

USER Friendly Cloning Coupled with Chitin-Based Natural Transformation Enables Rapid Mutagenesis of *Vibrio vulnificus*^{∇†}

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***Vibrio vulnificus* is a bacterial contaminant of shellfish and causes highly lethal sepsis and destructive wound infections. A definitive identification of virulence factors using the molecular version of Koch's postulates has been hindered because of difficulties in performing molecular genetic analysis of this opportunistic pathogen. For example, conjugation is required to introduce plasmid DNA, and allelic exchange suicide vectors that rely on sucrose sensitivity for counterselection are not efficient. We therefore incorporated USER friendly cloning techniques into pCVD442-based allelic exchange suicide vectors and other expression vectors to enable the rapid and efficient capture of PCR amplicons. Upstream and downstream DNA sequences flanking genes targeted for deletion were cloned together in a single step. Based on results from *Vibrio cholerae*, we determined that *V. vulnificus* becomes naturally transformable with linear DNA during growth on chitin in the form of crab shells. By combining USER friendly cloning and chitin-based transformation, we rapidly and efficiently produced targeted deletions in *V. vulnificus*, bypassing the need for two-step, suicide vector-mediated allelic exchange. These methods were used to examine the roles of two flagellin loci (*flaCDE* and *flaFBA*), the *motAB* genes, and the *cheY-3* gene in motility and to create deletions of *rtxC*, *rtxA1*, and *fadR*. Additionally, chitin-based transformation was useful in moving antibiotic resistance-labeled mutations between *V. vulnificus* strains by simply coculturing the strains on crab shells. The methods and genetic tools that we developed should be of general use to those performing molecular genetic analysis and manipulation of other gram-negative bacteria.**

Vibrio vulnificus is a halophilic bacterium present naturally in estuarine waters and often contaminates oysters and other shellfish (for a review, see reference 15). *V. vulnificus* is an opportunistic pathogen of humans, causing primary septicemia and wound infection in susceptible individuals, and is the leading cause of reported seafood-related deaths in the United States. In susceptible humans, *V. vulnificus* causes a rapid, fulminating disease process resulting in extensive tissue damage. Mortality rates for susceptible individuals who develop fulminating primary septicemia are greater than 50% (17). Skin infections can lead to severe cellulitis, necrotizing fasciitis, and myositis requiring surgical debridement of infected tissues or amputation of the limb (4, 29, 42). Therapeutic intervention is often difficult since death can occur in less than 24 h after contact with the bacteria. In a mouse model of infection, *V. vulnificus* replicates extremely rapidly in host tissues (40, 41) and kills host cells including neutrophils (41).

Over 20 years of genetic analysis, only a few virulence factors have been identified and confirmed by using the molecular version of Koch's postulates (15). Among the confirmed virulence factors are capsular polysaccharide (49), acquisition of iron (34, 52), type IV pilus (37), RTX toxins (21, 25, 30), and flagella (22, 26). Despite these advances, the full spectrum of virulence factors responsible for the rapid and destructive dis-

ease process has not been elucidated. A major hindrance to the molecular genetic analysis of *V. vulnificus* is the fact that the bacteria cannot be effectively electroporated, nor can the bacteria be chemically transformed. Therefore, the only effective means of introducing plasmid DNA is by conjugation. This limitation severely restricts the availability of plasmids for creating and complementing mutations for molecular genetic analysis.

A classical method to create mutants of *V. vulnificus* is the use of suicide vectors containing transposons, such as Tn*phoA* (31), mini-Tn5*phoA* (12), mini-Tn5*Km2* (12), mini-Tn5*lacZ1* (12), mini-Tn10/*kan* (23), and *Himar* (1). However, transposon mutagenesis can cause polar mutations and truncated genes. The most definitive genetic analyses can be performed by deleting the genes of interest. The standard method for deleting genes is to clone the flanking upstream and downstream sequences into an allelic exchange suicide vector and introducing the plasmid into the target strain. The suicide vector integrates into the target genome via one of the flanking DNA sequences by a single-crossover event. This process is selected using antibiotic resistance encoded on the vector. The single-crossover strain is then grown without selection to allow for a second crossover that excises the allelic exchange vector. The excision event is enriched using a counterselectable marker encoded on the allelic exchange vector. A commonly used counterselectable marker is sucrose sensitivity, encoded by the *sacB* gene (13). If the second crossover is in the same flanking sequence as the first, the wild-type genotype is restored; however, if the second crossover is in the opposite flanking sequence, the targeted gene is deleted.

This method of mutagenesis is problematic for *V. vulnificus*. First, one of the easiest ways to clone PCR amplicons is by TA

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TABLE 1. Bacterial strains used in this work

Strain	Relevant genotype or description ^a	Source or reference
<i>E. coli</i>		
S17-1 λ pir	RP4 2-Tc::Mu-Km::Tn7 <i>pro thi recA</i> HsdR ⁻ M ⁺ λ pir	39
Top10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
EC100Dpir ⁺	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i> <i>pir</i> ⁺ (DHFR)	Epicentre
<i>V. vulnificus</i>		
CMCP6	Wild type, biotype 1 clinical isolate	20
M06-24/O	Wild type, biotype 1 clinical isolate	49
CVD752	M06-24/O <i>wza</i> ::Tn <i>phoA</i>	48
FLA591	CMCP6 Δ <i>rtxC</i> ; constructed using pGTR265	This work
FLA614	CMCP6 Δ <i>fadR</i> :: <i>aph</i> ; constructed using pGTR2010	6
FLA674	CMCP6 Δ <i>motAB</i> :: <i>aph</i> ; constructed using pGTR1141	This work
FLA677	CMCP6 Δ <i>flaCDE</i> :: <i>aph</i> ; constructed using pGTR1136	This work
FLA680	CMCP6 Δ <i>flaFBA</i> :: <i>cat</i> ; constructed using pGTR1120	This work
FLA688	CMCP6 Δ <i>cheY-3</i> :: <i>aph</i> ; constructed using pGTR1171	This work
FLA711	Δ <i>flaCDE</i> :: <i>aph</i> Δ <i>flaFBA</i> :: <i>cat</i> ; constructed in FLA680 using pGTR1136 via conjugation and two-step allelic exchange mutagenesis	This work
FLA810	Δ <i>flaCDE</i> :: <i>aph</i> Δ <i>flaFBA</i> :: <i>cat</i> ; constructed using chitin transformation of genomic DNA from FLA677 into FLA680	This work
FLA900	CMCP6 Δ <i>rtxA1</i> :: <i>aph</i> ; constructed using pGTR274	This work
FLA1009	CMCP6 <i>wza</i> ::Tn <i>phoA</i> ; constructed by chitin transformation using genomic DNA of CVD752	This work

^a DHFR, dihydrofolate reductase.

topoisomerase-mediated ligation (TOPO TA cloning) with commercially available vectors such as pCR2.1 TOPO (Invitrogen, Carlsbad, CA). Unfortunately, these vectors are not mobilizable; hence, they cannot be used directly with *V. vulnificus*. Furthermore, there are no available TA topoisomerase allelic exchange vectors. Site-specific recombination vectors such as Gateway (Invitrogen) have facilitated PCR-mediated cloning and gene expression; however, none of the relevant vectors is mobilizable. Therefore, essentially all cloning for expression in or mutagenesis of *V. vulnificus* requires multiple subcloning steps. Second, sucrose sensitivity counterselection is not very efficient with *V. vulnificus*, as it is time-consuming and involves the screening of large numbers of colonies to find truly sucrose-resistant colonies. In some cases, *sacB* counterselection has completely failed. Another method for introducing mutations into target strains is the lambda phage red recombinase system (11). However, this procedure involves introducing additional helper plasmids into the target strain, and mobilizable helper plasmids have not been made. Finally, no generalized transducing phages and no methods for transformation have been described for *V. vulnificus*. Therefore, if one desires to move mutations between strains, the mutations must be cloned into suicide vectors, and the inefficient two-step allelic exchange process must be repeated for each strain.

We describe here the adaptation of USER (uracil-specific excision reagent) friendly cloning (3, 14) into allelic exchange and expression vectors that are commonly used with *V. vulnificus*. USER friendly cloning enables the creation of 3' overhangs in PCR amplicons by use of deoxyuridine in PCR primers and treatment with USER enzyme mix (uracil DNA glycosylase and DNA glycosylase-lyase endonuclease VIII). The overhangs are complementary to overhangs created in vectors. The method is very adaptable, with immense leeway in choosing the DNA sequences of the overhangs independent of

restriction enzyme cleavage sites. USER friendly cloning methods and vectors enable the rapid creation of upstream-downstream clones to delete genes or loci of interest. To alleviate inefficiencies of subcloning into *V. vulnificus* vectors, we created an array of USER friendly cloning allelic exchange and expression vectors that should be useful to many investigators.

Meibom et al. (32) recently described the ability of *Vibrio cholerae* to become naturally transformable during growth in the presence of chitin, as either chitohexose or crab shell fragments. We adapted their crab shell system and determined that *V. vulnificus* can also become naturally transformable during growth on chitin. The bacteria take up linear DNA; hence, as long as selectable markers are used, allelic exchange mutagenesis can be accomplished without the need for inefficient counterselection involving sucrose. We used these methods to create antibiotic resistance-marked deletions in *V. vulnificus* of the two loci encoding flagellins, *flaCDE* and *flaFBA*, and the genes encoding the following functions: the flagellar motor *motAB*; a critical element of signal transduction for chemotaxis, *cheY-3*; an RTX toxin, *rtxA1*; a putative RtxA-modifying enzyme, *rtxC*; and a regulator of fatty acid metabolism, *fadR*. These genetic tools and methods will facilitate the molecular genetic analysis of *V. vulnificus* as well as those of other gram-negative bacteria.

MATERIALS AND METHODS

Bacteria, plasmids, and oligonucleotide primers. The *Escherichia coli* and *V. vulnificus* strains used are listed in Table 1, plasmid constructs are listed in Table 2, and oligonucleotides are listed in Table 3.

