JOURNAL OF BACTERIOLOGY, Mar. 2008, p. 1638–1648 Vol. 190, No. 5
0021-9193/08/$08.00+0 doi:10.1128/JB.01484-07

A Common Virulence Plasmid in Biotype 2 *Vibrio vulnificus* and Its Dissemination Aided by a Conjugal Plasmid▽†

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Received 13 September 2007/Accepted 11 December 2007

Strains of *Vibrio vulnificus*, a marine bacterial species pathogenic for humans and eels, are divided into three biotypes, and those virulent for eels are classified as biotype 2. All biotype 2 strains possess one or more plasmids, which have been shown to harbor the biotype 2-specific DNA sequences. In this study we determined the DNA sequences of three biotype 2 plasmids: pR99 (68.4 kbp) in strain CECT4999 and pC4602-1 (56.6 kb) and pC4602-2 (66.9 kb) in strain CECT4602. Plasmid pC4602-2 showed 92% sequence identity with pR99. Curing of pR99 from strain CECT4999 resulted in loss of resistance to eel serum and virulence for eels but had no effect on the virulence for mice, an animal model, and resistance to human serum. Plasmids pC4602-2 and pR99 could be transferred to the plasmid-cured strain by conjugation in the presence of pC4602-1, which was self-transmissible, and acquisition of pC4602-2 restored the virulence of the cured strain for eels. Therefore, both pR99 and pC4602-2 were virulence plasmids for eels but not mice. A gene in pR99, which encoded a novel protein and had an equivalent in pC4602-2, was further shown to be essential, but not sufficient, for the resistance to eel serum and virulence for eels. There was evidence showing that pC4602-2 may form a cointegrate with pC4602-1. An investigation of six other biotype 2 strains for the presence of various plasmid markers revealed that they all harbored the virulence plasmid and four of them possessed the conjugal plasmid in addition.

Vibrio vulnificus is a gram-negative bacterial species that is ubiquitous in marine environments. This organism causes infections that are characterized by severe wound infection and primary septicemia with high mortality in humans, particularly those with underlying diseases such as liver cirrhosis and hemochromatosis (33, 40). It also causes systemic infections, called vibriosis, with high mortality in brackish-water-cultured eels (4, 16). This organism is considered an emerging pathogen, since cases in fish and human were first reported in 1976 and 1979, respectively (8, 34).

Strains of *V. vulnificus* are divided into three biotypes, biotype 1 (BT1), BT2, and BT3, based on their differences in phenotypic traits, serological type, and host range (6, 7, 42). All three biotypes can cause sporadic cases of human diseases and have been shown to be virulent for the mouse, an animal model; however, only the BT2 strains produce epizootic or outbreaks of vibriosis, mainly in eels (4, 34). The lesions on the body of a *V. vulnificus*-infected eel, similar to those caused by *Vibrio anguillarum*, include external skin ulcer, hemorrhagic fins, protrusion of the rectum, and hemorrhages and inflammation of the internal organs (4). The BT2 strains are further subdivided into several serovars (6, 16, 22), among which serovar E is not only the most virulent and prevalent but also the only one isolated from sporadic human infections associated with handling of contaminated fish (1).

The virulence mechanism of BT2 *V. vulnificus* strains in eels remains unclear, although some virulence factors have been proposed. The extracellular products of BT2 strains exhibit hydrolytic/toxic activities and lethality for the eel similar to those produced by the BT1 strains (3), suggesting that they may not be the sole virulence determinants. Nevertheless, the degree of virulence for eels is directly correlated with the ability to resist and grow in fresh eel serum (2).

To find out the genetic basis of eel virulence in BT2 *V. vulnificus* strains, the whole genome of one serovar E BT2 strain (tester strain) was compared with those of three BT1 clinical strains (driver strains) by suppression subtractive hybridization (SSH) (28). The distribution of the tester-specific DNA sequences thus identified was surveyed in a collection of more than 100 strains of the three biotypes by PCR. Three of these DNA sequences were present in all strains of BT2 but none of other biotypes. Further, these DNA sequences were all located in the plasmids, suggesting that the virulence for eels could be conferred by a plasmid. In fact, previous studies had shown that all BT2 strains harbored one or more plasmids of high molecular weight (6, 22, 30).

The purpose of this study was to investigate the role of plasmids in the virulence of BT2 *V. vulnificus* for eels. Toward this end, we determined and analyzed the nucleotide se-

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 21 December 2007.

quences of three plasmids in two serovar E BT2 strains, CECT4602 (containing two plasmids) and CECT4999 (containing one plasmid). We then isolated a strain that was cured of the plasmid from CECT4999 and examined its virulence for eels and mice. We found that these two strains shared a highly homologous plasmid that was essential for both the resistance to bactericidal activity of eel serum and virulence for eels but not for growth in human serum and virulence for mice. A gene in the virulence plasmid encoding a novel protein was further shown to be essential for virulence in the eel. We also demonstrated that the virulence plasmids of CECT4602 and CECT4999 could be transferred to the plasmid-cured strain by conjugation in the presence of the other plasmid of CECT4602, which was shown to be self-transmissible.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. The various *V. vulnificus* strains, plasmids, and primers used in this study are listed in Table 1.

Determination and analyses of plasmid nucleotide sequences. The plasmid DNA was extracted from bacterial cells harvested from a 100-ml overnight culture in Luria-Bertani (LB) medium (BD Biosciences) with 6 ml of TENS solution (0.09 N NaOH and 0.45% sodium dodecyl sulfate in Tris-EDTA buffer) followed by 3 ml of sodium acetate (3 M, pH 5.2). This mixture was centrifuged, and the plasmid DNA in the supernatant was extracted with equal volume of phenol-chloroform (1:1) several times, precipitated with ethanol, dissolved in 10 ml of Tris-EDTA buffer, and purified by CsCl gradient centrifugation (37).

The nucleotide sequences of the plasmids were determined as described previously (12). Briefly, the DNA sequences of clones in a shotgun library of the plasmid with over 10-fold coverage were determined with an autosequencer (Applied Biosystems Prism 3700) and then jointly assembled using Phred/Phrap/Consed software provided by University of Washington, Seattle (20, 21, 25). The sequence gaps were closed by editing the end sequences of each contig and primer walking on the linking clones. The assembled plasmid DNA sequence was confirmed by restriction mapping with a variety of enzymes.

The open reading frames (ORF) were predicted with two programs, Glimmer (36) and GeneMark (10). The predicted ORFs were then submitted to AutoFACT (27), a tool that combines the BLAST reports from UniRef90, UniRef100, NCBI's nrdb, COG, KEGG, PFAM, and SMART, for gene annotation. The graphic view of plasmid DNA sequence comparison was generated with the Artemis comparison tool (11) according to the results obtained from bl2seq (41). The percentage of DNA sequence identity between the plasmids was determined by ClustalW (13) via sequence alignment. For a more accurate result, the DNA sequences were rearranged with *traA* and *rtxE* as the initial sequences for the pC4602-1/pYJ016 and pC4602-2/pR99 alignments, respectively.

Isolation of deletion mutants and a plasmid-cured strain from CECT4999. A mutant, CT188, with a 225-bp in-frame deletion between the two HincII sites in ORFvep07 of pR99 and another mutant, CT095, with a chloramphenicol resistance (Cm^r) cassette (44) inserted between these two HincII sites in ORFvep07 were isolated from CECT4999 by allelic exchange as described previously (38).

