O Antigen Is the Receptor of *Vibrio cholerae* Serogroup O1 El Tor Typing Phage VP4

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Bacteriophage VP4 is a lytic phage of the *Vibrio cholerae* serogroup O1, and it is used in phage subtyping of *V. cholerae* biotype El Tor. Studies of phage infection mechanisms will promote the understanding of the basis of phage subtyping as well as the genetic differences between sensitive and resistant strains. In this study, we investigated the receptor that phage VP4 uses to bind to El Tor strains of *V. cholerae* and found that it infects strains through adsorbing the O antigen of *V. cholerae* O1. In some natural isolates that are resistant to VP4 infection, mutations were identified in the *wb** cluster (O-antigen gene cluster), which is responsible for the biosynthesis of O antigen. Mutations in the *manB*, *wbeE*, and *wbeU* genes caused failure of adsorption of VP4 to these strains, whereas the observed amino acid residue mutations within *wbeW* and *manC* have no effect on VP4 infection. Additionally, although mutations in two resistant strains were found only in *manB* and *wbeW*, complementing both genes did not restore sensitivity to VP4 infection, suggesting that other resistance mechanisms may exist. Therefore, the mechanism of VP4 infection may provide a basis for subtyping the phage. Elaborate mutations of the O antigen may imbue *V. cholerae* strains with resistance to phage infection.

Bacteriophages are found everywhere in the biosphere, and they outnumber prokaryotic cells by an estimated 10-fold (1). A specialized phage can infect only a narrow range of hosts, so phage typing schemes are used in epidemiological studies of many bacterial pathogens, such as *Staphylococcus aureus* (2), *Salmonella enterica* serovar Typhimurium (3), *Vibrio cholerae* (4, 5), and *Mycobacterium tuberculosis* (6–8). *V. cholerae* is the causative agent of the diarrheal disease cholera. Among the more than 208 O-antigen serogroups of *V. cholerae*, only the O1 and O139 serogroups have been found to cause epidemic cholera (9). The O1 serogroup is divided into the main serotypes Inaba and Ogawa and two distinct biotypes, designated classical and El Tor (10). Phage typing, as well as some other molecular subtyping methods, is also used for *V. cholerae*. A phage biotyping scheme has been used to subtype the Chinese O1 El Tor strains for 40 years (11), and it has played an important role in surveillance and source tracing of cholera. In the phage biotyping scheme, El Tor strains from patients in epidemics and from environmental samples during nonepidemic periods present different types and are therefore classified into "epidemic strains" and "nonepidemic strains" (11).

The study of phage typing mechanisms with various phage types may help to reveal genetic differences among the different bacterial strains, enhancing surveillance of the emergence of new phage-resistant clones in the environment and in epidemics (12, 13). In the natural environment, the phage-bacterium interaction promotes coevolution of both. The mutations in bacteria that lead to phage resistance allow these cells to dodge the phage attacks and have a survival advantage in their ecological niches. The mechanisms that bacteria use to avoid infection have hitherto been found in different steps of phage infection, i.e., adsorption resistance, prevention of host takeover, and abortive infection (14), which occur in the life cycles of phage adsorption, gene replication, and release, respectively (14, 15).

Adsorption to the host cell is the first step in the life cycle of phages. In this process, receptor-binding proteins (RBPs) (16) of the phage interact specifically with the receptors on the surface

of host cells. Different bacterial surface components have been identified as the receptors for phages, including flagella (17), pili (18), lipopolysaccharides (LPS), capsular polysaccharides (19), teichoic acids (20), and outer member proteins such as OmpA (21), OmpC (22), LamB (23), FepA (24), and TonA (25). LPS are common phage receptors in Gram-negative bacteria; T phage, especially T3, T4, and T7, adsorb LPS on the surface of *Shigella* (26) and *Escherichia* (27, 28). *Salmonella* phages φX174 (29) and P22 (30), *Klebsiella* phage FC3-10 (31), and *Yersinia enterocolitica* phage ΦYeO3-12 (32) also use LPS as receptors. More recently, the Hep/Glc-Kdo/Ko region of *Yersinia pestis* and *Yersinia pseudotuberculosis* LPS have been identified as the receptor for phage ΦA1122 (33), the core oligosaccharide (OS) region of LPS was found to be necessary for binding of *V. cholerae* typing phage VP3 (34), and the O side chain was found to serve as the receptor for temperate phage CP-T1 (35). In addition, recent studies have reported some novel receptors. The phase-variable *O*-methyl phosphoramidate moiety, which is a common component of the diverse capsular polysaccharides of *C. jejuni*, has been identified as a receptor for phages (36). OmpLC was also identified as a receptor for phage infecting *Edwardsiella ictaluri*, and the extracellular loop 8 of OmpLC was predicted to be vital to the process of adsorption (37). Some phages use at least two receptors on the cell envelope (38, 39). Phage SPP1 of *Bacillus subtilis* interacts with the glucosylated wall teichoic acids and the membrane protein YueB (40).