Chemicals, media, and culture. Unless noted otherwise, components for media were obtained from Difco (Franklin Lakes, NJ), chemicals were obtained from Sigma (St. Louis, MO), DNA extraction and purification kits were obtained from Qiagen (Valencia, CA), molecular genetics enzymes were obtained from New England Biolabs (Ipswich, MA), and oligonucleotides were obtained from IDT (Coralville, IA). Bacteria were grown in Luria-Bertani broth containing

TABLE 2. Plasmids used in this work

Plasmid	Vector	Description	Source and/or reference(s)
pCOS5	pCOS5	Cloning vector containing <i>cat</i> , <i>oriT</i> , ColE1, and <i>oriV</i> ; Ap ^r Cm ^r	10
pCR2.1-TOPO	pCR2.1	TOPO cloning vector for capturing PCR products using TA cloning	Invitrogen
pCVD442	pCVD442	R6K <i>ori mob sacB bla</i> ; suicide vector for allelic exchange; Ap ^r	13
pNEB206A	pNEB206A	USER friendly cloning vector; Ap ^r	New England Biolabs; 3
pRK437	pRK437	Cloning vector containing <i>tetAR</i> and <i>lacZα</i> ; deletion derivative of pRK404	38
pUC19	pUC19	Cloning vector; <i>lacZα</i> Ap ^r	51
pUC4K	pUC4K	Cloning vector containing <i>aph</i> and multiple-cloning site of M13mp7	43, 44
pGTR265	pCVD442	Δ <i>rtxC</i> ; <i>rtxC</i> upstream and downstream PCR amplicons digested with NotI and then USER cloned into pGTR1113; Ap ^r	This work
pGTR272	pCVD442	Δ <i>rtxA1</i> ; <i>rtxA1</i> upstream and downstream PCR amplicons USER cloned into pGTR1129; Ap ^r	This work
pGTR274	pCVD442	Δ <i>rtxA1::aph</i> ; <i>aph</i> -NotI cassette from pGTR1114 made blunt ended with Klenow fragment and inserted into the SmaI site between the <i>rtxA1</i> upstream and downstream fragments of pGTR272; Ap ^r Km ^r	This work
pGTR1090	pCR2.1-TOPO	<i>flaCDE</i> upstream sequences PCR amplified with primers <i>flaCDE-up5'</i> and <i>flaCDE-up3'</i> TOPO TA cloned into pCR2.1-TOPO; Ap ^r	This work
pGTR1091	pCR2.1-TOPO	<i>flaCDE</i> downstream sequences PCR amplified with primers <i>flaCDE-down5'</i> and <i>flaCDE-down3'</i> TOPO TA cloned into pCR2.1-TOPO; Ap ^r	This work
pGTR1092	pCR2.1-TOPO	<i>flaFBA</i> upstream sequences PCR amplified with primers <i>flaFBA-up5'</i> and <i>flaFBA-up3'</i> TOPO TA cloned into pCR2.1-TOPO; Ap ^r	This work
pGTR1093	pCR2.1-TOPO	<i>flaFBA</i> downstream sequences PCR amplified with primers <i>flaFBA-down5'</i> and <i>flaFBA-down3'</i> TOPO TA cloned into pCR2.1-TOPO; Ap ^r	This work
pGTR1106	pCR2.1-TOPO	Δ <i>flaFBA</i> ; pGTR1093 digested with XhoI and NotI and ligated with <i>flaFBA</i> upstream sequences from pGTR1092 removed with SalI and NotI; Ap ^r	This work
pGTR1107	pCR2.1-TOPO	Δ <i>flaCDE</i> ; pGTR1091 digested with XhoI and NotI and ligated with <i>flaCDE</i> upstream sequences from pGTR1090 removed with SalI and NotI; Ap ^r	This work
pGTR1108	pCVD442	Δ <i>flaFBA</i> sequences from pGTR1106 removed with SstI and XbaI inserted into pCVD442 digested with XbaI and SstI; Ap ^r	This work
pGTR1111	pCVD442	pCVD442 XbaI-SmaI Ω <i>lacZα</i> ; <i>lacZα</i> from HaeII fragment from pUC19; Ap ^r	This work
pGTR1112	pCVD442	Δ <i>flaCDE</i> from pGTR1107 removed with SstI and XbaI inserted into pGTR1111 digested with XbaI and SstI; Ap ^r	This work
pGTR1113	pCVD442	pGTR1111 <i>lacZα</i> ::USER; pGTR1111 digested with SphI and SstI and ligated with USER friendly cloning fragment created with oligonucleotides pCVD442-USER Linker top and pCVD442-USER Linker bottom; Ap ^r	This work
pGTR1114	pCVD442	<i>aph</i> gene PCR amplified from pUC4K with primers <i>aph5'</i> USER and <i>aph3'</i> USER and USER cloned into pGTR1113; Ap ^r Km ^r	This work
pGTR1116	pCR2.1	<i>cat</i> gene PCR amplified from pCOS5 with primers <i>cat-5'</i> and <i>cat-3'</i> and TOPO-TA cloned into pCR2.1-TOPO; Ap ^r Km ^r Cm ^r	This work
pGTR1117	pCR2.1	<i>tetAR</i> genes PCR amplified from pRK437 with primers <i>tetAR5'</i> and <i>tetAR3'</i> and TOPO-TA cloned into pCR2.1-TOPO; Ap ^r Km ^r Tc ^r	This work
pGTR1120	pCVD442	Δ <i>flaFBA::cat</i> ; <i>cat</i> gene from pGTR1116 inserted into the NotI site between the <i>flaFBA</i> upstream and downstream sequences of pGTR1108; Ap ^r Cm ^r	This work
pGTR1128	pCVD442	pGTR1111 EcoRV Ω <i>tetAR</i> ; <i>tetAR</i> genes from pGTR1117 removed with NotI, made blunt ended with Klenow fragment, and cloned into pGTR1111 at EcoRV; Ap ^r Tc ^r	This work
pGTR1129	pCVD442	pGTR1113 EcoRV Ω <i>cat</i> ; <i>cat</i> gene from pGTR1116 removed with NotI, made blunt ended with Klenow fragment, and cloned into pGTR1113 at EcoRV; Ap ^r Cm ^r	This work
pGTR1130	pCVD442	pGTR1113 EcoRV Ω <i>tetAR</i> ; <i>tetAR</i> genes from pGTR1117 removed with NotI, made blunt ended with Klenow fragment, and cloned into pGTR1113 at EcoRV; Ap ^r Tc ^r	This work
pGTR1132	pCVD442	pGTR1111 EcoRV Ω <i>cat</i> ; <i>cat</i> gene from pGTR1116 removed with NotI, made blunt ended with Klenow fragment, and cloned into pGTR1111 at EcoRV; Ap ^r Cm ^r	This work
pGTR1133	pCVD442	pGTR1111 NdeI Ω <i>aph</i> ; <i>aph</i> gene removed from pUC4K with BamHI, made blunt ended with Klenow fragment, and inserted into pGTR1111 digested with NdeI and made blunt ended with Klenow fragment	This work
pGTR1136	pCVD442	Δ <i>flaCDE::aph</i> ; <i>aph</i> -NotI cassette from pGTR1114 inserted at the NotI site between the <i>flaCDE</i> upstream and downstream sequences of pGTR1112; Ap ^r Km ^r	This work
pGTR1137	pCVD442	Δ <i>motAB</i> crossover PCR product USER cloned into pGTR1113; Ap ^r	This work
pGTR1141	pCVD442	Δ <i>motAB::aph</i> ; <i>aph</i> gene removed from pGTR1114 with NotI and inserted at the NotI site of pGTR1137; Ap ^r Km ^r	This work
pGTR1144	pCVD442	pGTR1113 EcoRV Ω <i>aph</i> ; <i>aph</i> gene from pGTR1114 removed with NotI, made blunt ended with Klenow fragment, and cloned into pGTR1113 at EcoRV; Ap ^r Km ^r	This work
pGTR1147	pNEB206A	Δ <i>cheY-3</i> crossover PCR product USER cloned into pNEB206A; Ap ^r	This work
pGTR1157	pNEB206A	Δ <i>cheY-3::aph</i> ; <i>aph</i> gene from pGTR1114 inserted into the NotI site between the <i>cheY-3</i> upstream and downstream sequences of pGTR1147	This work
pGTR1160	pRK437	pRK437-USER; pRK437 digested with BamHI and HindIII and ligated with a USER friendly cloning fragment created with oligonucleotides pRK437-USER Linker top and pRK437-USER Linker bottom; Tc ^r	This work
pGTR1171	pCVD442	Δ <i>cheY-3::aph</i> ; Δ <i>cheY-3::aph</i> sequences removed from pGTR1157 with EcoRI, made blunt ended with Klenow fragment, and cloned into pGTR1111 digested with SmaI; Ap ^r Km ^r	This work
pGTR1200	pCOS5	pCOS5 digested with HindIII and ClaI, made blunt ended with Klenow fragment, and religated; Cm ^r	This work
pGTR1202	pCOS5	pCOS5: <i>lacZα</i> ; <i>lacZα</i> HaeII fragment from pUC19 made blunt ended with Klenow fragment and inserted in a reverse direction relative to those of <i>bla</i> and <i>cat</i> of pGTR1200, digested with BamHI and XbaI, and made blunt ended with Klenow fragment; Cm ^r	This work
pGTR1204	pCOS5	pCOS5: <i>lacZα</i> ::USER; 47-bp USER friendly sequences from pGTR1113 removed by digestion with HindIII and SstI and ligated into pGTR1202 digested with HindIII and SstI; Cm ^r	This work

0.85% (wt/vol) NaCl (LB-N) or on LB-N plates containing 1.5% (wt/vol) agar. Strains were stored at -70°C in LB-N with 35% (vol/vol) glycerol. Antibiotics were included in media at the following concentrations: ampicillin at 100 $\mu\text{g/ml}$, tetracycline at 12.5 $\mu\text{g/ml}$, chloramphenicol at 30 $\mu\text{g/ml}$, and kanamycin at 40

$\mu\text{g/ml}$ for *E. coli* and ampicillin at 10 $\mu\text{g/ml}$, tetracycline at 6.25 $\mu\text{g/ml}$, chloramphenicol at 5 $\mu\text{g/ml}$, and kanamycin at 100 $\mu\text{g/ml}$ for *V. vulnificus*. To select for *V. vulnificus* and against donor *E. coli* cells during filter-mating conjugations, VVM agar (8) or LB-N agar containing 10^5 U/ml colistin and appropriate