To facilitate the isolation of a cured strain from CECT4999, a 255-bp in-frame deletion was introduced into *mazF* of CT095 by allelic exchange. To cure the plasmid, this CECT4999 derivative was cultured in LB broth containing 3 μg/ml of acridine orange (Sigma-Aldrich) at 39°C with agitation overnight and then plated on the LB plates. Among the 4,900 colonies tested, only one was Cm^s. This strain (designated CT218) was further confirmed to be plasmid free by plasmid extraction, restriction pattern analysis, and Southern hybridization (data not shown).

Cloning of ORFvep07. The entire ORFvep07 together with its putative promoter was amplified by PCR with primers C51F and C51R and then cloned into pIT009, a derivative of the broad-host-range plasmid pJRD215 (17), at the XbaI site. The resultant plasmid, pCT166, was transformed into *Escherichia coli* S17-1λ*pir* (38), from which it was transferred into the various *V. vulnificus* strains by conjugation.

Serum resistance assay and growth in whole blood. Eel blood and serum were prepared from European elvers (*Anguilla anguilla*) of 10 to 20 g as described previously (2). Human blood and serum were purchased from Sigma-Aldrich. To each sample, an equal volume of a bacterial suspension of 10^3 to ^{4} CFU/ml in phosphate-buffered saline was added, and the mixture was incubated at 28°C (for eel samples) or 37°C (for human samples) for 4 h. The viable counts were then determined on 1% NaCl-containing tryptic soy agar plates at 0 and 4 h after incubation. The significance of differences was analyzed by analysis of variance with the GraphPad Prism program.

Virulence assays. Bacterial virulence in eels was assayed as described previously (5). Briefly, nonimmunized European elvers with an average weight of 10 g were injected intraperitoneally with 0.1 ml of 10-fold serially diluted bacterial suspension in phosphate-buffered saline containing 1% NaCl, pH 7.4. Three doses were used for each strain, and six eels were injected per dose. The eels were maintained in aquaria (1% salinity) with aeration at 25°C. The mortality was recorded daily for 7 days after challenge. The bacteria recovered from the internal organs of dead eels were confirmed serologically.

Bacterial virulence in mice was determined in C3H/HeN mice (6 to 8 weeks old, purchased from the Animal Center of College of Medicine, National Cheng-Kung University) infected by intraperitoneal injection (26). Four 10-fold serially diluted doses were used for each strain, and five mice were challenged per dose.

The 50% of lethal dose (LD_{50}) of each strain was calculated from the number of confirmed deaths by a method described previously (35).

Conjugation. Two hundred microliters of an overnight culture of the donor strain and 20 μl of an overnight culture of the recipient strain were mixed in 5 ml of conjugation buffer (1% NaCl and 10 mM $MgSO_4$) and filtered through a membrane with 0.45-μm pores (Millipore). The membrane was left on the LB plate for 4 h at 30°C. The bacteria were then collected from the membrane with 5 ml of LB broth, incubated at 37°C with agitation for 1 h, and plated on an LB plate containing 2% 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) (MDBio) or appropriate antibiotics such as Cm (10 μg/ml) and streptomycin (Sm) (100 μg/ml).

PCR, RT-PCR, Southern hybridization, and colony hybridization. PCR was performed as described previously (28). *rtxA* transcripts were detected by reverse transcription-PCR (RT-PCR). Briefly, total RNA was extracted from a 4-h bacterial culture with Tri reagent (Molecular Research Center), treated with RQI DNase (Promega), and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) to prepare cDNA. PCR was then performed on the cDNA with primers P6 and P7 derived from *rtxA*.

In Southern hybridization, the uncut and restricted plasmid DNAs were separated on a 0.8% agarose gel, transferred onto a Hybond-N+ nylon membrane (Amersham Biosciences), and then hybridized with fluorescein-labeled PCR products prepared with the Gene Image random prime labeling kit (GE Healthcare Life Sciences). Primer pairs VF51/VR51, AD1-R/D1, Maz6/Maz7, JB02F/L1, and K1/JB02R (Table 1) were used to prepare the probes for seq51, *traH*, *mazF*, ID1, and ID2, respectively. The signals were visualized with the Gene Image CDP-Star detection module (GE Healthcare Life Sciences). Colony hybridization (37) was used to detect clones containing BT2 plasmids with the fluorescein-labeled probes prepared as described above.

Nucleotide sequence accession number. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AM293859 (pC4602-1), AM293860 (pC4602-2), and AM293858 (pR99).

RESULTS

Features of plasmid DNA sequences. Plasmids pC4602-1 and pC4602-2 of strain CECT4602 and plasmid pR99 of strain CECT4999 were 56,628 bp, 66,946 bp, and 68,446 bp in size, respectively, and their G+C contents were 45.2%, 43.3%, and 43.8%, respectively. The numbers of predicted ORFs for pC4602-1, pC4602-2, and pR99 were 69, 67, and 71. The main features of the plasmids are shown in Fig. 1 and 2. According to the annotation, the ORFs in these plasmids could be grouped into five categories: those related to conjugal transfer, those related to RTX toxin, those related to transposition, those with other functions, and those with unknown functions (Fig. 1 and 2; see Tables S1 to S3 in the supplemental material).

In pC4602-1 about half of the ORFs (32/69) had no known function, among which 20 were annotated as conserved hypothetical. Among the genes with known functions, a cluster of 15 genes (*traB*, *traC*, *traD*, *traE*, *traF*, *traG*, *traH*, *traI*, *traK*, *traL*, *traN*, *traU*, *traW*, *trbB*, and *trbC*) involved in conjugal transfer