The phage biotyping scheme includes five phages and some additional biochemical tests (11). VP4 is one of the five typing phages. In this study, we sought to investigate the receptor of VP4

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
N16961	O1 El Tor, Inaba	Laboratory stock
N16961-Sm	Spontaneous mutant of N16961; Sm^r	This study
919C	O1 El Tor	Laboratory stock
V07-16	O1 El Tor, Inaba	Laboratory stock
367	O1 El Tor, Ogawa	Laboratory stock
V06-92	O1 El Tor, Ogawa	Laboratory stock
465	O1 El Tor, Inaba	Laboratory stock
470	O1 El Tor, Ogawa	Laboratory stock
417	O1 El Tor, Inaba	Laboratory stock
136	O1 El Tor, Ogawa	Laboratory stock
427-2A1	N16961-Sm,vc0241::Tn, Sm^r Kan^r VP4^r	This study
514-2G8	N16961-Sm,vc0241::Tn, Sm^r Kan^r VP4^r	This study
514-3F2	N16961-Sm,vc0251::Tn, Sm^r Kan^r VP4^r	This study
513-2E4	N16961-Sm,vc0251::Tn, Sm^r Kan^r VP4^r	This study
516-8B8	N16961-Sm,vc0242::Tn, Sm^r Kan^r VP4^r	This study
506-1C4	N16961-Sm,vc0245::Tn, Sm^r Kan^r VP4^r	This study
514-4C2	N16961-Sm,vc0243::Tn, Sm^r Kan^r VP4^r	This study
427-2H5	N16961-Sm,vc0260::Tn, Sm^r Kan^r VP4^r	This study
506-2B11	N16961-Sm,vc2417::Tn, Sm^r Kan^r VP4^r	This study
N16961-Sm Δ*manB*	*manB* deletion of N16961-Sm	This study
N16961-Sm Δ*wbeU*	*wbeU* deletion of N16961-Sm	This study
N16961-Sm Δ*manB*/C*manB*	*manB* complementation of N16961-Sm Δ*manB*	This study
N16961-Sm Δ*wbeU*/C*wbeU*	*wbeU* complementation of N16961-Sm Δ*wbeU*	This study
95001 C*wbeE*/C*wbeU*	*wbeE* and *wbeU* complementation of 95001	This study
367 C*manB*	*manB* complementation of 367	This study
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-TC::Mu Km λ*pir*	47
Plasmids		
pSC123	Suicide plasmid carrying transposon; Kan^r Cm^r	9
pWM91	Suicide plasmid; *oriR oriT lacZ tetAR sacB*	40
pWM91Δ0242	pWM91 carrying upstream and downstream fragments flanking *manB*	This study
pWM91Δ2417	pWM91 carrying upstream and downstream fragments flanking *recJ*	This study
pCVD442	Suicide plasmid; R6K *ori mob* RP4 *bla sacB*	13
pCVD442-Δvc0260	pCVD442 carrying upstream and downstream fragments flanking *wbeU*	This study
pBR322	Cloning vector; ColE1 *ori* Amp^r Tet^r	TaKaRa
pBR322-c0260	pBr322 carrying *wbeU*	
pACYC184	Cloning vector; *rep* Cm^r Tc^r	6
pACYC184-c0242	pACYC184 carrying *manB*	This study
pACYC184-c0244	pACYC184 carrying *wbeE*	This study
pACYC184-c0261	pACYC184 carrying *wbeW*	This study
pUC18	Cloning vector; *ori lacZ* Amp^r	TaKaRa
pUC18-c0260	pUC18 carrying *wbeU*	This study

and to understand the typing mechanism of VP4 from the perspective of receptor gene differences. The O side chain of LPS was identified as the VP4 receptor. In addition, some mutations in the *wb** cluster (the O-antigen gene cluster that consists of open reading frames [ORFs] between VC0240 and VC0264 in El Tor strain N16961) of the natural strains confer resistance to VP4 infection.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and culture conditions. The phage, bacterial strains, and plasmids used in this work are described in Table 1. Phage VP4 was propagated on host strain 919c. The El Tor strain N16961 for which the whole genome has been sequenced (41), is sensitive to VP4. N16961-Sm, which is resistant to streptomycin (Sm) and sensitive to phage VP4, was selected by plating N16961 on Luria broth (LB) agar with 100 μg/ml of Sm. This strain was used in the conjugation test and was distinguished from *Escherichia coli* by its resistance to Sm. Unless otherwise stated, all strains were grown at 37°C in liquid or on solid (15 g/liter

agar) LB medium, which could be supplemented with 100 μg/ml of kanamycin (Kan), 100 μg/ml of Sm, 10 μg/ml of chloramphenicol (Cm), or 100 μg/ml of ampicillin (Amp).

Construction of transposon insertion library and selection of VP4-resistant mutants. Plasmid pSC123 (42) was transferred by conjugation from *E. coli* donor SM10λ*pir* (43) into N16961-Sm, and transconjugants were selected by streptomycin and kanamycin resistance (Sm^r and Kan^r). The resulting strains contained a chromosomal insertion caused by the integration of the plasmid, which carries Cm and Kan resistance genes. Single colonies were picked and incubated in 96-well plates until the optical density at 600 nm (OD$_{600}$) reached 0.1 to 0.2. The cultures were then mixed with a VP4 phage suspension (1×10^8 PFU/ml) at a ratio of 20:1 to 30:1 in new 96-well plates and incubated for 3 h. Cultures of strain N16961 with and without VP4 were used as negative and positive controls, respectively. The wells with an OD$_{600}$ significantly higher than that of the negative control and nearly as high as that of the positive control were selected as candidates for phage-resistant mu-

tants. These candidates were subsequently tested using a double-layer plaque assay (44).

Arbitrary PCR. To identify the transposon insertion site, arbitrary PCR (45, 46) was performed with two rounds. In the first round, portions of the chromosomal DNA preparations were used as templates, and primers ARB-1, ARB-6, and 123-3 (Table 2) were used in amplification. PCR was performed under the following conditions: 95°C for 5 min; six cycles of 94°C for 30 s, 30°C for 30 s, and 72°C for 1 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. The second round of PCR was performed with a portion of the PCR product of round 1 as the template, and primers 123-4 and ARB-2 (Table 2) were used. PCR was performed under the following conditions: 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. Amplicons were sequenced using the primer 123-4.