TABLE 3. Oligonucleotides used for PCRs

Primer and function	Sequence (5'–3') ^a
Antibiotic resistance	
cat-5'	GGCGGCCGCATGCCTGCAGGTCCAACCTTTGGCG
cat-3'	GGCGGCCGCTTACGCCCGCCCTGCCACTCATCGC
aph5'USER	<u>GGGAAAGUCGCGCCGCTTCTGATTAGAAAACTCATCGAGC</u>
aph3'USER	<u>GGAGACAUGCGGCCGCAAAGCCAGTTGTGTCTCAAATC</u>
tetAR-5'	GGCGGCCGCGCGTTCAATCGGACCAG
tetAR-3'	GGCGGCCGCTAGTGAGTGGGTTGCGCTCC
USER friendly constructions	
pCVD442-USER Linker top	CTGAGGGAAAGTCTAGATGTCTCCTCAGCATGAGCT
pCVD442-USER Linker bottom	CATGCTGAGGAGACATCTAGACTTTCCCTCAGCATG
pRK437-USER Linker top	AGCTTGGCTGAGGGAAAGTCTAGATGTCTCCTCAGCG
pRK437-USER Linker bottom	GATCCCCTGAGGAGACATCTAGACTTTCCCTCAGCCA
Flagellin deletion	
flaFBAup5'	GGTCGACAAAAACGCGCGGAAGTGGCG
flaFBAup3'	GGCGGCCGCGGCTTTGGATACTTATCGACCA
flaFBAdown5'	GGCGGCCGCGGTAGACAGTGAGCGTGCC
flaFBAdown3'	GGAGCTCCTGACGAGCTTGCGCCATT
flaCDEup5'	GGTCGACGTGAACATCGAAGATGTGCC
flaCDEup3'	GGCGGCCGCGCTCACAAAAACAAAAACGCTG
flaCDEdown5'	GGCGGCCGCTCTCTTTTCGAGTTCGCAAGC
flaCDEdown3'	GGAGCTCGTTGCAAGCTTCGCAAGCTCG
flaFBAup-out	TGATGGCACTGAAAAATCCGAAGC
flaFBAdown-out	CATTCATCTTTTCCACCATCTCAG
flaCDEup-out	ATTATCGACCTTTGTCTGGTGAG
flaCDEdown-out	TTGAATTCAATGAGAATTCACGCGC
Motility and chemotaxis deletion	
motABdown5'	TGTTTAGCGGCCGCGGATGGGCACTCCTCATGCTATTTCCG
motABdown3'	GGAGACAUCAGTTGATCCACCAAATTGTCC
motABup5'	GGGAAAGUTGGGTTGTGCAGTTTGACCG
motABup3'	CCATCCGCGGCCGCTAAACAGGGTACGTCAAGTTGAATAAAAG
cheYdown5'	TGTTTAGCGGCCGCGGATGGGAGATTACTTCGTCAGAGCTGC
cheYdown3'	GGAGACAUCAAAGGGCCTTGAATTCACTC
cheYup3'	CCCATCCGCGGCCGCTAAACATGTTTTAATCATTTTTGTGTCCAAG
cheYup5'	GGGAAAGUCGCGCCGCTAAAGGGCGCAAG
motABup-out	ATCCACCACCTTCAATCTTGATGG
motABdown-out	CGTCATCTAAAGCCAGATCACCG
cheYup-out	TCCAAGCGGGTATGATTGGCC
cheYdown-out	TTGAACTAAAAGATCTTCGATACG
RTX deletion	
rtxA1-down-3'	<u>GGGAAAGUCGCTTATGGCAACGGAATTTCG</u>
rtxA1-down-5'	<u>ACCCGGGUAACGAGGCACGAGTATAGAG</u>
rtxA1-up-3'	<u>ACCCGGGUTAAGCCAACTCTTCTTTAGGAG</u>
rtxA1-up-5'	<u>GGAGACAUGAGCTTGCAAGCGGAGAGTGA</u>
rtxC-up5'	<u>GGAGACAUTTGAGCTTGCAAGCGGAGAG</u>
rtxC-up3'	<u>CTTCTTTAGGGCGGCCGCTGCCCATAATCAACCAATATG</u>
rtxC-down5'	<u>GATATAGGGCAGAGCGGCCGCCCTAAAGAGTTTGGCTTATG</u>
rtxC-down 3'	<u>GGGAAAGUAGTACGGTCTAGTTTATTGCC</u>
RtxA1-deletion up	GAGCTTGCAAGCGGAGAGTGA
RtxA1-deletion down	CGCTTATGGCAACGGAATTTCG

^a Sequences in boldface type indicate engineered restriction enzyme sites, and underlined sequences indicate USER friendly ends for cloning.

antibiotics was used. Counterselection for the loss of suicide plasmids was done using LB-N plates containing 6% (wt/vol) sucrose.

sacB-assisted allelic exchange mutagenesis. pCVD442-based allelic exchange plasmids were electroporated into *E. coli* S17-1λpir cells for conjugation into *V. vulnificus* by filter mating (40). Transconjugants containing single crossovers of the allelic exchange plasmid integrated into the *V. vulnificus* genome were selected on VVM agar (8) or LB-N agar containing 10⁵ U/ml colistin and appropriate antibiotics. Our current protocol involves colistin and not VVM. The correct insertion of the plasmid was confirmed by PCR. Single-crossover strains were grown overnight for at least one passage in LB-N without selection for the vector but with selection for the antibiotic resistance of the mutation, if applicable. To enrich for cells in which a second crossover event occurred, the bacteria were plated onto LB-N agar containing 6% (wt/vol) sucrose and incubated at

25°C for 24 to 48 h. Sucrose-resistant colonies were passaged on LB-N-sucrose agar and screened for a loss of antibiotic resistance encoded by the vector. It was not uncommon to find colonies on LB-N-sucrose that were not truly sucrose resistant and that had maintained the allelic exchange vector. The correct allelic exchange of the mutant allele for the wild-type allele was confirmed by PCR.

Crab shell (chitin)-based transformation of *V. vulnificus*. Chitin-based natural transformation of *V. vulnificus* was performed as described previously by Meibom et al. (32). Carapaces from blue crabs (*Callinectes sapidus*) were washed in water, cut into approximately 1-cm² pieces, and autoclaved. Recipient *V. vulnificus* cultures were grown statically overnight in LB-N broth at room temperature. The cultures that were grown overnight were diluted 1:20 into fresh LB-N medium and grown with shaking at 37°C until an optical density at 600 nm of 0.4 was attained. The bacteria were washed once with filter-sterilized seawater (Uni-

versity of Florida Whitney Laboratory for Marine Bioscience) diluted to a salinity of 25 ppt, which is typical for estuarine water. The washed bacteria were suspended in a volume of seawater with a salinity of 25 ppt that was twice the starting culture volume. Sterile crab shells were placed into wells of a 12-well tissue culture plate (Costar, Corning, NY) with 2 ml of bacteria in seawater and incubated overnight at 30°C. The following day, the supernatant was aspirated, and 2 ml of fresh seawater with a salinity 25 ppt was added along with 1 µg of linearized plasmid DNA or 2 µg of genomic DNA. Plasmid DNA was linearized either by digestion with an enzyme that cut into the vector opposite the insert sequences (e.g., NdeI for pCVD442-based vectors) or by vortexing in a microcentrifuge tube on a setting of high for 5 min. Genomic DNA was prepared using a Qiagen DNeasy blood and tissue kit, which shears genomic DNA into 15- to 20-kb fragments. The plates were incubated overnight at 30°C. The following day, the supernatants were removed, diluted, and plated onto LB-N agar with and without antibiotics. Crab shells were washed once with phosphate-buffered saline, placed into a 50-ml conical tube containing 2 ml of phosphate-buffered saline, and vortexed to release bacteria, and the supernatant was diluted and plated with and without antibiotics. Transformation frequencies were calculated as the ratio of the numbers of antibiotic-resistant CFU to total CFU.

Construction of pCVD442::lacZα and pCVD442::lacZα::USER. The 445-bp *lacZα* gene fragment from pUC19 was excised with HaeII, purified by extraction from an agarose gel, and made blunt ended by treatment with the Klenow fragment of DNA polymerase I (see Fig. S1 in the supplemental material). pCVD442 (13) was digested with XbaI, made blunt ended with the Klenow fragment of DNA polymerase, digested with SmaI to remove sequences containing additional restriction sites (XbaI and SmaI are in the multiple-cloning site of pCVD442), and ligated with the pUC19 *lacZα* fragment. Ligated DNA was electroporated into *E. coli* EC100Dpir⁺ cells (Epicentre, Madison, WI) with selection for ampicillin resistance and screening for blue colonies in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The correct insertion of *lacZα* into the XbaI-SmaI site was confirmed by DNA sequencing (University of Florida Interdisciplinary Center for Biotechnology Research). The resulting plasmid was named pGTR1111.

To enable the capture of USER-treated PCR amplicons, pNEB206A-derived DNA sequences were inserted into the *lacZα* gene fragment of pGTR1111. The USER friendly cloning sequences of pNEB206A (New England Biolabs) (3) were reconstructed by annealing oligonucleotides pCVD442-USER Linker top and pCVD442-USER Linker bottom to create SphI and SstI overhangs. The oligonucleotides were annealed by mixing equal molar concentrations, heating to 95°C for 5 min, and cooling slowly to 25°C. The USER friendly cloning linker contained a single XbaI site with Nt.BbvCI (formerly marketed as N.BbvCIB) sites 8 bases away on each side of the XbaI site. The 8-bp sequences between each Nt.BbvCI-XbaI pair matched the corresponding sequences of USER friendly vector pNEB206A (the left and right sequences are different to enable directional cloning and to prevent self-annealing). The SphI and SstI overhangs were used to clone the fragment into pGTR1111, which was digested with SphI and SstI. This construction inserted the USER friendly vector sequences into *lacZα* and maintained the *lacZα* reading frame, exactly as was the case for pNEB206A. The ligation mixture was electroporated into *E. coli* EC100Dpir⁺ with selection for ampicillin resistance and screening for blue colonies in the presence of X-gal. The correct insertion of the USER friendly vector sequences into the *lacZα* site was confirmed by DNA sequence analysis. The resulting plasmid was named pGTR1113.

To increase the usefulness of the pGTR1111 and pGTR1113 allelic exchange vectors in *V. vulnificus*, we incorporated additional antibiotic resistance genes into unique restriction sites of the plasmids. The *cat*, *aph*, and *tetAR* antibiotic resistance sequences were PCR amplified from pCOS5, pUC4K, and pRK437, respectively, using the oligonucleotides detailed in Table 3. The PCR products were captured into pCR2.1TOPO (Invitrogen) using TA cloning and were electroporated into *E. coli* TOP10 cells (Invitrogen). The oligonucleotide primers included NotI sites at each 5' end, so the resulting pCR2.1 clones were digested with NotI, and the antibiotic resistance cassettes were recovered from agarose gels. The NotI overhangs were filled in with the Klenow fragment of DNA polymerase I, and the blunt-ended antibiotic resistance cassettes were cloned into either EcoRV-digested or NdeI-digested Klenow fragment-treated plasmids. Ligated plasmids were electroporated into *E. coli* EC100Dpir⁺ cells, plating with selection for the appropriate antibiotic resistance. The following plasmids were created: pGTR1111EcoRV*tetAR* (pGTR1128), pGTR1111EcoRV*aph* (pGTR1132), pGTR1111NdeI*aph* (pGTR1133), pGTR1113EcoRV*cat* (pGTR1129), pGTR1113EcoRV*tetAR* (pGTR1130), and pGTR1113EcoRV*aph* (pGTR1144) (Table 2).

