



# Natural Transformation in *Vibrio parahaemolyticus*: a Rapid Method To Create Genetic Deletions

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**ABSTRACT** The Gram-negative bacterium *Vibrio parahaemolyticus* is an opportunistic human pathogen and the leading cause of seafood-borne acute gastroenteritis worldwide. Recently, this bacterium was implicated as the etiologic agent of a severe shrimp disease with consequent devastating outcomes to shrimp farming. In both cases, acquisition of genetic material via horizontal transfer provided *V. parahaemolyticus* with new virulence tools to cause disease. Dissecting the molecular mechanisms of *V. parahaemolyticus* pathogenesis often requires manipulating its genome. Classically, genetic deletions in *V. parahaemolyticus* are performed using a laborious, lengthy, multistep process. Here, we describe a fast and efficient method to edit this bacterium's genome based on *V. parahaemolyticus* natural competence. Although this method is similar to one previously described, *V. parahaemolyticus* requires counterselection for curing of acquired plasmids due to its recalcitrant nature of retaining extrachromosomal DNA. We believe this approach will be of use to the *Vibrio* community.

**IMPORTANCE** Spreading of vibrios throughout the world correlates with increased global temperatures. As they spread, they find new niches in which to survive, proliferate, and invade. Therefore, genetic manipulation of vibrios is of the utmost importance for studying these species. Here, we have delineated and validated a rapid method to create genetic deletions in *Vibrio parahaemolyticus*. This study provides insightful methodology for studies with other *Vibrio* species.

**KEYWORDS** EspD, T2SS, *Vibrio parahaemolyticus*, natural transformation systems

*Vibrio parahaemolyticus* is a halophilic, Gram-negative bacterial pathogen, widely established as a causative agent of acute gastroenteritis associated with contaminated seafood (1, 2). Some of these infections, primarily in patients with preexisting conditions, lead to septicemia with fatal outcomes (3). Recently, this pathogen has been recognized as the leading cause of a devastating shrimp disease, acute hepatopancreatic necrosis disease (AHPND) (4). In light of recent findings, such as *V. parahaemolyticus*' adaptation to an intracellular life cycle and its ability to rewire host signaling pathways, there is a need to further understand the molecular mechanisms involved in its pathogenesis (5–7). Moreover, *V. parahaemolyticus* has emerged as a shrimp pathogen with consequent massive economic burden to the shrimp industry (8). Many of these pathogenic qualities can be attributed to the acquisition of new genetic material (4).

Investigating the various mechanisms involved in bacterial pathogenesis at the genetic level most commonly involves genetic engineering. The standard and most widely used technique for achieving genetic modifications in *V. parahaemolyticus* employs a multistep process, including cloning of a gene-flanking region into a nonreplicating suicide vector, such as pDM4, electroporation into an S17-1 ( $\lambda$ pir)