Construction of mutants. An in-frame *wbeU* deletion mutant of *V. cholerae* N16961-Sm was constructed by homologous recombination using the suicide plasmid pCVD442 (47). The deletion fragment inserted into pCVD442 was produced using overlap extension PCR. The ca. 700-bp regions upstream and downstream of the *wbeU* gene were amplified by PCR from N16961-Sm chromosomal DNA using primers pCVD442Δ0260F1/pCVD442Δ0260R2 and primers pCVD442Δ0260F3/pCVD442Δ0260R4 (Table 2), respectively. The two amplicons overlapped by 30 bp. The overlapping fragments were purified with the QIAquick gel extraction kit (catalog no. 28706; Qiagen, Germany) and mixed as templates to generate heteroduplexes that were extended by PCR (with primers pCVD442Δ0260F1 and pCVD442Δ0260R4). The fragment was then cloned into pCVD442 to generate plasmid pCVD442-Δ0260, which was conjugally transferred into N16961-Sm from the donor strain *E. coli* SM10λ*pir*. Transconjugants were selected on LB agar with Cm and Amp. Clones were streaked onto LB agar with 10% sucrose and without NaCl at 22°C. Colonies from the sucrose selection medium were then streaked on LB agar plates with Amp. Failure to grow indicated that the plasmid was absent and a double crossover had occurred. Clones were screened by PCR with special primers innerU-F and innerU-R (Table 2). PCR-negative clones were amplified with primers pCVD442Δ0260F1/pCVD442Δ0260R4 (Table 2), producing amplicons of 1.4 kb, which are shorter than the approximately 3.3 kb for the wild type N16961-Sm. Sequencing confirmed the *wbeU* deletion mutants.

The suicide plasmid pWM91 (40) was used to construct a *manB* deletion mutant of N16961-Sm in a similar way. Primer pairs pWM91Δ02421F/pWM91Δ02422R and pWM91Δ02423F/pWM91Δ02424R (Table 2) were used to amplify the 5′ and 3′ flanking sequences of *manB*, respectively. Primers pWM91Δ02421F and pWM91Δ02424R (Table 2) were used to amplify the heteroduplexes produced by mixing the two PCR products. The resulting fragment was subsequently cloned into pWM91 to construct the plasmid pWM91Δ0242. The construction of the *recJ* gene deletion mutation with pWM91 was performed using a similar protocol.

Complementation of mutations. Primers pBR322-U-F and pBR322-U-R, incorporating HindIII and BamHI restriction sites (Table 2), amplified a 2,666-bp region of the N16961 genome that included an 800-bp upstream sequence in addition to the ORF of *wbeU*. The amplicon was digested with BamHI and HindIII (New England BioLabs) and ligated into the BamHI- and HindIII-digested plasmid pBR322 (D3050; TaKaRa, Japan), producing plasmid pBR322-c0260, which was electroporated into the *wbeU* gene deletion mutant strain. The *manB* gene and the 800-bp upstream region were amplified with primers pBR322-B-F and pBR322-B-R and cloned into pBR322. This process created pBR322-c0251, which was electroporated into the *manB* gene deletion mutant and O1 El Tor strains (strain 367, strain v07-16, and strain v06-92). Using the same technique, we used pACYC184 (48) to construct plasmids pACYC184-c0244 and pACYC184-c0261 with primers pACYC184-E-F/pACYC184-E-R and pACYC184-W-F/pACYC184-W-R, respectively (Table 2). In addition, we constructed plasmid pUC18-c0260 from pUC18 using primers pUC18-U-F/pUC18-U-R (Table 2). The plasmids pACYC184-c0244 and

pUC18-c0260 were transformed into strain 95001, and pACYC184-c0261 and pBR322-c0251 were transformed into strains V07-16 and V06-92 via electroporation; transformants were selected by Cm and Amp resistance. All the recombinant clones described above were confirmed by PCR and sequenced to ensure that they contained the correct insert sequence. The VP4 sensitivity of the complemented mutants was examined by a double-layer plaque assay and compared with that of its respective mutant strain containing the empty vector.

Phage adsorption assay. Five hundred microliters of VP4 (approximately 10^7 PFU/ml) was mixed with 500 µl of bacterial culture (10^8 CFU/ml), incubated at 37°C for 10 min, and centrifuged at $10,000 \times g$ for 3 min. The phage titer remaining in the supernatant was determined by a double-layer plaque assay. LB broth was mixed with the phage without the bacteria and used as a control. Each assay was performed in duplicate and repeated three times.

Adsorption of SYBR gold-stained phage VP4 on the surface of bacteria. A phage lysate with a titer of at least 10^8 PFU/ml was mixed at 50,000:1 (vol/vol) with a SYBR gold nucleic acid gel stain stock solution (S11494; Invitrogen) and incubated for 30 min in the dark at room temperature. The mixture was filtered through 0.02-µm-pore-size filters (6809-5002; Waterman, Germany). The filters were washed with phosphate-buffered saline (PBS) three times and then backwashed with 1 ml of PBS to gather labeled phage. The gathered phage titer was 10^7 PFU/ml at a minimum. Bacterial cultures grown to an OD_{600} of 0.1 were mixed 1:1 with phage, incubated for approximately 10 min, and then centrifuged at $10,000 \times g$ for 3 min. The precipitate was resuspended with 50 µl of PBS. Phage VP4 adsorbed on the surface of bacteria was examined with a fluorescence microscope (BX51; Olympus, Japan) at a magnification of ×1,000 with blue-light (450 to 490 nm) excitation. For observation by confocal laser scanning microscopy (CLSM) (FV500; Olympus, Japan), the sample was mixed with an equal volume of 0.5% agarose and dropped on a slide in 1-µl aliquots.