Construction of pRK437::USER and pCOS5::USER vectors. We constructed USER friendly cloning derivatives of pRK437 (38) and pCOS5 (10), which are

conjugable plasmids that we commonly use for cloning and expression for *V. vulnificus* (see Fig. S1 in the supplemental material). To insert USER friendly vector sequences into the *lacZα* gene of pRK437, oligonucleotides pRK437-USER Linker top and pRK437-USER Linker bottom (Table 3) were annealed. BamHI and HindIII restriction site overhangs were engineered into the ends to keep the USER friendly vector *lacZα* sequence in frame with the *lacZα* gene of pRK437. pRK437 was digested with BamHI and HindIII and ligated with the annealed oligonucleotides. The ligation product was electroporated into *E. coli* EC100Dpir⁺ cells with selection for tetracycline resistance and screening for blue colonies in the presence of X-gal. The correct DNA sequence was confirmed by DNA sequence analysis. pRK437::USER was named pGTR1160.

To increase the utility of pCOS5, we added the pUC19 *lacZα* gene and USER friendly cloning sequences. To destroy the adjacent HindIII and ClaI restriction sites so that HindIII could be used in subsequent derivatives, pCOS5 was sequentially digested with HindIII and ClaI, made blunt ended by treatment with the Klenow fragment of DNA polymerase I, and ligated, yielding pGTR1200. The *lacZα* HaeII fragment from pUC19 was made blunt ended with Klenow fragment and ligated into pGTR1200, which had been sequentially digested with XbaI and BamHI and made blunt ended with Klenow fragment, thereby destroying both of these sites. The HindIII, ClaI, XbaI, and BamHI sites were not in the coding sequences. This pCOS5::*lacZα* plasmid with *lacZα* in the orientation opposite to the flanking *bla* and *cat* genes was named pGTR1202. To enable the capture of USER-generated PCR amplicons, pGTR1202 was digested with SstI and HindIII, which cut within the *lacZα* gene, and ligated with the USER friendly vector sequences removed from the pGTR1113 *lacZα* gene by digestion with SstI and HindIII. The ligation mixture was electroporated into *E. coli* EC100Dpir⁺ cells with selection for chloramphenicol resistance and screening for blue colonies in the presence of X-gal. pCOS5::*lacZα*::USER was named pGTR1204, and the correct insertion of the USER friendly vector sequences was confirmed by DNA sequence analysis.

Deletion of *flaCDE* and *flaFBA* flagellin loci from *V. vulnificus* CMCP6. The 0.5-kb upstream and downstream sequences flanking the *flaCDE* and *flaFBA* loci were PCR amplified using primers *flaCDEup5'* and *flaCDEup3'*, *flaCDEdown5'* and *flaCDEdown3'*, *flaFBAup5'* and *flaFBAup3'*, and *flaFBAdown5'* and *flaFBAdown3'* (see Fig. S2 in the supplemental material). These 0.5-kb amplicons were individually cloned into pCR2.1TOPO, yielding plasmids pGTR1090, pGTR1091, pGTR1092, and pGTR1093, respectively. The upstream sequences were excised using SalI and NotI, which had been engineered in the oligonucleotide primers, and were cloned next to the respective downstream sequences whose plasmids had been digested with XhoI and NotI, yielding plasmids pGTR1106 for *flaFBA* and pGTR1107 for *flaCDE*. The *flaFBA* upstream-downstream DNA sequence (Δ *flaFBA*) was excised from pGTR1106 with SstI and XbaI and cloned into pCVD442, which was digested with the same enzymes, yielding pGTR1108. The *flaCDE* upstream-downstream DNA sequence (Δ *flaCDE*) was excised from pGTR1107 with SstI and XbaI and cloned into pGTR1111 (pCVD442::*lacZα*), which was digested with the same enzymes, yielding pGTR1112. pGTR1112 was digested with NotI, separating the upstream and downstream *flaCDE* sequences, and ligated with the NotI-*aph* fragment from pGTR1114. Similarly, the NotI *cat* fragment from pGTR1116 was inserted into the NotI site between the upstream and downstream sequences of Δ *flaFBA* of pGTR1108. The suicide vectors carrying Δ *flaCDE*::*aph* and Δ *flaFBA*::*cat* were named pGTR1136 and pGTR1120, respectively, and were electroporated into *E. coli* S17-1λpir⁺ cells for conjugation into *V. vulnificus*. The deletions were recombined into the chromosome of *V. vulnificus* CMCP6 by using the standard two-step sucrose-resistance-assisted allelic exchange method described above. The use of *aph* and *cat* resistance cassettes to label the deletion mutations facilitated the identification of the recombinants. The deletion of the *flaCDE* and *flaFBA* loci was confirmed by a series of PCRs using primers *flaCDEup-out/flaCDEdown-out* and *flaFBAup-out/flaFBAdown-out*. The Δ *flaCDE*::*aph* and Δ *flaFBA*::*cat* *V. vulnificus* mutants were named FLA677 and FLA680, respectively. A double flagellin deletion mutant was made by the deletion of *flaCDE* from FLA680 using pGTR1136 with the two-step allelic exchange procedure. The Δ *flaCDE*::*aph* Δ *flaFBA*::*cat* mutant was named FLA711.

USER friendly cloning of single PCR amplicons into USER friendly vectors. The method described for the USER Friendly cloning kit (New England Biolabs) was followed exactly. Specially constructed vectors, such as pNEB206A (pUC19 containing USER friendly vector sequences in the *lacZα* gene) (3), or the plasmids that we describe here were treated with XbaI to linearize the vector in the USER friendly cloning sequence, followed by Nt.BbvCI, which created two different 8-bp 3' overhangs. To create USER friendly compatible ends of PCR amplicons, a single deoxyuridine was included 8 bases from the 5' end of each oligonucleotide primer, and the DNA sequences preceding the deoxyuridine were complementary to one of the two USER friendly cloning sequences in the

vector, either GGAGACAU or GGGAAAGU (Table 3). *Taq* DNA polymerase was used for PCRs because most other DNA polymerases do not recognize deoxyuridine in the template (3). The PCR product was treated with USER enzyme mix (New England Biolabs), which contains uracil DNA glycosylase to remove the uracil residue, and DNA glycosylase-lyase endonuclease VIII, which nicks the deoxyuridine-containing strand, thereby releasing the terminal single-stranded DNA fragment and creating an 8-bp 3' overhang designed to be complementary to the vector. Because of the 8-bp complementary overhangs, ligation was performed with T4 DNA ligase for as short as 15 min at room temperature. Ligation reaction mixtures were electroporated into an appropriate *E. coli* host strain such as EC100Dpir⁺.

Deletion of *motAB* and *cheY-3* using crossover PCR. *motAB* and *cheY-3* deletions were constructed by crossover PCR in a single cloning reaction to create joined upstream-downstream DNA sequences flanking the genes of interest for subsequent deletion by allelic exchange mutagenesis. Crossover PCR was performed essentially as originally described by Horton et al. (18) and as modified by Link et al. (28). Approximately 500-bp upstream and downstream sequences of *motAB* and *cheY-3* were amplified by PCR. The inside primers (*motAB*up3'/*motAB*down5' and *cheY-3*up3'/*cheY-3*down5') that were used to join the upstream and downstream sequences had 21 bp of overlap including a *NotI* site (Table 3). The outside primers (*motAB*up5'/*motAB*down3' and *cheY-3*up5'/*cheY-3*down3') for cloning into the vector included the extra 8-base USER friendly cloning sites at their 5' ends that corresponded to those of vector pNEB206A. The products of the initial PCR amplifications (the respective upstream and downstream sequences) were mixed together and amplified a second time using only the outside primers (the 5' primer of the upstream sequence and the 3' primer of the downstream sequence, e.g., *motAB*up5' and *motAB*down3'). An annealing temperature was chosen to allow for the annealing of the upstream and downstream overlapping complementary sequences prior to amplification. The resulting combined upstream/downstream fragment was purified by agarose gel electrophoresis and cleaned using Zymo Clean & Concentrator (Zymo Research, Orange, CA). For the deletion of *motAB*, the crossover PCR product was cloned into pGTR1113 (pCVD442:*lacZ*α::USER) using the USER friendly cloning method, creating pGTR1137. The *aph*-*NotI* cassette from pGTR1114 was inserted into the *NotI* site between the upstream and downstream sequences of *motAB* in pGTR1137, creating pGTR1141 (Δ *motAB*::*aph*). The *cheY-3* deletion was constructed by crossover PCR similarly to the *motAB* deletion except that the upstream-downstream sequence for Δ *cheY-3* was initially constructed in pNEB206A, yielding pGTR1147, and the insertion of an *aph*-*NotI* cassette yielded pGTR1157. The *cheY-3* upstream-*aph* downstream sequence was excised from pGTR1157 with *EcoRI*, made blunt ended with Klenow fragment, and ligated into pGTR1111 (pCVD442:*lacZ*α), which was digested with *SmaI*. The allelic exchange plasmid for Δ *cheY-3*::*aph* was named pGTR1171. pGTR1141 and pGTR1171 were used with *sacB*-assisted two-step allelic exchange mutagenesis to create CMCP6 Δ *motAB*::*aph* and CMCP6 Δ *cheY-3*::*aph* mutants, named FLA674 and FLA688, respectively. The mutants were confirmed by PCR using primers designed to detect the expected changes in size at the *motAB* or *cheY-3* locus (*cheY*up-out/*cheY*down-out and *motAB*up-out/*motAB*down-out) (Table 3).

Deletion of *rtxC* using crossover PCR. The allelic exchange plasmid for the *rtxC* deletion was designed to be constructed by crossover PCR, as described above. Approximately 500 bp upstream and downstream of *rtxC* were amplified separately by PCR. Inside primers (*rtxC*up3' and *rtxC*down5') contained 33 bp of overlapping sequences including a *NotI* site (Table 3). The outside primers (*rtxC*up5' and *rtxC*down3') included the extra 8-base USER friendly cloning sites at the 5' ends that corresponded to the USER friendly cloning sites of vector pNEB206A. The second PCR, which was supposed to anneal the upstream and downstream fragments to form a single joined fragment, was unsuccessful for unknown reasons. As an alternative to crossover PCR, the upstream and downstream amplicons were digested with *NotI* to create compatible 5' overhangs and were cleaned using Zymo Clean & Concentrator. The two *NotI*-digested PCR products were treated for 15 min at 37°C with USER enzyme mix to create the 3' overhang sequences at the USER friendly cloning ends and were mixed with allelic exchange vector pGTR1113, which had been treated with *XbaI* and *Nt.BbvCI*. The reaction mixture was then incubated at 42°C to allow for annealing of the *NotI* overhangs and subsequently ligated by adding ligase buffer and T4 DNA ligase and incubating at room temperature for 15 min. The ligation mixture was electroporated into *E. coli* EC100Dpir⁺ cultures, which were plated onto LB-N medium containing ampicillin. The resulting plasmid, pGTR265, was used for two-step *sacB*-assisted allelic exchange mutagenesis to create the CMCP6 Δ *rtxC* mutant, which was named FLA591. The mutant was confirmed by PCR using outside primers *rtxC*up5' and *rtxC*down3' (Table 3).