TABLE 1. *V. vulnificus* strains, plasmids, and primers used in this study

Bacterial strain, plasmid, or primer	Description or sequence (5′–3′)	Source or reference, or nt location (plasmid)[a]
Strains		
BT2		
CECT897	ATCC 33147, isolated from diseased eels in Japan, 1979; serovar E; API20E code 4006005	ATCC
CECT4602	Isolate from diseased eels in Spain, 1990; serovar E; API20E code 5206005	CECT
CECT4865	Isolate from a diseased shrimp in Taiwan; serovar E; API20E code 5206005	CECT
CECT4866	Clinical isolate from a patient in Australia; serovar E; API20E code 5306005	CECT
CECT4870	Isolate from diseased eels in Sweden, 1991; serovar E; API20E code 5306005	CECT
CECT4917	Isolate from diseased eels in Spain, 1997; serovar E; API20E code 4306005	CECT
CECT5198	Isolate from diseased eels in Spain, 1999; serovar A; API20E code 5346105	CECT
CECT4999	Isolate from diseased eels in Spain, 1990; serovar E	CECT
CT095	CECT4999 with a Cm^r cassette inserted between the two HincII sites in ORFvep07 of pR99	This study
CT188	CECT4999 Δ*vep07*	This study
CT218	Plasmid-free derivative of CECT4999	This study
CT223	CT218 transconjugant with pC4602-1	This study
CT225	CT218 transconjugant with pC4602-1 and pC4602-2	This study
CT226	CT218 transconjugant with rearranged plasmids	This study
CT227	CT218 transconjugant with pC4602-2	This study
CT237	Spontaneous Sm^r mutant of CT218	This study
CT239	CT188(pCT166)	This study
CT244	CT218(pCT166)	This study
CT295	CT095 transconjugant with pC4602-1	This study
BT1		
YJ016	Clinical isolate from a patient in Taiwan	38
SW058	Plasmid-free clinical isolate from a patient in Taiwan	Lab collection
LF044	Spontaneous Sm^r mutant of a plasmid-free derivative of strainYJ016	Lab collection
CT162	Spontaneous Sm^r mutant of SW058	Lab collection
CT167	YJ016(pCT166)	This study
Plasmids		
pJRD215	Broad-host-range plasmid	17
pIT009	Derivative of pJRD215 with the Sm^r gene between two XmnI sites replaced by the multiple-cloning-site-containing *lacZ* gene cloned from pUC19	This study
pCT166	pIT009 inserted with ORFvep07 cloned from pR99 at the XbaI site	This study
Primers		
K1	GCAGGATCAAGTGATCGG	56065–56082 (pC4602-1)
99P	GATGAGCTTGAGCGTTCAGG	2556–2575 (pC4602-2)
M2	GCTGAAAACGGTGTGATGG	3041–3059 (pC4602-1)
179R	GCAGTTTTATGCTTTAGCGGC	59690–59710 (pC4602-2)
VF10	CATCACTCAACTTCTCGACTCC	10241–10262 (pC4602-1)
VR10	AGCATCTCACCACGACGTC	10609–10627 (pC4602-1)
VF25	GCCAAGTGCTAATCCATCC	16716–16734 (pC4602-2)
VR25	TGCTCAAAGCCATACTCTCT	16324–16342 (pC4602-2)
VF51	GGACAGATACAAGGGCAAATGG	14985–15006 (pC4602-2)
VR51	AGAGATGGAAGAAACAGGCG	15309–15328 (pC4602-2)
AD1-R	GCTGGTTAAGTGTTTCAATGG	33788–33808 (pC4602-1)
D1	CAAGCTCAGCATGAATGTGG	33088–33107 (pC4602-1)
Maz6	CAGTGGGATAAGTCTCAGC	3012–3030 (pC4602-1)
Maz7	GGCTAAGTACATTCCCAAGC	3314–3333 (pC4602-1)
CT39-R	ATGCGCCTGACTAGATTGC	53142–53160 (pC4602-1); 66038–66056 (pC4602-2)
JB02R-4	GAAACAGCCTAACGCACAG	684–702 (pC4602-1); 684–702 (pC4602-2)
JB02R	CACCATCATCAACTTTCAACC	2096–2116 (pC4602-1); 2096–2116 (pC4602-2)
L1	GAAGCTGACCGCAGCGTAAC	52777–52796 (pC4602-1); 65673–65692 (pC4602-2)
JB02F	GATTCGCAAGGAAATAGAGAC	47558–47578 (pC4602-1); 60454–60474 (pC4602-2)
P1	GGGCAACTAACTCATCGTGTG	27987–28007 (pR99); 38203–38223 (pC4602-2)

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TABLE 1—*Continued*

Bacterial strain, plasmid, or primer	Description or sequence (5′-3′)	Source or reference, or nt location (plasmid)[a]
P2	CTTGGTCAACACTGGATGTGCTGG	33028–33051 (pR99); 43244–43267 (pC4602-2)
P3	CGGGAATGAACCAAGTGATGTC	33175–33196 (pR99); 43391–43412 (pC4602-2)
P4	GTTGGCTTTACCGAACATCGCCG	37968–37990 (pR99); 48184–48206 (pC4602-2)
P5	GAAACACGCAAAGCCGATGC	40241–40260 (pR99); 50457–50476 (pC4602-2)
P6	ATCCGCTGCGTTCGAACCACA	48324–48344 (pR99); 58540–58560 (pC4602-2)
P7	AATTCAACGGTGGCGAAGGGC	48150–48170 (pR99); 58366–58386 (pC4602-2)
C51F	GCACGGACTTTTCCTTGC	7107–7124 (pR99); 16227–16244 (pC4602-2)
C51R	GCAATGGTCATTCGTGGG	4963–4980 (pR99); 14083–14100 (pC4602-2)

[a] ATCC, American Type Culture Collection, Manassas, VA; CECT, Spanish Type Culture Collection, Valencia, Spain.

was found in a 27.2-kb region (nucleotides [nt] 20993 to 48163). This cluster shared high homology with those of F plasmid (23) and pYJ016, a conjugal plasmid of 48.5 kb in the BT1 *V. vulnificus* strain YJ016 (12), in DNA sequence and gene organization (see Fig. S1A in the supplemental material). The overall identity between pC4602-1 and pYJ016 was 81%, and the regions with a DNA sequence identity of over 84% contained mainly the transfer genes (see Fig. S1B in the supplemental material). An operon homologous to *mazEF*, a member of the toxin-and-antitoxin (TA) modules involved in maintenance of plasmids by postsegregational killing of the host (32), was also found in this plasmid. seq10, a BT2-specific DNA sequence identified previously by SSH (28), was found spanning two ORFs (ORFvpa21 and ORFvpa22), both of which were annotated as transposase (Fig. 1A; see Table S1in the supplemental material).

Half of the ORFs (34/67) in pC4602-2 were annotated as hypothetical, and 21 of them were conserved. A notable feature of this plasmid was a cluster of RTX (repeats-in-toxin) genes homologous to those in the chromosomes of *Vibrio cholerae* and *V. vulnificus*, including those encoding RTX toxin (*rtxA*), toxin transporter (*rtxB*, *rtxD*, and *rtxE*), and an enzyme for toxin activation (*rtxC*) (9, 12, 45). A comparison of the *rtx* gene cluster DNA sequence of pC4602-2 with those of *V. cholerae* and BT1 *V. vulnificus* revealed dissimilar regions that were all located in *rtxA*. As shown in Fig. 3, the *rtxA* gene of *V. cholerae* ($rtxA_{Vc}$) contains an actin-cross-linking domain (ACD) (39) that is not present in BT1 *V. vulnificus rtxA* ($rtxA_{Vvbt1}$). On the other hand, $rtxA_{Vvbt1}$ contains two regions, U1 and U2, that are absent in $rtxA_{Vc}$, with U1 being at the same location as the ACD in $rtxA_{Vc}$. Surprisingly, the *rtxA* gene of pC4602-2 ($rtxA_{Vvbt2}$) showed a mosaic structure composed of DNA fragments from $rtxA_{Vc}$ and $rtxA_{Vvbt2}$. In $rtxA_{Vvbt2}$ the region corresponding to U1 of rtx_{Vvbt1} was replaced by the entire ACD of $rtxA_{Vc}$, and only the 5′ portion (1,078 bp) of U2 was retained in $rtxA_{Vvbt2}$. Moreover, a 1,872-bp DNA sequence immediately downstream of the ACD was replaced by another copy (identity, 95%) of this 5′ portion of U2.

seq25 and seq51, two BT2-specific sequences previously identified by SSH in our laboratory (28), were found to be close to and overlapping with, respectively, ORFvpb22, which was annotated as RTX toxin and related Ca^{2+}-binding protein (Fig. 1B; see Table S2 in the supplemental material).

Forty-five percent (32/71) of the ORFs in pR99 were annotated as hypothetical, among which 21 were conserved. This plasmid showed 92% identity with pC4602-2. Like pC4602-2, pR99 carried the *rtx* gene cluster and the ORF (ORFvep07) that contained part of seq51 (Fig. 2), both of which were 100% identical to those of pC4602-2. However, the *mazEF* operon, which was not present in pC4602-2, was found in pR99.