Antiserum agglutination assay. Clones grown to mid-logarithmic phase on LB plates were picked up with a toothpick and mixed thoroughly into a drop of O1 antiserum (S&A Reagents Lab, Bangkok, Thailand). Bacteria expressing O1 antigen produced a granular agglutination within seconds, while O1-antigen-negative bacteria stayed in homogenous suspension.

LPS pattern analysis. Strains N16961-Sm, N16961-Sm Δ*wbeU*, and N16961-Sm Δ*manB*, as well as complemented strains N16961-Sm Δ*wbeU*/C*wbeU* and N16961-Sm Δ*manB*/C*manB*, were cultured in LB broth for 12 h. A 100-ml sample of each culture was used for LPS extraction as described previously (49). The purified LPS were separated on a sodium dodecyl sulfate-polyacrylamide gel (18.5 cm by 20.0 cm by 1.0 mm) containing 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Electrophoresis was performed at 10 mA in the stacking gel and 30 mA in the separating gel until the tracking dye ran to the end of the gel. The gel was visualized by silver staining using the method described by Tsai and Frasch (50).

Sequencing of the *wb cluster in the naturally VP4-resistant and -sensitive strains.** Thirteen primer pairs (wbe-1F/wbe-1R to wbe-13F/wbe-13R) (Table 2) were designed to PCR amplify the *wb** cluster. Each amplicon was approximately 1,800 bp in size and shared a 200-bp overlapping region with the adjacent amplicons. The *wb** gene clusters of naturally VP4-resistant strains and some genes of VP4-sensitive strains were sequenced, including four resistant strains (strain 367, strain V07-16, strain V06-92, and strain 95001) and four sensitive strains (strain 465, strain 470, strain 417, and strain 136) isolated from two different areas at different times. Among these strains, only 95001 carries the cholera toxin genes. The sequence data were assembled using DNAStar SeqMan (version 5.01; DNASTAR Inc.) and aligned using BLAST with Vector NTI Advance 10 (version 10; Invitrogen).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *wb** gene clusters of strains 95001, V07-16, V06-92, and 367 are KC152957, KC152956, KC152955, and KC152954, respectively.