Three-fragment USER friendly cloning. To clone upstream and downstream flanking DNA sequences together in a single step, the vector plasmid containing

pNEB206A USER friendly vector sequences was prepared for USER friendly cloning by treatment with *XbaI* and *Nt.BbvCI* to create the 8-bp overhangs. The oligonucleotides used to amplify the outside ends of the upstream and downstream flanking sequences included one of the USER friendly vector sequences. The inside ends of the upstream and downstream fragments that were to be joined together were amplified with oligonucleotides that created compatible USER friendly ends containing a restriction enzyme site, either *SmaI* or *NotI* (Table 3). For example, to create an *SmaI* USER friendly cloning end shared between the upstream and downstream sequences, the 5' ends of the primers were ACCCGGGU, followed by the sequences corresponding to the target DNA. The initial A is complementary to the U that is present in the opposing primer of the other fragment. After PCR amplification, treatment of the upstream and downstream PCR products with the USER enzyme for 15 min at 37°C generated three different pairs of complementary ends: the two different ends of the vector and the end shared between the upstream and downstream fragments. Therefore, there was only one way in which the vector and the two insert PCR amplicons could join to form a closed plasmid. The joining of the upstream and downstream fragments also created an *SmaI* site between them. The PCR products were mixed with the *XbaI*-*Nt.BbvCI*-digested USER friendly vector and were ligated by adding ligase buffer and T4 DNA ligase at room temperature for 15 min. Ligation reaction mixtures were electroporated into *E. coli* EC100Dpir⁺ cells with selection for the appropriate antibiotic resistance.

Deletion of *rtxA1* using three-fragment USER friendly cloning. To construct the allelic exchange plasmid to delete the 15.6-kb *V. vulnificus* *rtxA1* gene, 1.0-kb upstream and downstream sequences were amplified with the USER friendly cloning sequences complementary to pNEB206A on the outside ends (*rtxA1*up-5' and *rtxA1*down-3') (see Fig. S3 in the supplemental material). The inside ends where the upstream and downstream sequences would join were amplified with ACCCGGGU at the 5' end to create a common *SmaI* restriction site (*rtxA1*up-3' and *rtxA1*down-5') (Table 3). These upstream and downstream *rtxA1* fragments were cloned into USER friendly allelic exchange vector pGTR1129 exactly as described immediately above for three-fragment USER friendly cloning, yielding pGTR272 (Δ *rtxA1*). To enable the selection of *rtxA1* deletion mutants of *V. vulnificus*, the *aph*-*NotI* kanamycin resistance cassette from pGTR1114 was made blunt ended with Klenow fragment and cloned into the *SmaI* site between the upstream and downstream sequences of pGTR272, yielding plasmid pGTR274, carrying Δ *rtxA1*::*aph*. This allelic exchange plasmid was used to recombine the mutation into *V. vulnificus* CMCP6 in the two-step *sacB*-assisted allelic exchange process, yielding strain FLA900. The correct recombination events were confirmed by PCR using oligonucleotides *RtxA1*-deletion up and *RtxA1*-deletion down and Southern blot analyses.

Analysis of motility and flagella. Bacteria from a static culture grown overnight were stabbed into LB-N soft-agar plates containing 0.3% (wt/vol) agar, and the plates were incubated overnight at 37°C. The diameter of the zone of growth was measured after 15 to 18 h of incubation (consistent within each experiment). Triplicate plates were examined for each strain. The presence or absence of flagella was visualized by staining bacterial cells with Nano Orange (Invitrogen). Bacteria were removed from an LB-N soft-agar plate with a toothpick and mixed into 2 μ l of phosphate-buffered saline on a microscope slide. A total of 0.5 μ l of Nano Orange dye was mixed in with a pipette tip, and a coverslip was placed over the bacteria. The stained cells were examined at a \times 1,000 magnification under oil immersion using a Nikon Eclipse 600 fluorescence microscope with a Nikon B-2 E/C filter, which has an excitation filter wavelength of 465 to 495 nm and an emission filter wavelength of 515 to 535 nm. Photomicrographs were made using a Nikon DXM1200C charge-coupled-device camera.

Statistical analysis. The Student *t* test was used to determine significant differences between groups. Values were considered significant at *P* values of <0.05. All quantitative experiments were repeated at least once.

RESULTS

Construction of pCVD442:*lacZ*α and use in deleting flagellin loci from *V. vulnificus*. Because *V. vulnificus* possesses six flagellin genes, an important question is which of them is essential for the production of flagella and motility. We therefore deleted the two unlinked flagellin loci *flaCDE* and *flaFBA* separately and combined using the allelic exchange suicide vector pCVD442. To facilitate cloning into pCVD442 by blue-white screening for inserted DNA, we inserted the pUC19 *lacZ*α multiple-cloning site into pCVD442, yielding pGTR1111

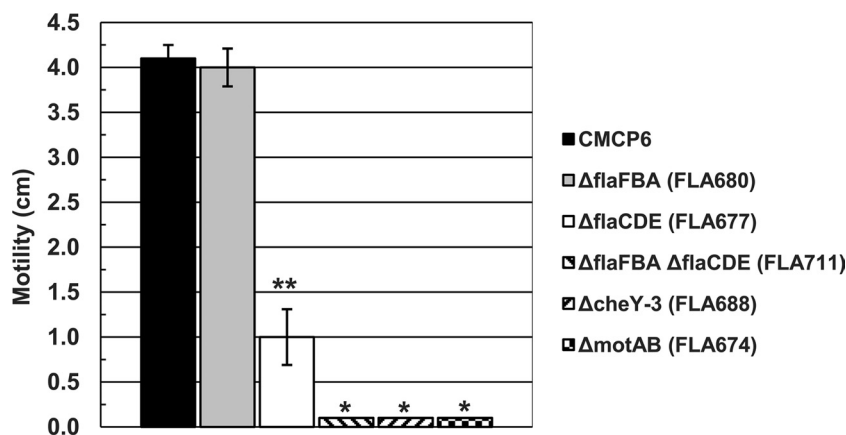


FIG. 1. Motilities of Δfla , $\Delta motAB$, and $\Delta cheY-3$ mutants. *V. vulnificus* strains were stabbed into soft LB-N agar, and the diameters of growth from motility after overnight growth were measured as described in Materials and Methods. Strains examined were wild-type strain CMCP6, $\Delta flaFBA::cat$ strain FLA680, $\Delta flaCDE::aph$ strain FLA677, $\Delta flaCDE::aph \Delta flaFBA::cat$ strain FLA711, $\Delta motAB::aph$ strain FLA674, and $\Delta cheY-3::aph$ strain FLA688. *, $P \leq 1 \times 10^{-5}$ compared with CMCP6 and FLA680 and $P = 0.007$ compared with FLA677; **, $P \leq 0.0002$ compared with CMCP6 and FLA680 ($n = 3$).

(see Fig. S1 in the supplemental material). As described in Materials and Methods (see Fig. S2 in the supplemental material), upstream and downstream sequences flanking the *flaCDE* and *flaFBA* loci were individually cloned into pCR2.1TOPO, and the upstream sequences were subsequently combined with the downstream sequences. These $\Delta flaCDE$ and $\Delta flaFBA$ sequences were then subcloned into pGTR1111 and pCVD442, respectively, and were conferred with different antibiotic resistance genes to facilitate subsequent genetic manipulations, yielding $\Delta flaCDE::aph$ in pGTR1136 and $\Delta flaFBA::cat$ in pGTR1120. The *lacZ α* multiple-cloning site of pGTR1111 made the cloning of the $\Delta flaCDE$ sequences more straightforward. The deletions were recombined into the chromosome of *V. vulnificus* CMCP6 by using the two-step allelic exchange method involving the selection of a single-crossover integration event followed by sucrose resistance-mediated counterselection for the excision of the vector by a second crossover event, as described in Materials and Methods. *V. vulnificus* CMCP6 $\Delta flaCDE::aph$ and *V. vulnificus* $\Delta flaFBA::cat$ strains were named FLA677 and FLA680, respectively. pGTR1136 was similarly used to create a $\Delta flaCDE::aph$ mutation in the $\Delta flaFBA::cat$ background of FLA680, yielding a double flagellin locus mutant ($\Delta flaCDE::aph \Delta flaFBA::cat$) named FLA711.

The deletion of *flaFBA* had no significant effect on motility (Fig. 1) or the presence of flagella (Fig. 2). In contrast, the deletion of *flaCDE* significantly decreased but did not abolish motility, and the bacteria were still flagellated. As expected, the $\Delta flaCDE::aph \Delta flaFBA::cat$ double mutant was nonmotile and nonflagellated. Therefore, the *flaFBA* locus appeared to be completely dispensable for motility in vitro but could partially compensate for the deletion of the *flaCDE* locus, which had the greater effect on motility in vitro. These flagellin deletion constructions demonstrated the usefulness of incorporating the *lacZ α* -complementing sequences into allelic exchange suicide vector pCVD442 but also exemplified the tedious nature of constructing deletion mutations by separately cloning upstream and downstream fragments into TOPO TA vectors.

Construction of USER friendly vectors for use with *V. vulnificus*. TOPO TA cloning and Gateway cloning (Invitrogen) are very useful tools for cloning of PCR-generated DNA sequences. Unfortunately, in our studies of *V. vulnificus*, none of the commercially available vectors was directly useful because they were not mobilizable. However, the USER Friendly cloning system (New England Biolabs) appeared to be a suitable alternative method of efficiently capturing PCR products. Any plasmid vector can be converted into a USER friendly vector simply by the inclusion of short DNA sequences. Capturing PCR amplicons into USER friendly cloning vectors simply involves the inclusion of 8 nucleotides at the 5' end of the oligonucleotides for PCR with a deoxyuridine in the place of a thymidine at the eighth nucleotide. USER friendly cloning vector pNEB206A is a pUC19 derivative (3) and is not mobilizable in *V. vulnificus*. We therefore constructed USER friendly derivatives of the two most useful cloning vectors for our work with *V. vulnificus*, pCOS5 (10) and pRK437 (38), yielding plasmids pGTR1204 and pGTR1160, respectively (see Materials and Methods; also see Fig. S1 in the supplemental material). Similarly, we constructed a USER friendly derivative of vector pCVD442:*lacZ α* , pGTR1111, yielding pGTR1113 (see Fig. S1 in the supplemental material). Because selection for ampicillin resistance is inefficient for working with *V. vulnificus*, we also constructed chloramphenicol-, kanamycin-, and tetracycline-resistant derivatives of pGTR1113, named pGTR1129, pGTR1144, and pGTR1130, respectively (Table 2; see Fig. S1 in the supplemental material).