We further detected a copy of the *rtx* gene cluster exhibiting a restriction pattern indistinguishable from that of pR99 (plasmid-safe DNase-treated total DNA of CECT4999), but not the BT1 strain YJ016, in the plasmid-free derivative of CECT4999, CT218 (Fig. 4A). By using RT-PCR, we showed that both CECT4999 and CT218 expressed *rtxA* when they were cultured in rich medium (Fig. 4B). PCR products showing restriction patterns identical to those of products amplified from CECT4999 or CT218 were also obtained from strain CECT4602 with primer pairs P1/P2 and P3/P4 (Fig. 4A). However, an additional restriction fragment of 1.7 kb, which could be derived from the chromosomal *rtxA* gene, was observed in the PCR product amplified from CECT4602 with primers P5 and P6 (Fig. 4A).

Virulence of a CECT4999 derivative that had been cured of plasmid. To determine whether the plasmid was involved in virulence for eels, we isolated a plasmid-free derivative, CT218, from strain CECT4999. The growth of CT218 in LB medium was similar to that of the parent strain, CECT4999 (data not shown). The LD_{50} of this cured strain in eels was at least 4 logs higher than that of CECT4999 and close to that of a BT1 strain, YJ016 (Table 2). However, the virulence of CT218 in mice was comparable to that of CECT4999 or YJ016 (Table 2). The resistance of CT218 to eel serum and growth in whole blood were also greatly impaired compared to those of the parent strain (Table 2).

Involvement of ORFvep07 in pR99 with virulence for eels. ORFvep07, which contained a BT2-specific sequence, seq51,

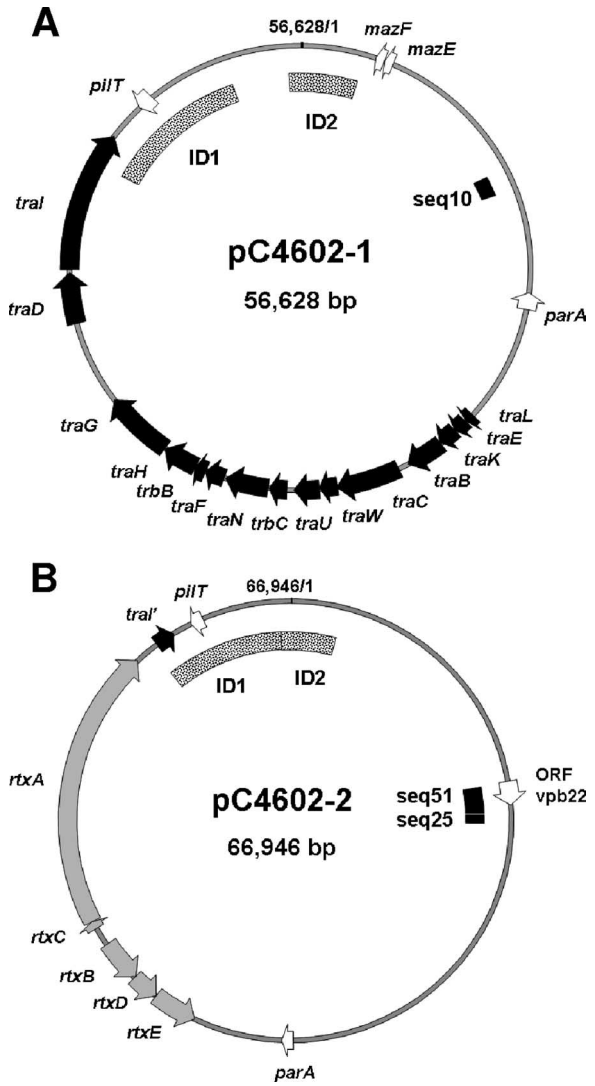

FIG. 1. Main features of the plasmids pC4602-1 (A) and pC4602-2 (B) in *V. vulnificus* strain CECT4602. Some of the predicted ORFs are indicated with arrows. The ORFs associated with production of an RTX toxin, conjugative transfer of plasmids, and other functions are indicated in gray, black, and white, respectively. Regions ID1 and ID2, which are present in both pC4602-1 and pC4602-2, are indicated with stippled bars. seq10, seq25, and seq51 are shown as black bars.

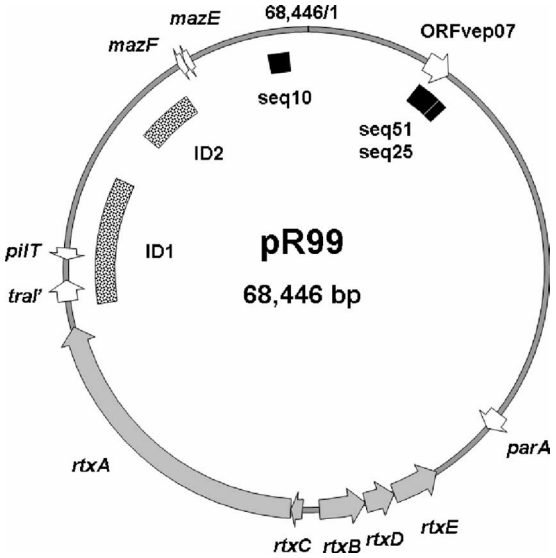

FIG. 2. Main features of the plasmid, pR99, in *V. vulnificus* strain CECT4999. Some of the predicted ORFs are indicated with arrows. The ORFs related to the production of an RTX toxin and those with other functions are labeled in gray and white, respectively. seq10, seq25, and seq51 are shown as black bars. Regions ID1 and ID2, identical to those in pC4602-1 and pC4602-2, are indicated with stippled bars.

and shared low sequence homology with a protein annotated as RTX toxin and related Ca^{2+}-binding protein, was tested for its involvement in virulence for eels. The Δ*vep07* deletion mutant, CT188, was isolated by allelic exchange and confirmed by Southern hybridization (see Fig. S2 in the supplemental material) and nucleotide sequence determination. The growth of this mutant in LB medium was not affected (data not shown). However, its survival in eel whole blood and serum as well as virulence in the eel was greatly reduced to a level comparable to that of plasmid-cured strain CT218 (Table 3). Reintroduction of ORFvep07 carried by a broad-host-range plasmid into this mutant restored these abilities (Table 3). However, introduction of ORFvep07 alone enhanced, but did not fully restore, the survival of CT218 in eel blood and its virulence for eels and had little effect on those of the BT1 strain YJ016 (Table 3).

