boxA" and "boxB" constitute the putative *nutL* and *nutR* loci, respectively; the pairs of inward-facing arrows indicate the inverted repeat sequences of *boxB*. The putative repressor-binding sites *OL1*, *OL2*, *OL3*, *OR1*, *OR2*, and *OR3* are boxed, and the presumed -35 and -10 regions of the pL, pRM, pR and pRE promoters are underlined with broken lines. The regions labeled "tRM" and "tR" constitute the putative terminators for pRM and pR, respectively. The CII-binding site indicated in the pRE promoter is located in the position corresponding to the -35 region. The three large arrows indicate the locations and directions of the *H0092*, *H0093* and *cI* genes.

this region is not similar to those of other phages. The operators, which may be recognized by CI and Cro of VT2-Sakai, overlap the putative rightward promoter, pR, as well as the putative leftward promoter, pRM. By analogy to lambda, the former may transcribe *cro* and other early genes of VT2-Sakai; the latter may be responsible for transcription of *cI* for maintaining the repressed state. The six operators described above give a consensus sequence with inverted symmetry (Fig. 5).

Lambda, P22 and ϕ 21 contain cryptic promoters activated by their respective CII proteins (Wulff and Rosenberg, 1983; Wulff and Mahoney, 1987). They lack conserved -35 regions, but have the typical -10 regions. One such lambda promoter, pRE, is responsible for transcription of the *cI* gene after infection to establish lysogeny (Wulff and Rosenberg, 1983). It is located downstream of the *cro* gene. A search for the corresponding sequence in this region of VT2-Sakai revealed a sequence that shares the consensus TTGC repeat of the CII recognition sequence with lambda: TTGCN6TTGC (Fig. 4) (Wulff and Mahoney, 1987). Downstream of the putative recognition sequence, there is a reasonable -10 sequence. It is likely that these sequence elements constitute the pRE promoter of VT2-Sakai. Like in lambda phage, the promoter overlaps the coding sequence for CII. Also like in lambda, the putative antitermination signal, *nutR*, and the putative terminator, *tR1*, were found between the *cro* and *cII* (Fig. 4). Transcription antitermination in lambda requires the phage *nut* sites (Friedman and Gottesman, 1983). N protein attaches to RNA polymerase at the *nut* sites to form a termination-resistant complex (Friedman and Court, 1995; Das, 1992). The *nut* sites can be divided into a promoter-proximal sequence, *boxA*, conserved among several lambdoid phages, and *boxB*, a non-conserved region of hyphenated dyad symmetry (Friedman and Gottesman, 1983; Friedman et al., 1990). The variations in *boxBs* are related to the different *N* genes and, in

<i>O</i> _{L1}	AAGTACATA TAGTACGA
<i>O</i> _{L2}	TAGAACAAA CAATACAA
<i>O</i> _{L3}	AAATACAAA TACAACGC
<i>O</i> _{R1}	AAGTACATA AAATACAA
<i>O</i> _{R2}	ATGCACAAT AAATACAC
<i>O</i> _{R3}	TATTACGCT CAGTACAG

CONSENSUS aAgTACAa

Fig. 5. The putative recognition sequences (operators) of VT2-Sakai. To obtain a consensus sequence for the repressor-binding site, the 6 sequences of *O*_{L1}, *O*_{L2}, *O*_{L3}, *O*_{R1}, *O*_{R2}, and *O*_{R3} were broken near the midpoints to give 12 half-sites. The sequence of one member of each pair of half-sites was inverted with respect to the other, and the sequences of the top (5' to 3') strands of the half-sites were aligned with each other. A consensus sequence was obtained from the alignment of the 12 half-sites, and is indicated at the bottom.

part, account for their specificities (Friedman and Court, 1995; Das, 1992). Lambda carries two nearly identical *nut* sites: *nutL*, located between pL and *N*, and *nutR*, which is downstream of *cro* (Friedman and Gottesman,

	<i>box A</i>	<i>box B</i>
VT2-Sakai <i>nutR</i>	CGCTCTTTG	taacaacggacaTTCGTCTACGTCGCTGAA
VT2-Sakai <i>nutL</i>	GGCTCTTTA	aacaacgtcgacTCTCGACTACGTGGCTGAAA
lambda <i>nutR</i>	CGCTCTTA	cacattccaGCCCTGAAAAAGGGC
lambda <i>nutL</i>	CGCTCTTA	aaaattaaGCCCTGAAGAAGGGC
21 <i>nutR</i>	TGCTCTTTA	acagttctggccttTCACCTCTAACC
21 <i>nutL</i>	GGCTCTTTA	acatcgacggacTCTCAACCTAACC
H-19B <i>nutR</i>	CGCTATTTT	cacaatggacattcgctctacgTCGCTGACAAAGCGA
H-19B <i>nutL</i>	CGCACTACT	caccagggcggtgatatacaacgattcgaatatgaatctacgCGCTGACAAAGCGC

Fig. 6. Comparison of the sequences of the *nut* sites of VT2-Sakai with those of other lamboid phages.