Use of crossover PCR and USER friendly cloning to construct *motAB* and *cheY-3* deletions. The *motAB* genes (VV1_0312 and VV1_0311) are the only genes in the *V. vulnificus* CMCP6 genome annotated as encoding the motor complex for flagella. To confirm that these adjacent genes are essential for motility, we targeted VV1_0312 and VV1_0311 for deletion. In contrast, there are 10 genes annotated as “*cheY*” or “*cheY*-like receiver” in the *V. vulnificus* genome. In the *V. cholerae* N16961 genome, among the four genes annotated as *cheY*, the VC2_065 gene, annotated as *cheY-3*, has been shown to be essential for mediating chemotaxis (19, 27).

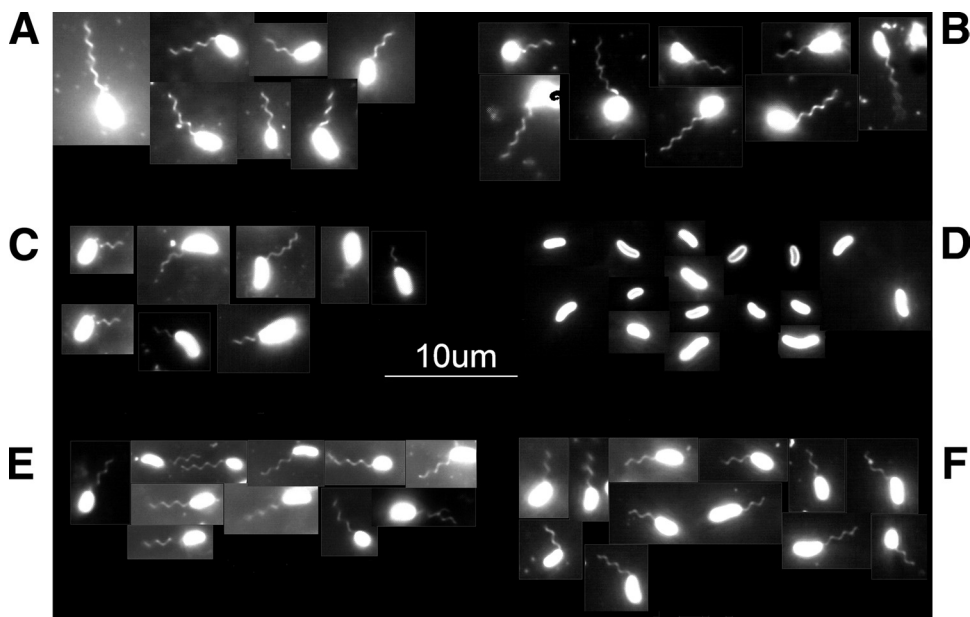


FIG. 2. Flagellation of Δfla , $\Delta motAB$, and $\Delta cheY-3$ mutants. Bacteria from LB-N soft-agar plates were stained with Nano Orange and examined using epifluorescence at a $\times 1,000$ magnification as described in Materials and Methods. (A) Wild-type CMCP6; (B) $\Delta flaFBA::cat$ strain FLA680; (C) $\Delta flaCDE::aph$ strain FLA677; (D) $\Delta flaCDE::aph \Delta flaFBA::cat$ strain FLA810; (E) $\Delta motAB::aph$ strain FLA674; (F) $\Delta cheY-3::aph$ strain FLA688.

The protein encoded by the VV1_1953 gene of the *V. vulnificus* CMCP6 genome has the highest homology to the *V. cholerae* CheY-3 protein, so we named VV1_1953 *cheY-3*. To determine if *cheY-3* is essential for the chemotaxis of *V. vulnificus*, we targeted VV1_1953 for deletion.

To delete *motAB* and *cheY-3*, rather than cloning the upstream and downstream flanking sequences separately and then combining them into the allelic exchange suicide vector, as was done for the *flaCDE* and *flaFBA* loci, we used crossover PCR (28) to amplify the joined upstream and downstream sequences and clone them in a single ligation, as described in Materials and Methods. The $\Delta motAB$ sequences were USER cloned directly into the *sacB* allelic exchange plasmid pGTR1113, and an *aph*-NotI cassette was inserted between the upstream and downstream sequences, yielding pGTR1141. In contrast, the $\Delta cheY-3$ sequences from crossover PCR were first USER cloned into pNEB206A, and an *aph* cassette was then inserted between the upstream and downstream sequences. $\Delta cheY-3::aph$ sequences were subcloned into allelic exchange vector pGTR1111, yielding pGTR1171. The $\Delta motAB::aph$ and $\Delta cheY-3::aph$ mutations were recombined into the *V. vulnificus* CMCP6 genome using the two-step allelic exchange sucrose resistance-assisted procedure described in Materials and Methods, yielding strains FLA674 and FLA688, respectively. The mutations were confirmed by PCR (data not shown).

As expected, the $\Delta motAB::aph$ mutation resulted in a complete lack of motility of *V. vulnificus* (Fig. 1) but did not affect the presence of flagella (Fig. 2). When stabbed into motility agar plates, $\Delta cheY-3::aph$ strain FLA688 had the same appearance as $\Delta motAB::aph$ strain FLA674, i.e., no spreading (Fig. 1), which is consistent with a nonchemotactic phenotype (7). To confirm that FLA688 was motile, we examined bacteria from the motility agar in a wet preparation under bright-field mi-

croscopy. As opposed to the swimming-and-tumbling behavior of parental strain CMCP6, the $\Delta cheY-3::aph$ mutant constantly swam but did not tumble (data not shown). Furthermore, the $\Delta cheY-3::aph$ mutant possessed flagella (Fig. 2). These constructions demonstrated the usefulness of USER friendly cloning in capturing PCR-generated DNA sequences into both commercially generated and laboratory-generated vectors as well as the potential usefulness of crossover PCR to clone upstream and downstream flanking sequences in a single step.

Three-part USER friendly cloning is a highly efficient method to produce allelic exchange plasmids. In using crossover PCR to generate the allelic exchange plasmids described above, we experienced a great difficulty in obtaining sufficient yields of the combined upstream-downstream fragment during the second PCR. As part of a study examining the roles of RTX toxins of *V. vulnificus* in virulence, we wanted to delete the *rtxC* gene (VV2_0480) associated with the *rtxA1* locus of *V. vulnificus* CMCP6. As was done for *motAB* and *cheY-3*, the upstream and downstream sequences of *rtxC* were amplified individually and then mixed for crossover PCR. However, we were unable to amplify a crossover PCR product joining the upstream and downstream sequences. Because we had included a NotI restriction site in the common sequences between the upstream and downstream fragments for crossover PCR, we reasoned that we could digest the PCR products with NotI and the USER enzyme mix to create three sets of complementary overhangs (viz., the left and right USER friendly vector sequences and the NotI sequence). This would enable the cloning of the PCR products into USER friendly allelic exchange vector pGTR1129. The three-part cloning reaction yielded allelic exchange plasmid pGTR265, carrying $\Delta rtxC$. pGTR265 was used to recombine $\Delta rtxC$ into the genome of *V. vulnificus* CMCP6 by the two-step *sacB*-assisted process, yield-

ing FLA591, without the use of an added antibiotic resistance gene cassette between the upstream and downstream sequences. In agreement with data described previously by Liu et al. (30), FLA591 was not attenuated for virulence in subcutaneously inoculated, iron dextran-treated mice (J. L. Joseph et al., unpublished data). This construction demonstrated the usefulness of combining USER friendly cloning with engineered restriction sites in accomplishing three-part cloning.

Considering the success of the three-part cloning by combining USER friendly sequences on the outside ends and a common restriction site on the inside ends of the upstream and downstream sequences, we reasoned that an additional unique USER friendly sequence could be constructed into the sequence common to the upstream and downstream fragments. This is possible because the principal aspect of USER friendly cloning is the incorporation of a deoxyuridine into the oligonucleotides used for PCR. As long as the oligonucleotides to be joined in a USER friendly cloning reaction have a complementary sequence 5' to the deoxyuridine so as to generate compatible overhangs after USER enzyme treatment, essentially any DNA sequence can be used. The advantage of designing USER friendly sequences at all three junctions (vector-upstream, upstream-downstream, and downstream-vector) is that only a single enzyme treatment, the USER enzyme mix, is required to generate the overhangs. We used this strategy to create a $\Delta rtxA1::aph$ mutation in *V. vulnificus* CMCP6, as described in Materials and Methods (see Fig. S3 in the supplemental material), and a $\Delta fadR::aph$ mutation (6). The deletion of the 15.6-kb *rtxA1* gene was confirmed by PCR (Fig. 3) and Southern blot (data not shown) analyses. In agreement with the results described previously by others (21, 25, 30), the $\Delta rtxA1::aph$ mutant was attenuated for virulence in mice and cytotoxicity in cell culture (Joseph et al., unpublished). The $\Delta fadR::aph$ mutant was extremely attenuated for virulence in iron dextran-treated mice (6). In our experience, three-part USER friendly cloning was the most efficient method for generating the allelic exchange plasmids containing the joined upstream-downstream fragments for deletional mutagenesis.

Crab shell (chitin)-mediated natural transformation of *V. vulnificus*. The studies described above detail our development of methods to capture PCR-generated DNA sequences to construct allelic exchange plasmids to create deletions and insertions in *V. vulnificus* using pCVD442-derived vectors. A central aspect of using such vectors is the *sacB*-encoded susceptibility to sucrose in gram-negative bacteria as a counterselection against the vector to select for the second crossover event (13). However, we and our colleagues have found that sucrose selection is not as effective for *V. vulnificus* as it is for *V. cholerae* and other gram-negative bacteria (e.g., in our experience, *E. coli* and *Salmonella enterica* serovar Typhimurium) (46). Most often, in spite of our attempts at optimizing the system, colonies growing on the initial sucrose-containing plates were not truly sucrose resistant. An alternative to plasmid-mediated two-step allelic exchange would be to have the recipient bacteria take up linear sequences of DNA encoding the mutation labeled with a selectable marker so that the only way that the marker could be acquired would be by a double-crossover event and marker rescue. Meibom et al. (32) previously identified and characterized the ability of *V. cholerae* to become competent for transformation with linear DNA during growth

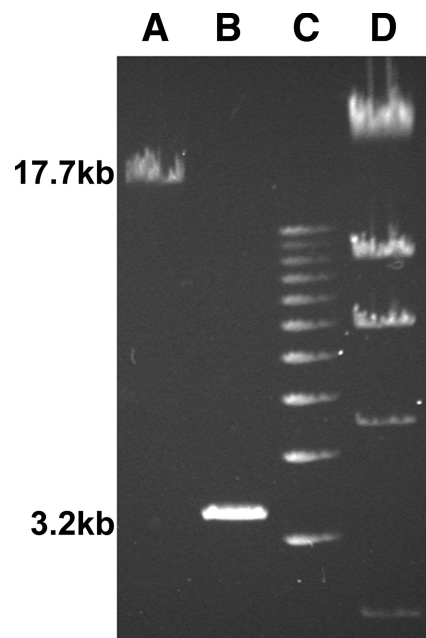


FIG. 3. PCR confirmation of the deletion of 15.6-kb *rtxA1* and creation of $\Delta rtxA1::aph$. Genomic DNAs from *V. vulnificus* CMCP6 (lane A) and $\Delta rtxA1::aph$ strain FLA900 (lane B) were PCR amplified using oligonucleotide primers *rtxA1*-deletion-up and *rtxA1*-deletion-down, which amplify 1 kb upstream and downstream of the start and stop codons of the *rtxA1*. The sizes of the products are shown to the left, based on the 1-kb ladder (lane C) and lambda phage-HindIII digestion (lane D). Southern blot analysis also confirmed the absence of *rtxA1* sequences (data not shown).

in the presence of chitin. Chitin could be provided as a soluble substrate or by growing the bacteria in the presence of crab shells. We therefore attempted to reproduce the results from *V. cholerae* with *V. vulnificus*.