Formation of a cointegrate between pC4602-1 and pC4602-2. Two DNA sequences, ID1 (nt 46873 to 53550) and ID2 (nt 56129 to 2511), separated by a region of about 2.6 kb in pC4602-1, were found to be identical to two adjacent DNA sequences (nt 59769 to 66446 and nt 66447 to 2511, respectively) in pC4602-2 (Fig. 1A and B). Thus, these two plasmids might form two types of cointegrate via homologous or site-specific recombination between the common regions, ID1 or ID2, in these plasmids (Fig. 5A). However, no band with the predicted size of the cointegrate was observed either directly in agarose gels (Fig. 6A, lane 3) or after Southern hybridization with probes derived from the plasmids (data not shown). Nevertheless, when we performed PCR with two pairs of primers, K1/99P and M2/179R, derived from the unique DNA se-

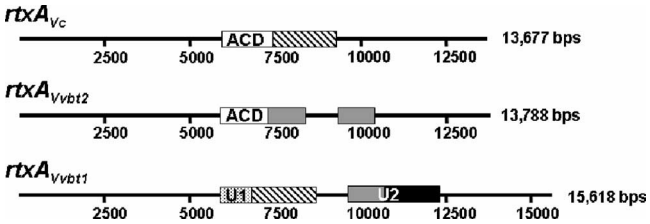

FIG. 3. Comparison of the *rtxA* nucleotide sequences in *V. cholerae* strain N16961 ($rtxA_{Vc}$), *V. vulnificus* BT1 strain YJ016 ($rtxA_{Vvbt1}$), and pC4602-2 ($rtxA_{Vvbt2}$). The comparison was done with MUSCLE, a tool for multiple-sequence alignment (19). DNA sequences shared (with >85% identity) by all the three *rtxA* genes are indicated by lines, and those that are unique or shared by any two of them are indicated in boxes. Boxes with the same pattern are regions showing >80% sequence identity. U1, unique region 1; U2, unique region 2.

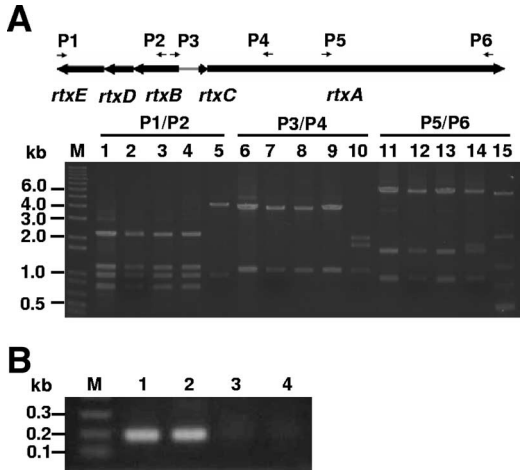

FIG. 4. Analyses of the *rtx* gene clusters in the chromosomes of strains CECT4999 and CECT4602. (A) Restriction patterns of PCR products amplified from the *rtx* gene clusters. The DNA fragments amplified by PCR with the indicated primer pairs were digested with HindIII and then fractionated by electrophoresis on a 0.8% agarose gel. The DNA templates used in PCR were the total DNAs of CECT4999 (lanes 1, 6, and 11), CT218 (lanes 3, 8, and 13), CECT4602 (lanes 4, 9, and 14), and YJ016 (lanes 5, 10, and 15) and the plasmid-safe DNase (Epicenter)-treated total DNA of CECT4999 (lanes 2, 7, and 12). (B) Detection of *rtxA* transcripts in CT218 (lane 1) and CECT4999 (lane 2) by RT-PCR. PCR with total RNA from CT218 (lane 3) and CECT4999 (lane 4) were included as the negative controls. Lane M, 1-kb plus DNA ladder (Invitrogen).

quences in each plasmid, we did obtain PCR products of the predicted sizes from strain CECT4602 (Fig. 5B, lane 5). The DNA sequences of these PCR products were further shown to be as predicted by nucleotide sequence determination or restriction pattern analysis (data not shown). These results demonstrated that the cointegrates coexisted with pC4602-1 and pC4602-2, although in an amount too small to be detected unless PCR was used.

Transfer of pC4602-1 and pC4602-2 from strain CECT4602 to B1 and B2 strains by conjugation. The colonies of CT218 and its transconjugants could be easily distinguished from those of strain CECT4602 by their colors on a plate containing X-Gal: CECT4602 was blue, while CT218 was white. After conjugation, the bacteria were plated on X-Gal-containing LB agar and the white colonies were tested for the presence of plasmids by colony hybridization with the probes derived from pC4602-1 and pC4602-2, respectively. It was found that both pC4602-1 and pC4602-2 could be transferred to CT218 at different frequencies by conjugation (Table 4). Among the 229 colonies tested, 64 hybridized only with the probe derived from pC4602-1, 3 hybridized only with that derived from pC4602-2, and 9 hybridized with both probes (Table 4).

All the transconjugants identified were tested for the activity of ornithine decarboxylase (ODC) to ensure that they were derived from CT218, an ODC-positive strain, but not CECT4602, an ODC-negative strain (data not shown). They were further subjected to plasmid profile analysis (some of the results are shown in Fig. 6), and the presence of either or both plasmids was confirmed. Similar to strain CECT4602, the transconjugants containing both plasmids gave PCR products (Fig. 5B) with the primer pairs K1/99P and M2/179R, which amplified DNA fragments only from the cointegrates as mentioned above.

Two of the nine transconjugants that contained two plasmids exhibited a plasmid profile different from those of the other seven transconjugants and strain CECT4602 (the results for one of them are shown in Fig. 6). The plasmid sizes and two of the DNA fragments produced by BglII digestion

TABLE 3. Association of ORFvep07 with bacterial virulence in eels and growth in eel serum and whole blood

Strain	LD_{50} in eel[a]	Fold growth (mean ± SD) in eel blood[b]	
		Whole	Serum
CECT4999	1.7×10^2	70.0 ± 2.0	6.5 ± 0.5
CT218	$>1.0 \times 10^{7c}$	0.7 ± 0.1[c]	0.03 ± 0.02[c]
CT188	$>1.0 \times 10^{7c}$	0.15 ± 0.02[c]	0.40 ± 0.05[c]
CT239	2.3×10^2	65.0 ± 1.0	6.4 ± 1.2
CT244	8.0×10^{5c}	11.0 ± 1.4[c]	1.2 ± 0.1[c]
CT167	$>1.0 \times 10^{7c}$	0.90 ± 0.03[c]	<0.01[c]
YJ016	$>1.0 \times 10^{7c}$	0.10 ± 0.01[c]	<0.01[c]

[a] Expressed as CFU per fish.
[b] Bacterial growth after 4 h of incubation with respect to the inoculated dose ($n = 3$).
[c] Significant difference from strain CECT4999 ($P < 0.01$).

TABLE 2. Association of BT2 plasmids with bacterial virulence in eels and mice and growth in eel and human sera and whole blood

Strain	Plasmid content	LD_{50} in[a]:		Fold growth (mean ± SD)[b] in:			
		Eel	Mouse	Eel blood		Human blood	
				Whole	Serum	Whole	Serum
CECT4999	pR99	4.1×10^2	7.0×10^4	70.00 ± 2.00	81.10 ± 1.60	1.30 ± 0.10	0.20 ± 0.03
CT218	None	$>1.0 \times 10^{7c}$	4.3×10^5	0.70 ± 0.10[c]	0.03 ± 0.02[c]	0.80 ± 0.10	0.18 ± 0.02
CT223	pC4602-1	$>1.0 \times 10^{6c}$	ND[e]	ND	0.01 ± 0.00[c]	ND	ND
CT225	pC4602-1 and pC4602-2	3.1×10^{4d}	ND	ND	82.50 ± 0.70	ND	ND
CT226	pC4602-1 and pC4602-2	6.3×10^{4d}	ND	ND	79.10 ± 2.60	ND	ND
CT227	pC4602-2	2.3×10^{4d}	ND	ND	83.50 ± 0.60	ND	ND
YJ016	pYJ016	$>1.0 \times 10^{7c}$	3.7×10^5	ND	<0.01[c]	1.30 ± 0.15	0.21 ± 0.03

[a] Expressed as CFU per fish or mouse.
[b] Bacterial growth after 4 h of incubation with respect to the inoculated dose ($n = 3$).
[c] Significant difference from strain CECT4999 ($P < 0.005$).
[d] The LD_{50} decreased to around 10^2 CFU per fish after one passage through the eels.
[e] ND, not done.