TABLE 2 Primers used in the study

Use and primer name	Sequence	Restriction site
Mutagenesis of VC0260 of N16961-Sm		
pCVD442Δ0260F1	CGCGTCGACTTTTACTCTTTCCGGACCGTC	SalI
pCVD442Δ0260R2	ATGTTTACCCCTATTTCGCCACAAAAATGA	
pCVD442Δ0260F3	TCATTTTTGTGGCGAAATAGGGGTAAACAT	
pCVD442Δ0260R4	GCGGCATGCTTAGCCTTAACCCCTTCG	SphI
InnerU-F	GATGGCTAGTGCCTTCCACATTCTC	
InnerU-R	CTTTATTCCCCGCAGTGTCCCCTTTA	
Mutagenesis of VC0242 of N16961-Sm		
pWM91Δ02421F	CCGCTCGAGAATTGTTATATCTACGCCCC	XhoI
pWM91Δ02422R	TTAAATATCCAATTTAGTTAACTCTTTCAC	
pWM91Δ02423F	GTGAAAGAGTTAACTAAATTGGATATTTAA	
pWM91Δ02424R	AAGGAAGCGGCCGCATACGCATACATCTTTGCCACA	NotI
Mutagenesis of *recJ* of N16961-Sm		
pWM91ΔrecJ1F	CCGCTCGAGCTAGGCATGAATAGCTACCGT	XhoI
pWM91ΔrecJ2R	TTGGCCTGTCACAGTCTGGAAGCGAAATGA	
pWM91ΔrecJ3F	TCATTTCGCTTCCAGACTGTGACAGGCCAA	
pWM91ΔrecJ4R	ATAGCGGCCGCGCCACTCAACGCCAAGA	NotI
Construction of complementary plasmids		
pACYC118-E-F	ACATGCATGCCTAAAGATTACGTTAAGATGCA	SphI
pACYC118-E-R	ACGCGTCGACTCAAATACAGTTGAAATAATTT	SalI
pACYC184-W-F	CGCGGATCCTTTGTTGCCAGCACCCCA	BamHI
pACYC184-W-R	ACATGCATGCTTATGGCTTAACACGATCAC	SphI
pBR322-U-F	CGCAAGCTTTACACCAGACTACGCA	HindIII
pBR322-U-R	CGCGGATCCTCATTTTTGTGGCGA	BamHI
pBR322-B-F	ATAGGATCCGCTTCCGTGTTCTTA	BamHI
pBR322-B-R	CGCGTCGACTACTTCATACCCTTTTTC	SalI
pUC18-U-F	CGGGGTACCTACACCAGACTACGCA	KpnI
pUC18-U-R	CGCGGATCCTCATTTTTGTGGCGA	BamHI
Arbitrary PCR		
ARB-1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	
ARB-6	GGCCACGCGTCGACTAGTACNNNNNNNNNNNACGCC	
ARB-2	GGCCACGCGTCGACTAGTAC	
123-3	ACGCACTGAGAAGCCCTTAG	
123-4	GCCCGGGAATCATTTGAAGG	
Sequencing of *wb∗* cluster		
wbe-1F	ATAATTAAGATGGCTATAAACTACC	
wbe-1R	ATTGGGTTATGACTTGCGGTA	
wbe-2F	CAGATAAAACAGTTGTGGTAGGTG	
wbe-2R	GGGATTCGTGGTTAAAAAGTAT	
wbe-3F	ACTAAGTTTTATCAAGCCTCAA	
wbe-3R	GTTGCAAATTTCTTTTACTTGATCA	
wbe-4F	ATGGGTTGATGACCTTCTTAGA	
wbe-4R	AATAATCATCTACTCCCATTCTCCA	
wbe-5F	TTGGGAATGAAAATGCTTAAAGA	
wbe-5R	GCATCGATAATATCTTCAACAT	
wbe-6F	ATCTTATTGCAGGAATTCTTGA	
wbe-6R	CTTCAAGCTCTCCAATAACTCCA	
wbe-7F	ATGGCGTTAAAAGGTTTAGTAGC	
wbe-7R	ACATCATCAGGAGAAATACCCAAT	
wbe-8F	ATTTTTGGATTCACGTTTGTACTT	
wbe-8R	CACAGCGGTCCTCCTAGAGTTAC	
wbe-9F	CGACAGTGTACATGAAGATATCCA	
wbe-9R	CCTTTGACCGCCAATAAATAAT	
wbe-10F	GTGAACGCTCTTGCTACAGCA	
wbe-10R	ATCTTGAAAACGTTAAGCTAGTAG	
wbe-11F	CGCCATTGATGTTCACATAAGAT	
wbe-11R	AAATAGTTGCAGCACTTGGC	
wbe-12F	TTTTACGCCAGCTTCTATAGCT	
wbe-12R	CCGCCTCATATTTACTTAAGCC	
wbe-13F	GATGCCCTTAAAGCGTATCGTGA	
wbe-13R	CTTCCATGTTGTTTCTCCTGTTTTG	
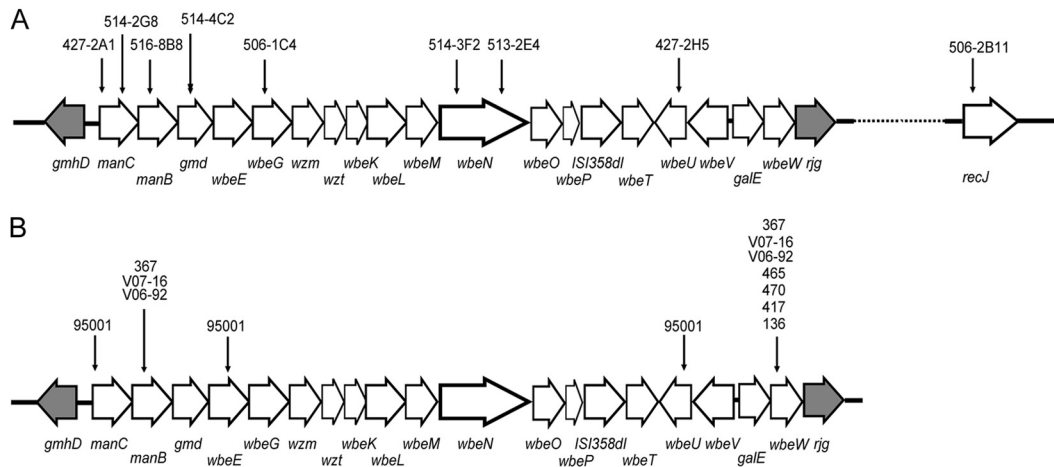
FIG 1 Mutations in the transposon insertion and VP4-resistant natural isolates. (A) Strains and transposon insertion sites in the *wb** gene cluster and *recJ* gene. The large white arrows represent the *wb** cluster, and the *recJ* gene and the transposon insertion positions are marked with small black arrows. The *gmhD* and the *rjg* junction genes are indicated by gray arrows. (B) Locations of mutations in the *wb** gene clusters of VP4-resistant and -sensitive natural isolates. The large white arrows represent the *wb** gene cluster, and the mutation sites are marked with small black arrows. The *gmhD* and the *rjg* junction genes are indicated by gray arrows.

RESULTS

Transposon insertion in the *wb** gene cluster and *recJ* cause resistance to VP4 infection. In order to search for the possible gene(s) related to resistance to VP4 infection, we first screened a transposon insertion library which was generated by plasmid pSC123, containing approximately 12,000 mutants. Nine mutants (427-2A1, 427-2H5, 514-3F2, 514-2G8, 514-4C2, 516-8B8, 506-2B11, 506-1C4, and 513-2E4) (Table 1) showed growth rates when coincubated with phage VP4 that were comparable to that of the control strain (N16961-Sm) without VP4, suggesting that they were resistant to VP4 infection. The resistances were further confirmed by a double-layer plaque assay. Seven transposon insertion mutants were confirmed, and the insertion sites were identified using arbitrary PCR. The *manC* gene was interrupted in two mutants (427-2A1 and 514-2G8), and insertions in *wbeN* were found in mutant strains 514-3F2 and 513-2E4. Mutants 516-8B8, 506-1C4, 514-4C2, 427-2H5, and 506-2B11 had single insertions in the genes *manB*, *wbeG*, *gmd*, *wbeU*, and *recJ*, respectively (Fig. 1A).

Six of the genes from the screen (all except *recJ*) are located within the *wb** cluster, which consists of ORFs between VC0240 (*gmhD*) and VC0264 (*rjg*) (41) in El Tor strain N16961 and is responsible for the biosynthesis of the O antigen. In this study, we focused on the *wb** cluster. To further confirm the correlation between *wb** gene mutations and VP4 infection resistance, the *manB* and *wbeU* genes were selected for in-frame deletion by homologous recombination (mediated by the suicide plasmid). These new *manB* and *wbeU* mutants lost sensitivity to VP4 infection as well. When complemented with the intact genes (*manB* and *wbeU*), these deletion mutants regained the VP4-sensitive phenotype.