As described in Materials and Methods, *V. vulnificus* CMCP6 was grown in diluted seawater in the presence of a fragment of autoclaved crab shell. We initially tested the chitin transformation system by reconstructing a $\Delta flaCDE::aph$ $\Delta flaFBA::cat$ double mutant strain. FLA680, which contains the $\Delta flaFBA::cat$ mutation, was chosen as the recipient strain, and allelic exchange plasmid pGTR1136 was the donor of the $\Delta flaCDE::aph$ mutation. When intact pGTR1136 was added to the *V. vulnificus* culture growing with the crab shell, no kanamycin-resistant, chloramphenicol-resistant recombinants were obtained. However, when pGTR1136 was sheared by sonication or linearized by digestion with EcoRV, the addition of 1 μ g of DNA to the crab shell culture yielded kanamycin-resistant, chloramphenicol-resistant recombinants with a frequency of 1.2×10^{-7} . Apparently, only linear DNA is capable of being taken up by the chitin-competent *V. vulnificus* recipients. We confirmed this hypothesis by attempting to transform uncut vector plasmids pUC19, pRK437, and pCOS5 into *V. vulnificus*, and we failed to obtain any antibiotic-resistant isolates. We also examined if the linear fragment of DNA encoding the homologous flanking sequences and internal antibiotic resistance cassette for chitin transformation could be obtained directly by PCR amplification without first being cloned into a plasmid vector. When we used the same PCR amplicons that

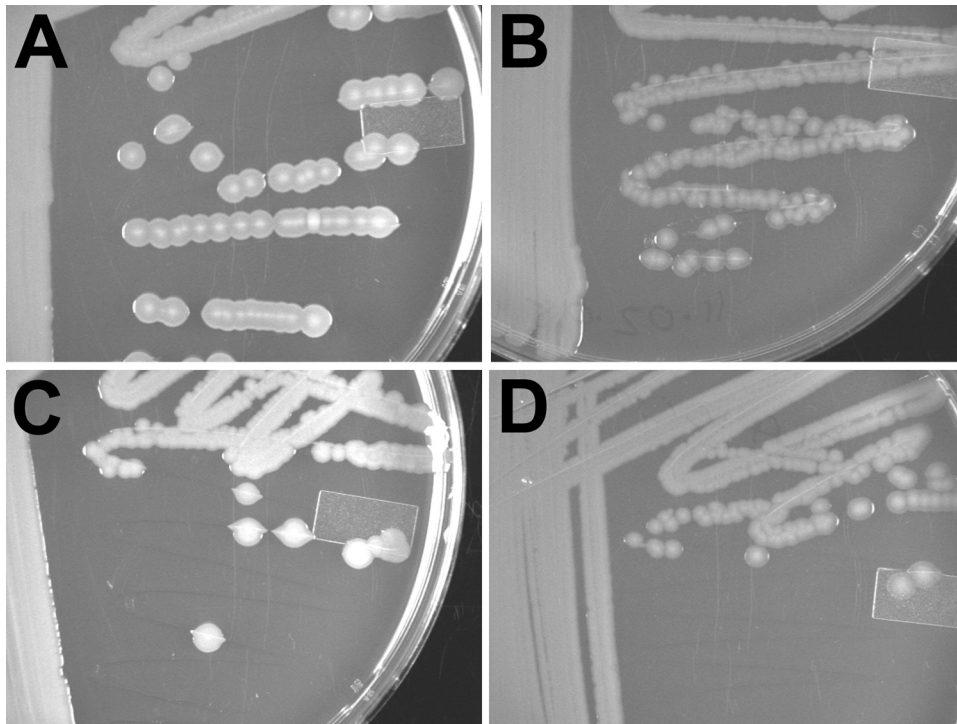


FIG. 4. Chitin transformation of *wza::TnphoA* from *V. vulnificus* CVD752 into CMCP6 yields a translucent-colony morphotype. Genomic DNA from CVD752 (M06-24/O *wza::TnphoA*) was chitin transformed into CMCP6, selecting for kanamycin resistance and yielding FLA1009. Kanamycin-resistant colonies acquired the translucent-colony morphotype (B and D). (A) M06-24/O; (B) CVD752; (C) CMCP6; (D) FLA1009.

had been cloned into the suicide allelic exchange vectors for chitin-based transformation, the frequencies of recombinants were much lower and sometimes were undetectable (data not shown). Given that the lengths of the homologous flanking sequences were the same in the linearized allelic exchange plasmids and the PCR-generated fragments, it appeared that the length of total flanking DNA is critical for determining transformation efficiency, possibly because of the exonuclease activity of *V. vulnificus* (50).

Given the optimal efficiency of chitin transformation with longer linear pieces of DNA, we considered the possibility that chitin-based transformation could be used to move mutations with selectable markers between *V. vulnificus* strains. We tested this by moving the Δ *flaCDE::aph* mutation of FLA677 into Δ *flaFBA::cat* strain FLA680 to create a double deletion of the flagellin loci. Two micrograms of genomic DNA from FLA677, either untreated or sheared by sonication, was added to a FLA680 culture growing on a crab shell. Kanamycin-resistant recombinants were readily obtained and were confirmed by PCR. The phenotype of the double flagellin deletion strain FLA810 was confirmed by the lack of motility (data not shown) and the lack of flagella (Fig. 2). This genomic DNA-mediated recombination was performed using the same strain CMCP6 background for the donor and recipient strains. To determine if chitin-based transformation could be accomplished between different strain backgrounds, we moved the *wza::TnphoA* mutation of *V. vulnificus* M06-24/O derivative CVD752 (48, 49) into CMCP6. The *wza* gene encodes an essential component of capsule biosynthesis, and the mutation of *wza* would be expected to yield a translucent-colony mor-

photype. Genomic DNA from CVD752 was added to CMCP6 cells growing on a crab shell, and recombinants were selected by plating onto LB-N agar with kanamycin. The *wza::TnphoA* phenotype of the resulting strain, FLA1009, was obvious by the translucent colonies, which no longer expressed capsular polysaccharide (Fig. 4). Furthermore, FLA1009 was significantly attenuated for virulence in subcutaneously inoculated iron dextran-treated mice (6a). These results demonstrated that chitin transformation is useful for moving selectable mutations between *V. vulnificus* strains.

Cocultivation of two *V. vulnificus* strains on crab shell enables chitin-based transformation. Meibom et al. (32) previously reported that chitin-based transformation could be accomplished by simply mixing two *V. cholerae* strains together during growth on crab shells. Apparently, sufficient DNA is released in the coculture to enable chitin-based transformation. To examine if coculture could mediate the chitin-based transformation of *V. vulnificus*, Δ *flaCDE::aph* strain FLA677 and Δ *flaFBA::cat* strain FLA680 were mixed for growth overnight on crab shells. The next day, the frequency of kanamycin- and chloramphenicol-resistant CFU was 2.3×10^{-9} . Eighteen colonies were verified to be nonmotile by stabbing into motility soft-agar plates. To determine if increasing the incubation time could increase transformation frequencies by possibly causing a greater release of genomic DNA and/or an increase in competency, we sampled the coculture for up to 3 days. The transformation frequencies on days 2 and 3 were 6.0×10^{-8} and 3.4×10^{-7} , respectively. Therefore, it appears that *V. vulnificus* can exchange genomic DNA during growth in the presence of

chitin, and an increased incubation time increases the frequency of recombination.

DISCUSSION

We report here a progression of the development of genetic methods that have vastly improved our ability to manipulate the genome of *V. vulnificus*. Many of the methods and certainly most of the plasmid vectors should be useful to others examining other gram-negative bacteria. Most trivially, we produced derivatives of commonly used plasmid vectors to improve their usefulness, such as the inclusion of *lacZ* α into pCVD442 (13) and pCOS5 (10) to enable blue-white screening of the insertion of cloned DNA sequences using appropriate *E. coli* hosts and the inclusion of alternative antibiotic resistance markers (Table 2; see Fig. S1 in the supplemental material). However, since our preferred method of constructing mutations in specific genes or loci is allelic exchange mediated by cloning and joining sequences upstream and downstream of the targeted locus, we examined methods to improve the efficiency of cloning of dual fragments of DNA.

The greatest obstacle in our molecular genetic manipulation is the fact that the transformation and electroporation of intact plasmids into *V. vulnificus* are difficult, if not impossible. Therefore, all of the plasmids that we use for genetic manipulations must be mobilizable for transfer by conjugation. One of the most efficient ways to clone PCR-amplified DNA fragments is TA cloning coupled with topoisomerase ligation (Invitrogen). Unfortunately, none of the commercially available TOPO TA vectors, such as pCR2.1 TOPO, are mobilizable. Therefore, as described above for the deletions of *flaCDE* and *flaFBA*, we TOPO TA cloned the upstream and downstream sequences separately into pCR2.1 TOPO, followed by the joining of the fragments into a pCR2.1-vectored clone. This was cumbersome and time-consuming (see Fig. S2 in the supplemental material). These difficulties led us to refine cloning methods for PCR-generated products. Converting our commonly used vectors to TOPO TA vectors was not feasible. Upon investigating alternative means to capture PCR amplicons, we found USER friendly cloning from New England Biolabs (3). USER friendly cloning had several advantages for our use. First, by simply incorporating specific deoxyuridine-containing nucleotide sequences into PCR primers, we generated 3' overhangs compatible with vector pNEB206A for efficient cloning. Second, we constructed USER friendly cloning *lacZ* α derivatives of our most useful expression and allelic exchange vectors, pRK437, pCOS5, and pCVD442 (Table 2; see Fig. S1 in the supplemental material). Therefore, we could capture upstream and downstream sequences for mutagenesis and entire genes for complementation or reversion.