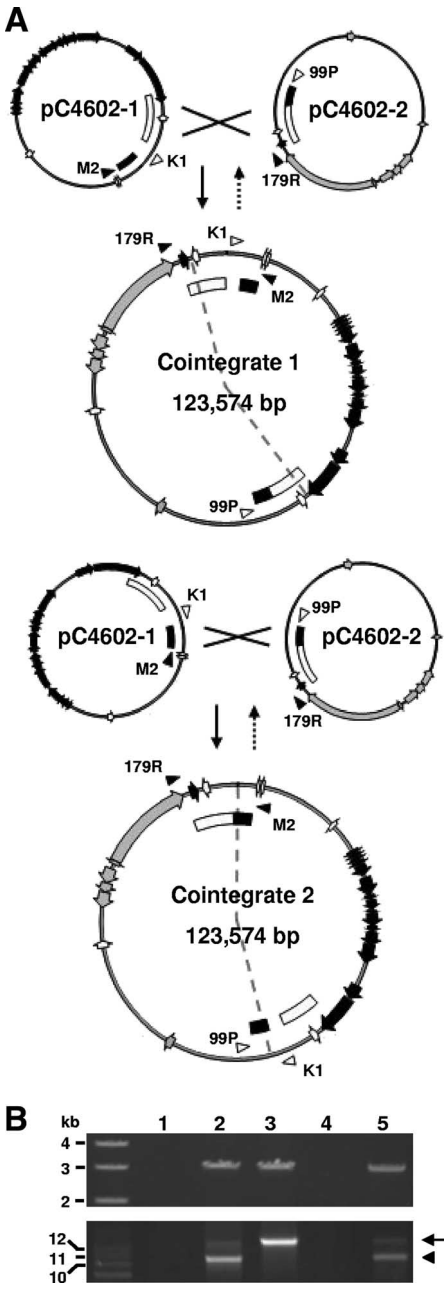

FIG. 5. Formation of cointegrates of plasmids pC4602-1 and pC4602-2. (A) Model of cointegrates formation (solid arrows) and resolution (broken arrows) via recombination between the identical regions, ID1 (white bar) or ID2 (black bar), of pC4602-1 and pC4602-2. The regions corresponding to pC4602-1 and pC4602-2, respectively, in the cointegrate are separated by dashed lines. The locations of DNA sequences hybridized by the primer pairs K1/99P (white arrowheads) and M2/179R (black arrowheads) used to amplify DNA fragments from the cointegrates are indicated. The predicted size of the PCR product amplified from cointegrate 1 with M2/179R is 12.8 kb, while no PCR product is expected with K1/99P because the DNA sequences hybridized by these two primers are too far apart. The predicted sizes of the PCR products amplified from cointegrate 2 with K1/99P and M2/179R are 3.2 kb and 10.3 kb, respectively. (B) PCR products amplified from the plasmid DNAs of strain CECT4602 and various transconjugants of strain CECT4999 with primer pairs K1/99P (upper panel) and M2/179R (lower panel). Lanes 1 to 5, PCR products amplified from CT223, CT225, CT226, CT227, and CECT4602, respectively. Left lane, 1-kb plus DNA ladder. Arrow, PCR product amplified from cointegrate 1; arrowhead, PCR product amplified from cointegrate 2.

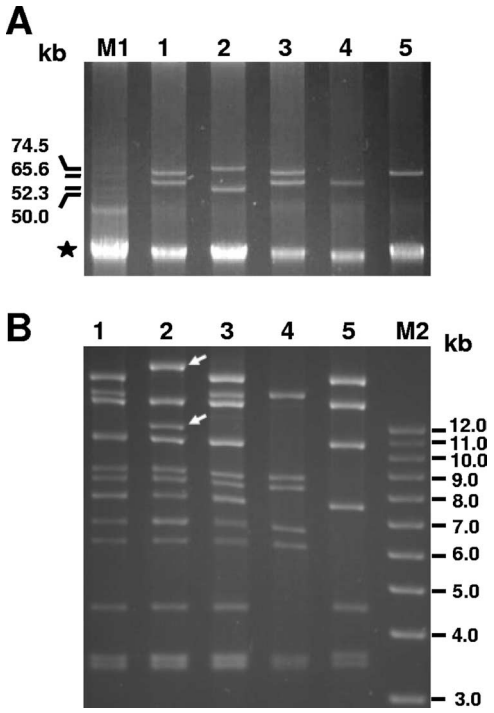

FIG. 6. Profiles of uncut (A) and BglII-restricted (B) plasmids of various transconjugants of strain CECT4999. Lanes 1 to 5, plasmids extracted from CT225, CT226, CECT4602, CT223, and CT227, respectively. Lane M1, uncut plasmids from *Pantoea stewartii* SW2 (15); lane M2, 1-kb plus DNA ladder. The arrows indicate the bands possibly generated by DNA rearrangement; the asterisk denotes chromosomal DNA.

had apparently been changed in these two transconjugants (Fig. 6A and B).

The transfer of plasmids from strain CECT4602 to two spontaneous Sm^r BT1 mutants, LF044 and CT162, was also investigated. The colonies grown on Sm-containing LB plates after mating were tested for the presence of plasmids by colony hybridization. None of the 1,000 randomly picked colonies hybridized with the probe derived from pC4602-1 or pC4602-2 for either LF044 or CT162 (Table 4).

Association of pC4602-1 and pC4602-2 with virulence for eels. Four transconjugants of CT218 containing either or both pC4602-1 and pC4602-2 were further tested for their virulence in eels and ability to grow in eel serum. As shown in Table 2, CT225 and CT226, containing both pC4602-1 and pC4602-2, as well as CT227, which contained pC4602-2 only, were virulent

TABLE 4. Frequencies of transfer of pC4602-1 and pC4602-2 from strain CECT4602 to CT218 and biotype 1 strains by conjugation

Plasmid content	Frequency of transfer (%) to strain[a]:		
	CT218	LF044	CT162
pC4602-1 only	27.9 (64/229)	<0.1 (0/1,000)	<0.1 (0/1,000)
pC4602-1 and pC4602-2	3.9 (9/229)	<0.1 (0/1,000)	<0.1 (0/1,000)
pC4602-2 only	1.3 (3/229)	<0.1 (0/1,000)	<0.1 (0/1,000)

[a] Transconjugants were detected by colony hybridization with probes derived from seq10 in pC4602-1 and seq25 in pC4602-2. LF044 and CT162 are biotype 1 strains. The number of positive colonies with respect to the total is indicated in parentheses.

for eels, exhibiting an LD_{50} of around 1×10^4 CFU/fish. The virulence of bacteria recovered from the internal organs of killed eels was further shown to be enhanced to the wild-type level (LD_{50} of around 1×10^2 CFU/fish) for all of these strains (Table 2). In contrast, the transconjugant that contained pC4602-1 only was not virulent for eels (Table 2).