Deletions of the *wb** gene and transposon insertions cause LPS change and O-antigen loss. Based on the results from the transposon mutation screen, we further constructed in-frame deletions of some *wb** genes. The *manB* gene was assumed to be responsible for the biosynthesis of perosamine, which composes the backbone of the O antigen (51). Insertion mutations in the *wbeU* gene may cause O-antigen loss (52). The mutants

N16961-Sm Δ*wbeU* and N16961-Sm Δ*manB* showed a rough colony phenotype and were not agglutinated by anti-O1 antiserum (data not shown). When these strains were analyzed by LPS electrophoresis, as shown in Fig. 2, LPS from N16961-Sm Δ*wbeU* and N16961-Sm Δ*manB* strains were missing the O-antigen ladder with the highest molecular weight (53). In contrast, when complemented with *wbeU* and *manB*, the corresponding deletion mutants had the smooth colony phenotype and agglutination with anti-O1 antiserum restored, and their LPS regained the same ladders as with N16961-Sm (Fig. 2). Similar O side chain loss was observed in the transposon mutant strains with insertions in *manC*, *gmd*, *wbeG*, and *wbeN* (data not shown).

Loss of O antigen causes nonadsorption of VP4 to the mutants. To observe the adsorption of VP4 to the wild-type strain and the mutants lacking O antigen, we performed quantitative phage adsorption assays and qualitatively observed labeled phage absorbing on the bacterial surfaces by microcopy and CLSM. The results are shown in Fig. 3. After the phages were incubated with N16961-Sm Δ*wbeU* and N16961-Sm Δ*manB*, VP4 infection titers
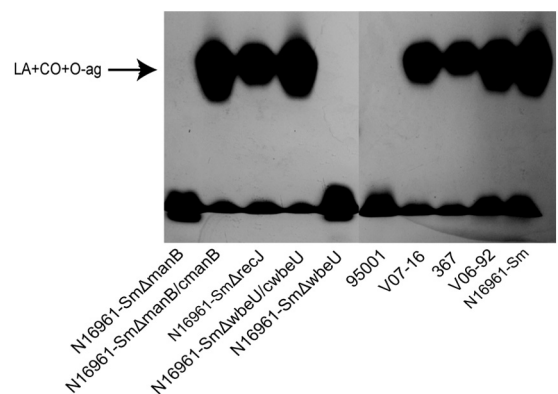
FIG 2 SDS-PAGE and silver staining of LPS from strain N16961-Sm and its mutants and the naturally VP4-resistant isolates. The top ladder indicates the integrated LPS. LA, lipid A; CO, core oligosaccharide; O-ag, O polysaccharide.
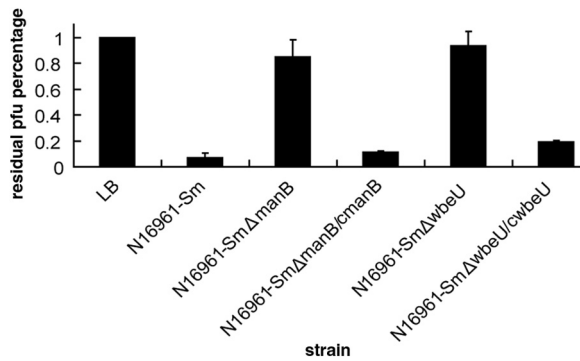
FIG 3 VP4 adsorption to *manB* and *wbeU* gene deletion mutants and complemented strains, shown as residual PFU percentages. LB was used as the no-bacterium control, and the phage titer in the control supernatant was set to 100%. Error bars indicate ranges.

FIG 4 Binding of SYBR gold-labeled VP4 on the surfaces of different strains observed by CLSM.

in the supernatants were similar to those after incubation with LB and much higher than those seen with strain N16961-Sm. When VP4 was incubated with the complemented strains of N16961-Sm Δ*wbeU* and N16961-Sm Δ*manB*, its titers declined sharply, although they remained slightly higher than that with N16961-Sm. These data indicate that the deletion mutants are deficient for VP4 adsorption, while the complemented strains have the phage's ability to adsorb restored. In addition, when SYBR gold-stained phage VP4 was incubated with N16961-Sm and both of the complemented strains, the cells were coated with green spots visible under the fluorescence microscope (data not shown). A similar result was more clearly observed under CLSM, which showed VP4 adsorption on the surface of the wild-type strain and complemented strains of N16961-Sm Δ*wbeU* and N16961-Sm Δ*manB* (Fig. 4). In contrast, no fluorescence was observed around the cells of the N16961-Sm Δ*wbeU* and N16961-Sm Δ*manB* mutants (Fig. 4). All of these results suggest that the phage VP4 adheres to the sensitive strains by binding to the O antigen and using it as a cell surface receptor.

Mutations in the *wb** gene clusters of the naturally resistant isolates. To investigate the mechanism by which natural *V. cholerae* strains are resistant to VP4 infection, four resistant strains (strain 367, strainV07-16, strain V06-92, and strain 95001) and four sensitive strains (strain 465, strain 470, strain 417, and strain 136) were selected for analysis. Their *wb** gene clusters between *gmhD* and *rjg* were amplified to overlapping PCR fragments that were sequenced. Variances of tens of nucleotide were found in the *wb** cluster for each strain, but most were silent mutations. With the exception of strain 95001, only the genes *manB* and *wbeW* had amino acid residue changes in these strains. However, all four sensitive strains had three amino acid residues that varied compared to those in the control strain N16961 (Table 3 and Fig. 1B), indicating that these residue changes have no effect on VP4 adsorption and infection. These residue changes in *wbeW* were also found in the four VP4-resistant strains that we analyzed. We did not pursue this gene any further.