1983). Despite the lack of similarity of the DNA sequence in these regions between lambda and VT2-Sakai, both sequences carry putative *nut* sites at the corresponding positions (Fig. 4). In VT2-Sakai, the sequences of the *boxA* and *boxB* sites of *nutR* and *nutL* are similar to each other. The sequences of the *boxAs* are similar to those of the *nut* sites of other phages, while the sequences of the *boxBs* are unique to VT2-Sakai. This fact suggests that the N protein of VT2-Sakai probably specifically recognizes these *nut* sites (Fig. 6).

DISCUSSION

We have determined the complete nucleotide sequence of the prophage, VT2-Sakai carrying *stx2* genes, of EHEC O157:H7 (RIMD 0509952). To our surprise, the DNA sequence of this prophage was different in several regions from that of a VT2 carrying phage 933W which was isolated from another EHEC O157:H7 (EDL933), although the rest of the sequence were nearly identical. These two phages showed little homology in the sequences related to the expression of immediate early genes, which define the immunity, and therefore they should possess different immune specificities. In addition, VT2-Sakai carries an *IS629*, which may contribute to causing the defectiveness of this prophage.

It has been reported that transcription of the *stx* genes is induced by treatment with mitomycinC only when these genes are encoded by prophages (Takeda et al., 1979; Yee et al., 1993). Production of VT2 by EHEC O157:H7 (RIMD 0509952) was also induced by treatment with mitomycinC or with UV irradiation (unpublished data). During the lytic cycle, the late regulator protein, Q, counteracts the termination of transcription initiated from the late promoter pR' using the *qut* sequence that partially overlaps the promoter. 933W and H-19B phages are known to have different immune specificities and homologous Q systems (Plunkett et al., 1999; Neely and Friedman, 1998a; Neely and Friedman, 1998b). The Q proteins of H-19B and 933W are exchangeable for the induction of each other's *stx* genes and late genes, including those for lysis and for head and tail proteins (Neely and Friedman, 1998a). Because the Q, pR' and *qut* sequences of VT2-Sakai are identical or similar to those of 933W and H-19B, the regulation of the expression of VT2 (*Stx2*) in VT2-Sakai may be similar to those reported (Plunkett et al., 1999; Neely and Friedman, 1998a; Neely and Friedman, 1998b).

VT2-Sakai has an *IS629* insertion upstream of the genes that encode lysis proteins. Transcription initiated from the late promoter pR' may be attenuated or blocked by the insertion of *IS629*. As a result, sufficient amount of late proteins, including lysis proteins and structural proteins for the phage particle, to produce infective phage may not be produced. Although it is still unclear how toxins are

released from bacteria to cause disease, it has been proposed that death of the bacteria with associated autolysis must occur for the release of toxins. It has also been proposed that expression of the lysis genes is an important factor for the release of Stx toxins. If this is true, since the lysis proteins in VT2-Sakai may not be produced due to the *IS629* insertion, lysis proteins must be supplied in *trans* by other prophages after induction of lytic growth. It is possible that the lysis protein may be provided by some other prophage(s) lysogenized in the strain.

In the sequence data bases, there are only two cases of *stx* genes described as chromosomal gene sequences: one is *Shigella dysenteriae* (Kozlov et al., 1988) and the other is *E. coli* O111:H⁻ (Paton et al., 1993). Although the entire DNA sequences with their surrounding regions are not available, there are common features in these cases: the existences of *IS* elements and phage-like sequences near the toxin genes (Mizutani et al., 1999). These features are very similar to those of the VT2-Sakai prophage in the Sakai strain. These data suggest that the *stx2* genes previously thought to be chromosomal genes will turn out to be encoded by prophages and insertion of an *IS* element might have been responsible for making them defectiveness. The ongoing genome project to analyze the genome of the Sakai strain has also revealed that more than ten phage-like sequences exist in the genome (unpublished data). The apparent chimeric nature of VT2-Sakai might have resulted from multiple recombination events with other prophages residing in the genome.

Recently, Miyamoto et al. (1999) have published the complete sequence of the *Stx2*-converting phage VT2-Sa derived from another O157:H7 Sakai strain RIMD 0509894. The VT2-Sa phage does not carry the *IS* element and *bor* (*H0126*) gene that exist in VT2-Sakai, and has an amber mutation in the *cI* gene.

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