Transfer of pR99 in the presence of pC4602-1. We further tested the transfer of virulence plasmid pR99 in the presence or absence of pC4602-1 to confirm that the virulence plasmid by itself is not transmissible but that its transfer can be achieved with the aid of a conjugal plasmid. Plasmid pC4602-1 was transferred from CECT4602 to CT095, a CECT4999 derivative containing a Cm^r cassette in pR99, by conjugation, and the plasmid compositions of a transconjugant, CT295, were confirmed by PCR with specific primer pairs. The frequencies of transfer of pR99::Cm^r from CT095 and CT295 to CT237, a spontaneous Sm^r mutant of CT218, by conjugation were then determined. Colonies resistant to both Cm and Sm were obtained after conjugation at frequencies of 1.1×10^{-1} and $< 10^{-5}$, respectively, for CT295 and CT095. The frequencies of spontaneous mutation that gave rise to colonies resistant to both Cm and Sm for CT095, CT295, and CT237 were determined in parallel as negative controls, and they were all found to be $<10^{-5}$.

Distribution of seq51, *traH*, *mazF*, ID1 and ID2 in the plasmids of various B2 strains. To investigate the prevalence of virulence and conjugal plasmids of strains CECT4602 and CECT4999 in BT2 *V. vulnificus*, six other BT2 strains from various sources were examined by Southern hybridization with a number of probes derived from these plasmids. Among them, two contained one plasmid and the other four contained two plasmids (Fig. 7A). seq51, a virulence plasmid marker, and *mazF*, the toxin module associated with plasmid stability, were detected in all of the tested strains; seq51 was located in the large plasmids, while *mazF* was located in either the large or small plasmid in those with two plasmids (Fig. 7B). The *traH* gene, a conjugal plasmid marker, was detected only in the small plasmid of strains with two plasmids (Fig. 7B). ID1 and ID2, the common DNA sequences in pC4602-1, pC4602-2, and pR99, were detected in every plasmid of the tested BT2 strains (Fig. 7B). The presence of a gap between ID1 and ID2 was further investigated by PCR with a pair of primers derived from ID1 and ID2, respectively. As shown in Fig. 7C, PCR products corresponding to those amplified from pC4602-1 and pC4602-2, respectively, were obtained from strains containing two plasmids, except for strain CECT4865. All the strains with only one plasmid and strain CECT4865 gave a PCR product corresponding to that amplified from pC4602-1.

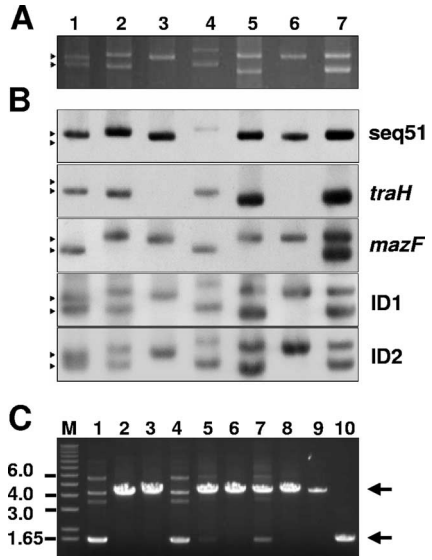

FIG. 7. Distribution of genes and regions involved in virulence for eels, plasmid transfer, and plasmid stability in the plasmids of various B2 strains. (A) Profiles of uncut plasmids. Lanes 1 to 7, plasmids from strains CECT4602, CECT4865, CECT4917, CECT897, CECT4870, CECT4866, and CECT5198, respectively. (B) Southern hybridization of the uncut plasmids with probes derived from seq51, *traH*, *mazF*, and regions ID1 and ID2. The arrowheads in panels A and B indicate the positions of pC4602-1 (lower band) and pC4602-2 (higher band). (C) PCR products amplified from various plasmids with primers CT39-R and JB02R-4 derived from ID1 and ID2, respectively. Lanes 1 to 10, strains CECT4602, CECT4865, CECT4917, CECT897, CECT4870, CECT4866, CECT5198, CECT4999, CT223, and CT227. Lane M, 1-kb plus DNA ladder. The arrows indicate PCR products amplified from pC4602-1 (higher band, about 4.2 kb) and pC4602-2 (lower band, about 1.6 kb).

DISCUSSION

BT2 of *V. vulnificus* is unique among the three biotypes of this species in its ability to infect and cause septicemia in eels. Two previous findings, the presence of large plasmids in all BT2 strains (6, 16, 30) and the identification of BT2-specific plasmid DNA sequences found by SSH (28), suggested that the plasmids might be involved in virulence for eels. To confirm this, we selected two highly virulent serovar E strains, CECT4999 and CECT4602, that exhibited different plasmid profiles and determined the nucleotide sequences of their plasmids, followed by isolation of a plasmid-cured strain and testing of its virulence.

The completed plasmid nucleotide sequences revealed that both strains possessed a common plasmid (nucleotide sequence identity, 92%) of about 68 kb in size. Like many other virulence plasmids (14, 18, 24), the common plasmid of BT2 strains appeared to be a mosaic composed of regions from multiple sources that might be formed via horizontal gene transfer between the aquatic bacteria.

Based on the plasmid DNA sequences, we were able to identify and disrupt *mazEF*, a TA module, on the plasmid to facilitate the isolation of a plasmid-free derivative from strain CECT4999. Curing of pR99 resulted in loss of virulence for eels, and introduction of pC4602-2 into the cured strain restored the virulence (Table 2), indicating that both pR99 and pC4602-2 were virulence plasmids.

The virulence conferred by pR99 or pC4602-2 is different from that conferred by pJM1, the common virulence plasmid of *V. anguillarum*, another causative agent of vibriosis, which has been shown to encode the ability to take up iron for growth (18). The loss of virulence for eels of the plasmid-cured strain and CT188, a mutant with a deletion in ORFvep07 of pR99, was associated with diminished resistance to killing by eel serum and, consequently, an inability to grow in the eel blood. However, plasmid loss did not affect either the virulence in

mice or growth in human blood, suggesting that different sets of virulence genes might be used in different hosts.

The virulence plasmid may be required for resistance to a bactericidal factor functioning in eel, but not human, serum. The identification of this bactericidal factor would be crucial for better understanding of the pathogenesis of vibriosis in eels. Previous studies on serovar E BT2 strains have suggested that different surface antigens may be involved in resistance to sera from different hosts: lipopolysaccharide for resistance to eel serum (2) and capsule for resistance to human serum (5, 46). We had checked the structure of lipopolysaccharide in CT218 and found that it was not altered (43), implying that the virulence plasmid might encode another factor(s) important for the resistance to eel serum.

The *rtx* genes encoding the production, export, and activation of an RTX family member (9, 45) were the only known virulence genes in the virulence plasmids of BT2 strains. An *rtx* gene cluster has also been found in the small chromosome of BT1 *V. vulnificus* (12) and has been shown to be associated with virulence in the mouse (29, 31). The *rtxB*, *rtxC*, *rtxD*, and *rtxE* genes and the promoter region of this gene cluster in pC4602-2 or pR99 were highly homologous to those of *V. cholerae* and BT1 *V. vulnificus* (80% and 94% identical, respectively). To our surprise, the *rtxA* gene in pC4602-2 or pR99 was a mosaic composed of DNA fragments from those of *V. cholerae* and BT1 *V. vulnificus*. A copy of the *rtx* gene cluster showing a restriction pattern identical to that of the virulence plasmid was also detected in the chromosome of CECT4999, suggesting a redundancy of this gene cluster in this strain. Such a redundancy was also observed in strain CECT4602, with only a minor difference in *rtxA*. Using RT-PCR, we demonstrated that the chromosomal *rtxA* gene of the plasmid-cured strain, CT218, was transcribed when the organism was cultured in rich medium (Fig. 4B). Further, CT218, but not CT218 Δ*rtxA*, could lyse HEp-2 cells (our unpublished data), indicating that a functional RTX could be produced from the chromosome. While the role of RTX toxin in virulence for eels needs to be further defined, we feel that the *rtx* gene cluster in the virulence plasmid might not be indispensable, since there is another copy in the chromosome that is functional.