The *manB* genes in strain 367, strain V07-16, and strain V06-92 had three amino acid residue changes in common compared to N16961 and the four assayed sensitive strains (Table 3 and Fig. 1B). CLSM showed that no fluorescence appeared around the cells of these strains when SYBR gold-stained VP4 phage were incubated with them (Fig. 4) (only strain 367 is presented as an exam-
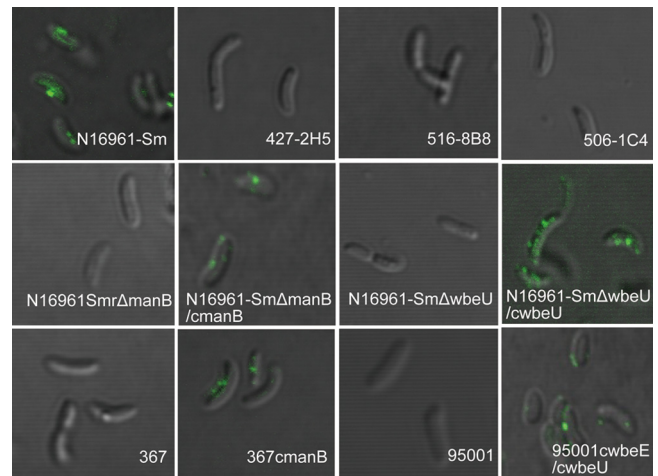
ple). When the plasmid pACYC184-c0242, which carries a copy of *manB* from N16961, was complemented into these strains, strain 367c*manB* showed sensitivity to VP4 infection, and VP4 adsorption to this strain was also observed under CLSM (Fig. 4). Nevertheless, strains V07-16 and V06-92 that were complemented with pACYC184-c0242 still showed resistance to VP4 infection, suggesting that additional mutations that cause resistance to VP4 infection may be present in both strains.

In strain 95001, amino acid changes were found within genes *manC*, *wbeE*, and *wbeU*. One residue change was found in *manC* (Table 3 and Fig. 1B). A single-nucleotide deletion within *wbeE* resulted in a frameshift and truncated translation of this gene. Two independent single-nucleotide deletions and a 10-bp fragment deletion in *wbeU* caused frameshifts leading to truncated translation of this gene as well (Table 3 and Fig. 1B). No VP4 adsorption to 95001 was observed with CLSM (Fig. 4). We complemented genes *wbeE* and *wbeU* first with the individual gene plasmids and then with both. Individual complementation of either *wbeE* or *wbeU* did not convert 95001 to VP4 sensitivity, but when exogenous copies of both genes were provided, the resulting strain (95001 C*wbeE*/C*wbeU*) was sensitive to VP4, and VP4 adsorption to the surface of this strain was observed in the CLSM assay (Fig. 4). Wild-type strain 95001 is the rough type, but it became smooth when complemented with the combination of *wbeE* and *wbeU*. It could also be agglutinated with serogroup O1 antiserum, showing that its LPS structure could bind effectively to VP4 and the O1 serogroup antibody.

DISCUSSION

Phages have important roles in microbial genetics and evolution as well as applications in the diagnosis of disease, the treatment of infection, the food industry, and biotechnology. Phages are also used in bacterial subtyping because they are able to specifically recognize and infect their host bacteria. In this study, we found that *V. cholerae* El Tor typing phage VP4 uses the O antigen as its receptor, and we further found that some naturally VP4-resistant El Tor strains have variation in the O-antigen genes and cause failure of adsorption of VP4 to the host cell. These findings suggest that the resistance of these natural isolates to VP4 infection is related to some aspect of the receptor, possibly structure changes.

TABLE 3 Mutations and gene complementation of natural isolates resistant or sensitive to phage VP4

Strain	Sensitivity[a]	Amino acid substitution in *wb** cluster genes	Gene(s) used to complement	Sensitivity after complementation
367	R	*manB*, I72V, I77L, Y91H; *wbeW*, A13V, A118V, S122A	*manB*	Restored
V07-16	R	*manB*, I72V, I77L, Y91H; *wbeW*, A13V, A118V, S122A	*manB*; *manB+wbeW*	Not restored
V06-92	R	*manB*, I72V, I77L, Y91H; *wbeW*, A13V, A118V, S122A	*manB*; *manB+wbeW*	Not restored
95001	R	*wbeU*, 12-base fragment deletion of nt[b] 1670–1681; *wbeE*, single-nucleotide deletion of nt 393, codon TAA occurs in nt 413; *manC*, S54P	*wbeE+wbeU*	Restored
465	S	*wbeW*, A13V, A118V, S122A		
470	S	*wbeW*, A13V, A118V, S122A		
417	S	*wbeW*, A13V, A118V, S122A		
136	S	*wbeW*, A13V, A118V, S122A		

[a] R, resistant to VP4; S, sensitive to VP4.
[b] nt, nucleotide.

Some phages that infect *V. cholerae* and other bacterial species use LPS as their receptors. These glycosyl structures are the sites with which RBPs of phage interact. Genes within the *wb** cluster are responsible for the biosynthesis of the O antigens. Through transposon mutations and in-frame deletions, we identified the *wb** genes whose mutations had resulted in O-antigen changes and thereby caused failure of adsorption of phage VP4.