To create the upstream-downstream deletion cassettes in a single cloning step, we first examined crossover PCR (18, 28). We had some success by coupling crossover PCR with USER friendly cloning to create deletions of *motAB* and *cheY-3*; however, the low fidelity of obtaining the joined upstream and downstream fragments by crossover PCR was problematic. We were unsuccessful in using crossover PCR to generate the deletion construct for the *rtxC* gene, which encodes a putative acyl transferase that is required in other bacteria for the modification and functional activity of associated RtxA toxins (45).

We do not know why crossover PCR failed in this attempt, but the inconsistency of the method was noted previously by others (3). Therefore, we took advantage of the fact that we had engineered NotI sites into the overlapping sequences for crossover PCR between the upstream and downstream *rtxC* sequences. By treating the fragments with NotI and then performing a three-part ligation using the USER friendly ends of the fragments to join to the vector and the NotI ends between the insert fragments, we constructed the desired upstream-downstream deletion clone for *rtxC* in our USER friendly vector pGTR1129. We used *sacB*-assisted allelic exchange to recombine the Δ *rtxC* mutation into the *V. vulnificus* CMCP6 genome. In agreement with results described previously by Liu et al. (30), our *V. vulnificus* *rtxC* mutant exhibited wild-type levels of virulence in our animal model of disease (Joseph et al., unpublished).

Given the ease of the three-part cloning used for constructing the Δ *rtxC* mutation with a shared NotI site between the insert fragments, we created the common overlapping sequence with USER friendly cloning sites to construct a deletion of the *rtxA1* gene. Up to this point, we had used only the two USER friendly sequences that were complementary to cloning vector pNEB206A and the USER friendly vectors that we had constructed with the pNEB206A sequences. However, USER enzyme mix can be used to create 3' overhangs by simply incorporating a deoxyuridine within the PCR primer used to generate the fragment. To create a deletion of the *rtxA1* gene, we amplified 1-kb upstream and downstream flanking sequences with a SmaI restriction site shared between them. By flanking the SmaI site with a deoxyadenine residue and a deoxyuridine residue, treatment with USER enzyme generated TGGGCCCA overhangs. By incorporating the USER friendly sequences complementary to pNEB206A at the left end of the upstream sequence and the right end of the downstream sequence, the fragments were captured into our USER friendly allelic exchange vector pGTR1129 very efficiently (see Fig. S3 in the supplemental material). The SmaI restriction site was used to insert an *aph* kanamycin resistance gene cassette between the upstream and downstream fragments to enable the selection of the deletion mutations during *sacB*-assisted allelic exchange into the *V. vulnificus* genome. In agreement with results described previously by others (21, 25, 30), the Δ *rtxA1::aph* mutant *V. vulnificus* strain was defective for cytotoxicity to epithelial cells and was attenuated for virulence in iron dextran-treated mice (Joseph et al., unpublished). We similarly used three-part USER friendly cloning and *sacB*-assisted allelic exchange to rapidly construct a Δ *fadR::aph* mutant and determined that FadR-mediated regulation of fatty acid metabolism is critical for the virulence of *V. vulnificus* (6).

Three-part USER friendly cloning coupled with our modified USER friendly vectors used to create the Δ *rtxA1::aph* and Δ *fadR::aph* mutations enabled the creation of allelic exchange plasmids for the unlabeled deletion of genes in a single day, including PCRs, USER enzyme treatment, ligation, and electroporation into *E. coli*. The inclusion of the antibiotic resistance cassette into the deletion mutation plasmids added a couple of days to the plasmid constructions. The three-part USER friendly cloning system for creating allelic exchange plasmids is highly efficient and should be useful to many others working with gram-negative bacteria. As opposed to the diffi-

culties in creating TOPO TA cloning vectors, the ability to incorporate USER friendly sequences into any vector is particularly useful. The only limitation to creating one's own USER friendly cloning site is that a restriction enzyme site that leaves an A residue at the 5' end (such as XbaI) is used in conjunction with the Nt.BbvCI nicking site. This A is complementary to the T that takes the place of the U in the USER friendly primer.

After the relative ease of creating the allelic exchange plasmids with USER friendly cloning, working with two-step *sacB*-assisted allelic exchange was problematic. After conjugation into *V. vulnificus* and selection for the single-crossover integration event, counterselection against the *sacB* gene of our pCVD442-based allelic exchange vectors in *V. vulnificus* was inefficient. Blomfield et al. (5) previously reported that eliminating sodium chloride from the sucrose-containing plates improved counterselection in *E. coli*; however, the strict requirement for *V. vulnificus* to have salt in the medium precludes this modification of the procedure. We examined numerous concentrations of sucrose, different growth media, and different growth temperatures to optimize counterselection; however, we still found it necessary to screen over 100 putative sucrose-resistant colonies to find a true excision event that had lost the allelic exchange vector, and in some cases, we never isolated truly sucrose-resistant excision events. The use of an antibiotic resistance gene cassette between the upstream and downstream sequences to label the mutation enabled selection for the mutant genotype; however, when no antibiotic resistance marker was used, we had to screen considerably more sucrose-resistant isolates, since many of them had reverted to the wild type.

A solution to this problem was found by applying the results described previously by Meibom et al. (32), who demonstrated that *V. cholerae* becomes naturally transformable upon growth in the presence of chitin, to our studies of *V. vulnificus*. Those authors examined both purified chitin and crab shells; we used only crab shells in our studies. By simply growing the recipient *V. vulnificus* strain in seawater with a salinity of 25 ppt with a small piece of sterile crab shell, we could readily obtain transformants with linear DNA. Intact plasmids, including cloning vectors pCOS5 and pRK437, could not be taken up and/or maintained by the recipient *V. vulnificus* cells. Even if the plasmids encoded *V. vulnificus* DNA sequences to enable the integration of the plasmids into the recipient genome, no antibiotic-resistant transformants were obtained. However, if the vectors containing *V. vulnificus* sequences flanking an antibiotic resistance cassette were linearized by restriction enzyme digestion or shearing (sonication or vortexing), single-step allelic exchange events were obtained with usable frequencies, on the order of 1.2×10^{-7} . The use of the PCR amplicons consisting of *V. vulnificus* DNA sequences flanking an antibiotic resistance cassette was not efficient or reliable for creating recombinants. However, if the same sequences were incorporated into a plasmid vector, followed by linearization, the transformation and recombination were efficient. Apparently, PCR product-based allelic exchange via transformation suffers from the ends of the transformed DNA fragment being too short, possibly being degraded by the potent nuclease of *V. vulnificus* (50). However, the presence of vector sequences outside of the cloned upstream and downstream *V. vulnificus*

sequences apparently protects the ends long enough to enable recombination into the homologous sequences of the recipient *V. vulnificus* strain. The use of chitin-based transformation for allelic exchange has obviated the need to use sucrose resistance as a counterselectable marker when a selectable genetic marker is included with the mutation.

Until now, if one had created an antibiotic resistance-labeled mutation in one *V. vulnificus* strain and wanted to create the same mutation in a different strain, either to combine mutations (as was the case for the $\Delta flaCDE::aph$ and $\Delta flaFBA::cat$ mutations described here) or to examine mutations in different strain backgrounds, the allelic exchange plasmid and the tedious two-step *sacB*-assisted allelic exchange had to be used again for each strain. However, the fact the chitin-based natural transformation of *V. vulnificus* works best with relatively long pieces of linear DNA led us to examine the possibility of using genomic DNA of strains with antibiotic resistance-labeled mutations to move the mutations from one strain to another or to replace the mutation in the same strain background to preclude the effects of unlinked secondary mutations that could occur during strain construction. We used chitin-based transformation with genomic DNA to combine the $\Delta flaCDE::aph$ and $\Delta flaFBA::cat$ mutations and showed that this method was an effective means of moving established mutations within the CMCP6 strain background. Similarly, we transformed the *wza::TnphoA* mutation, which abolishes capsular polysaccharide synthesis (49), from the *V. vulnificus* M06-24/O background into the CMCP6 background in a single step by selecting for the acquisition of kanamycin resistance. As expected, the kanamycin-resistant transformants acquired the translucent-colony morphotype typical of capsule mutants and were attenuated for virulence in our subcutaneously inoculated iron dextran-treated mouse model. Therefore, once an antibiotic resistance-labeled mutation has been created in one *V. vulnificus* strain, there is no further need for the difficult two-step *sacB*-assisted counterselection allelic exchange procedure to move the mutation into different strains.

In their studies of chitin-based transformation of *V. cholerae*, Meibom et al. (32) previously showed that simply growing different *V. cholerae* strains together in the presence of crab shells enabled horizontal gene transfer between the strains. We similarly asked if the coculture of *V. vulnificus* strains on crab shells could enable horizontal gene transfer. By growing the $\Delta flaCDE::aph$ and $\Delta flaFBA::cat$ strains together on a crab shell in seawater, we readily obtained double mutants that were confirmed by PCR and by their nonmotile phenotype. Horizontal gene transfer between *V. vulnificus* cells growing on chitin has implications for population genetics of *V. vulnificus*. There are two clades of *V. vulnificus* based on multilocus sequence typing, ribotyping, and other PCR-based measures (2, 9, 33). However, in their multilocus sequence typing analysis, Bisharat et al. (2) recently detailed a mosaic pattern between the clades, suggesting that horizontal gene transfer was occurring in nature. Similarly, we have examined relationships among diverse genotyping strategies examining different loci in the *V. vulnificus* genome, and we have observed strains that appear to have acquired sequences from different clades or genotypes (P. C. Thiaville et al., unpublished data). We have examined 19 *V. vulnificus* phages for their abilities to transduce a spontaneous rifampin resistance mutation between *V. vulni-*

ficus strains, but we never identified a generalized transducing phage (J. L. Martin et al., unpublished data). There is very little known about plasmids of biotype 1 *V. vulnificus* strains and even less so about the possible Hfr-mediated exchange of chromosomal DNA sequences. However, conserved conjugative and mobilizable plasmids have been identified for biotype 2 *V. vulnificus* strains (24). Because *V. vulnificus* is believed to grow as biofilms on chitin-containing planktonic organisms in the environment (16, 35, 36, 47), it is conceivable that chitin-based transformation occurs in the wild and could significantly contribute to the mosaic pattern of the genomic structure of *V. vulnificus*.

In summary, we believe that the vectors that we have described here will be useful to others for capturing PCR amplicons for both expression and allelic exchange mutagenesis. Similarly, our adaptation of USER friendly three-part cloning for the single-step creation of allelic exchange sequences should facilitate the construction of such plasmids for use with a variety of bacteria. USER friendly cloning can be adapted to essentially any plasmid vector. Finally, with relevance to *V. vulnificus* and likely other species of *Vibrio*, chitin-based transformation and recombination should make allelic exchange mutagenesis, at least that employing antibiotic resistance cassettes to label mutations, considerably more rapid and efficient than the standard two-step selection for single-crossover and counterselection for second crossover events.

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