On the other hand, ORFvep07 of pR99 appeared to be essential for virulence in eels, because disruption of this gene in strain CECT4999 resulted in greatly reduced virulence in eels. ORFvep07 and its equivalent, ORFvpa22, in pC4602-2 shared low nucleotide sequence identity (21% in a 279-amino-acid region of this 388-amino-acid protein) with a gene annotated as the RTX toxin and related Ca^{2+}-binding protein, whose function requires further experimental validation. These two genes may encode an outer membrane or extracellular protein, as was suggested from the result of computational analysis with CELLO, a subcellular localization predictor (47). However, ORFvep07 by itself was not sufficient for causing disease in eels, as the BT1 strain or plasmid-cured BT2 strain remained avirulent or became weakly virulent at most in the presence of this ORF alone. This suggested that other genes in the virulence plasmid might be required for the expression of full virulence in eels. The mechanism of resistance of BT2 strains to killing by eel blood and serum, in which ORFvep07 plays a role, is currently under investigation in our laboratories.

The third plasmid, pC4602-1, was shown to be an F-like conjugal plasmid that shared high sequence homology with pYJ016 of the BT1 strain YJ016. Interestingly, in a mating between strains CECT4602 and CT218, we obtained three groups of transconjugants, which contained pC4602-1 alone, both pC4602-1 and pC4602-2, and pC4602-2 alone, respectively. The frequency of transfer of pC4602-2, which did not carry any known transfer gene, was about one-sixth of that of transfer of pC4602-1. Transfer of pC4602-2 may occur in a form of cointegrate with pC4602-1 that could be generated by a recombination between the common regions, ID1 or ID2, in these plasmids. The existence of the cointegrate was suggested by the obtaining of predicted PCR products with two primer pairs that were both composed of primers hybridizing specifically to pC4602-1 and pC4602-2, respectively (Fig. 5B). Once entering the recipient, the cointegrate may rapidly resolve into two plasmids, making detection of the cointegrate difficult. The fact that two of the nine transconjugants with both plasmids showed altered plasmid sizes and restriction profiles (Fig. 6) as well as PCR products amplified with primer pair M2/179R (Fig. 5B), which could result from aberrant resolution, is consistent with this suspicion.

The three transconjugants with only the virulence plasmid may be derived from those containing both plasmids by losing the conjugal plasmid or, alternatively, may be a result of incomplete transfer of the cointegrate when the mating process was interrupted. The former could be readily ruled out because we found, by colony hybridization, that the conjugal plasmid was stably maintained in the transconjugants containing either the conjugal plasmid alone or both plasmids after overnight growth in rich medium (data not shown). Nevertheless, the possibility that the virulence plasmid may be mobilizable and that its transfer could be achieved once the mating pair is formed with the aid of a conjugal plasmid cannot be excluded. Although it demands experimental validation, a putative transfer origin (*oriT*), CGTGGTGTGTG, of F-like plasmids enabling transfer of the plasmid in the presence of a conjugal plasmid with the same *oriT*, has been found residing the ID1 regions of pC4602-1, pC4602-2, and pR99 between *traI* and *pilT*.

When we compared the nucleotide sequence of pR99 with those of pC4602-1 and pC4602-2, we found a common DNA sequence (100% identical), which contained ID1, ID2, the 2.6-kb sequence between ID1 and ID2, and four more ORFs, including *mazEF*, in pR99 and pC4602-1. The plasmids of two other single-plasmid-containing serovar E strains, CECT4917 and CECT4866, were also shown to contain ID1, ID2, and the DNA sequence between these two regions. These findings provided additional clues for the transmission of a virulence plasmid by conjugation with the aid of a conjugal plasmid. The single-plasmid-containing strains could have originated from a mating between a two-plasmid-containing donor and a plasmid-free recipient, as had been demonstrated in the conjugation between CECT4602 and CT218. Indeed, by introducing pC4602-1 into CECT4999, we were able to transfer pR99 into the plasmid-cured strain, even though pR99 per se was not transferable.

The frequency of transferring pC4602-1 from CECT4602 to CT218 by conjugation was close to 30%, but that of transferring it to two BT1 strains, LF044 and CT162, was less than 10^{-3}, if any. This finding may partly explain why only a subset

of *V. vulnificus* strains can cause disease in eels: the dissemination of the virulence plasmid is an uncommon process because the conjugation efficiency of the conjugal plasmid enabling its transfer is very low. The difficulty of transferring pC4602-1 to BT1 strains by conjugation may be due to defects in forming a mating pair between the two biotypes and/or replication of this plasmid in the BT1 strains or to rapid degradation of this plasmid by a restriction enzyme(s) in BT1 strains.

The *mazEF* operon, encoding a member of TA modules that contribute to the stability of plasmids (32), was found in pC4602-1, pR99, and the plasmids of all six other BT2 strains checked. This suggests that the BT2 strains might retain the plasmids via this system. In strain CECT4602, the *mazEF* operon was located in the conjugal plasmid but not the virulence plasmid. It is possible that *mazEF* of pC4602-1 stabilizes both plasmids in a form of cointegrates.

All of the tested BT2 strains, irrespective of their serotypes (E or A), phenotypic characteristics, sources (environments, fish, or humans), geographic origins (Europe, Asia, or Australia), or isolation dates (1979 to 1999), harbored a plasmid similar to the virulence plasmids characterized in this study. The majority harbored an additional plasmid that carried the transfer genes and might be needed for the transmission of virulence plasmid. Intriguingly, even though the size of the putative virulence plasmid differed among the strains (Fig. 7A), the distances between ID1 and ID2 that were detected in all the plasmids appeared to be highly restricted: either none or 2.6 kb.

Collectively, our data demonstrated that the ability to colonize and develop disease in fish has emerged in *V. vulnificus* by acquisition of a virulence plasmid. The original virulence plasmid was probably a mosaic of genes from different aquatic bacterial species, including fish pathogens. This virulence plasmid might have been acquired by different ancestors (polyphyletic origin) to give rise to BT2 of the species, as phenotypically and serologically this biotype is composed of heterogeneous strains (1, 16, 28). Once it was acquired, the virulence plasmid could be stably maintained, via a *mazEF* TA module, in the BT2 strains and be disseminated with the aid of a conjugal plasmid. The virulence and conjugal plasmids can form a cointegrate, and the recombination events occurring during formation and resolution of the cointegrates could contribute to the diversification of the plasmids.

ACKNOWLEDGMENTS

This work was supported by grants NSC 93-3112-B-006-007, NSC 94-2320-B-006-066, and NSC 95-3112-B-010-008 from the National Science Council of Taiwan and AGL2005-04688 from the Ministry of Science and Education of Spain and by the NCKU Project of Promoting Academic Excellence & Developing World Class Research Centers, Taiwan. We thank I. T. Lai for construction of pIT009 during a summer research program funded by the National Science Council of Taiwan (NSC-2815-C-006-058-B).

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