LPS chemically consists of O antigen, core oligosaccharide, and lipid A. The *wb** gene cluster (and a newly recognized gene important for perosamine biosynthesis) and a complex pathway are required for O1 antigen synthesis (54). It could be speculated that some mutations in these genes may result in the changes or loss of O antigen, including the chemical group modification on the residues caused by some of the *wb** gene mutations, and then the phages may fail to bind and infect the strains or may reduce the infection efficiencies. An example is the finding that O1 antigen variation mediated by three homonucleotide tracts in two genes in *V. cholerae* results in a lower molecular weight of O1 antigen and infection failure of O1-specific phage ICP1 afterwards, or lower ICP1 infection efficiency without macroscopic change in LPS pattern, possibly because of less O1 antigen-substituted LPS in such a case (54). In our study, the naturally VP4-resistant strain 367 is still of the O1 serogroup and has an O-antigen pattern similar to that of the VP4-sensitive O1 strain, and complementation of its mutant *manB* converted its sensitivity to VP4, suggesting that some group modifications of O antigen are responsible for the phage infection (binding) but do not affect recognition of O1 antiserum. In *V. cholerae* O1, serotypes Ogawa and Inaba differ by the presence of a 2-*O*-methyl group in the nonreducing terminal sugar of the Ogawa O antigen but its absence in that of Inaba (55). *V. cholerae* O1 strains sensitive to phage VP4 include both Ogawa and Inaba serotypes; therefore, the 2-*O*-methyl group may not be the binding site of VP4.

In these VP4-resistant and -sensitive strains, most of the observed nucleotide variation in the *wb** cluster led to silent mutations, showing the conservation of O-antigen biosynthesis genes. In this study, *manB* gene mutations occurred in three wild-type VP4-resistant strains, preventing VP4 adsorption. *manB* which is predicted to produce phosphomannomutase, as well as *manA*, *manC*, *manB*, and *gmd*, is involved in GDP-perosamine biosynthesis (56, 57). The *manB* of the natural strain 367 has only three amino acid mutations but not the gene deletion; no obvious change was found in its LPS staining (Fig. 2), and this strain still agglutinates with O1 antiserum. These observations suggest that small modifications or other changes have occurred in its O antigen, but these changes still effectively block the binding of VP4. Additionally, amino acid mutations occurred in three O-antigen biosynthesis genes in resistant strain 95001, including obvious mutations in genes *wbeE* and *wbeU*. Restoration of both genes demonstrated that its VP4 resistance mechanism results from an O-antigen deficiency.

Phages specifically recognize and infect a narrow range of hosts. The resistance mechanisms of bacteria have evolved in the multiple steps of phage infection. These strategies include blocking receptors or altering receptor structure to prevent phage adsorption, preventing phage DNA entry, digesting phage nucleic acid, inhibiting replication of the phage genome, causing abortive phage infection, etc. (15). In this study, we identified receptor genes and another gene (*recJ*) in a *V. cholerae* O1 strain that are related to VP4 infection, also showing that multiple mechanisms have evolved to provide VP4 resistance. Two other strains, V07-16 and V06-92, have the same amino acid mutations within their *wb** clusters as strain 367, and no obvious change was found in the LPS staining (Fig. 2), but complementation of *manB* did not restore the sensitivity to VP4 infection, suggesting that some other mechanism may be involved in VP4 resistance, possibly in a different step of infection. In fact, in addition to the O-antigen receptor genes, we found that *recJ* may also be related to VP4 infection, because complementing the transposon mutant of N16961 with intact *recJ* restored sensitivity to VP4 infection (data not shown). The *recJ* gene is recognized as being involved in base excision repair, mismatch repair, homologous recombination (58), and the rescue of arrested replication forks (59), and it is also the only 5′-3′ exonuclease specific for single-stranded DNA (ssDNA) in most prokaryotic species (60, 61). In *Acinetobacter baylyi*, the *recJ* DNase strongly suppresses short foreign DNA fragments from integrating into genomic DNA (62). The resistance may be related to its role in restriction of VP4 DNA. Further studies are necessary to clarify the VP4 resistance mechanism relevant to *recJ*.

Phage infection is host specific; different sensitivities to the phage of strains that belong to the same species and serogroups can be determined by their genetic differences, such as whether a receptor exists or not, different receptor structures of these strains, or other differences which play roles in multiple steps of phage infection. Understanding infection mechanisms of the typing phages can lead to the determination of genomic differences be-

tween phage-sensitive and -resistant strains. Additionally, the evolution of resistance mechanisms against phages may enhance the ability of bacteria to survive in various environments. The phage resistance may protect against phage predation in environment, emergence of new clones of pathogens, and subsequent epidemics. From the aspect of biology, studies have shown that phages in the environment may lead to the collapse of cholera epidemics because phage predation leads to the decline of the sensitive bacteria. Moreover, this process can also lead to the emergence of a new clone that is resistant to a phage (12, 13), although this change does not happen frequently in the wild (63). Therefore, it may be speculative to suggest that the strains that have VP4 resistance (similar to strain 367) have a stronger ability to survive in an environment containing VP4 phages.

In summary, we identified the O antigen as the receptor of the *V. cholerae* El Tor typing phage VP4, and we confirmed that mutations in the O-antigen biosynthesis genes were responsible for VP4 adsorption failure in some wild-type resistant strains. This may be due to interaction between and coevolution of phage and host bacteria. Moreover, the study of phage resistance mechanisms can also be a strategy employed to find genomic and functional genome differences between sensitive and resistant strains, not only differences in their receptors, but also differences in other functions for which the host bacteria have evolved, such as preventing phage DNA entry, blocking DNA replication, or causing abortive infection.

ACKNOWLEDGMENTS

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