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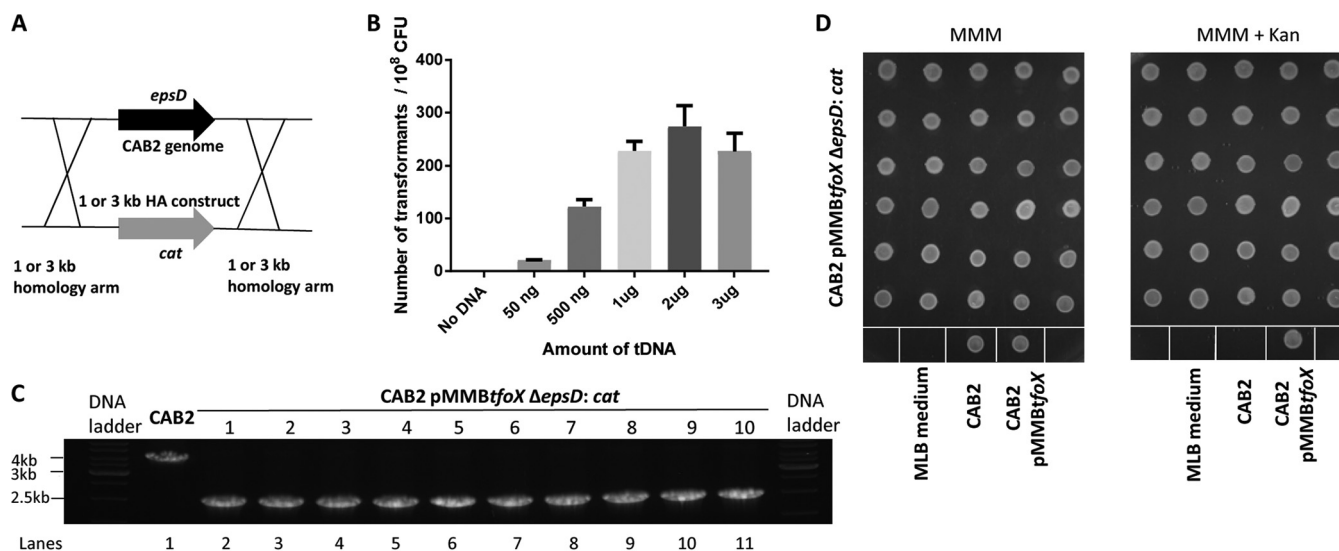
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**FIG 1** Natural transformation of *V. parahaemolyticus* expressing *V. cholerae* *tfoX* improves transformation frequency; however, plasmid curing is inefficient. (A) Schematic showing the homologous recombination between the 1-kb or 3-kb HA construct and the *epsD* locus in the CAB2 genome. (B) *V. parahaemolyticus* CAB2 strain expressing *V. cholerae* *tfoX* gene via pMMBtfoX was used in transformation with various concentrations of transforming DNA (tDNA). (C) PCR analysis using primers A and B confirms the deletion of *epsD* gene in 10 randomly chosen CAB2 pMMBtfoX  $\Delta$ *epsD*::*cat* transformants using primers designed to amplify the region spanning 1 kb upstream to 1 kb downstream of *epsD* with the amplification of a 2.8-kb band (0.8-kb *cat* plus 2-kb flanking *epsD*) in all of the transformants (lanes 2 to 11) and a 4-kb band in CAB2 (2-kb *epsD* and 2-kb flanking, lane 1). (D) CAB2 pMMBtfoX  $\Delta$ *epsD*::*cat* transformants were subcultured 10 times in MLB medium without the antibiotic to cure the plasmid pMMBtfoX and further plated to obtain single colonies. Thirty of these colonies were chosen randomly and patched onto MMM plates with kanamycin (right) and MMM plain plates (left) to check for curing. CAB2 and CAB2pMMBtfoX serve as negative and positive controls, respectively, for growth on MMM<sup>+</sup> kanamycin plates.

*Escherichia coli* strain for propagation, transfer of the plasmid into *V. parahaemolyticus* by conjugation, mutant selection in antibiotics, and final curing of plasmid (1, 9–14). This process is laborious and can take up to 2 to 3 weeks before mutant strains are validated and useful for downstream applications.

Natural competence is the ability of a bacterium to take up foreign DNA from the surroundings, and natural transformation is the ability to integrate the foreign DNA into the genome based on homologous recombination. Meibom et al. first reported natural competence induced by chitin in *Vibrio cholerae*, proposing a transformation model based on chitin, cell density, and nutrient limitation for natural competence (15). Recently, other members of the *Vibrionaceae* family have been shown to be naturally competent and transformable in the presence of chitin (16, 17).

In this study, we have investigated the use of the natural competence phenomenon and here show that natural transformation can be successfully used to create genetic modifications in *V. parahaemolyticus*. However, the method for *V. parahaemolyticus* is unlike the one used for *Vibrio natriegens*, as it requires plasmid curing via counterselection because of *V. parahaemolyticus*' recalcitrant nature of retaining extrachromosomal plasmid DNA (18).

## RESULTS AND DISCUSSION

**Chitin does not induce natural transformation in *V. parahaemolyticus*.** To test whether chitin can induce natural transformation in *V. parahaemolyticus*, we attempted to delete the *epsD* gene (VP0133). *epsD* encodes secretin, an integral part of the type 2 secretion system (T2SS) in *V. parahaemolyticus*. For these experiments we used CAB2, a *V. parahaemolyticus* strain derived from the clinical isolate RIMD 2210633, containing deletions in TDH hemolysins (lacking *tdhA* and *tdhS*) and in the first type III secretion system (T3SS1) transcriptional regulator (*exsA* mutant) (7). A linear PCR construct, consisting of a chloramphenicol acetyltransferase gene (*cat*) cassette flanked by sequences homologous to upstream and downstream regions of *epsD*, was used to replace the *epsD* gene with *cat* via homologous recombination (Fig. 1A). Transformation reactions were plated onto minimal marine medium (MMM) plates with chloram-

**TABLE 1** Chitin does not induce natural transformation in *V. parahaemolyticus*<sup>a</sup>

Amt of tDNA (ng)	No. of transformants/10 <sup>8</sup> total CFU			
	<i>V. parahaemolyticus</i>		<i>V. cholerae</i>	
	1-kb homology	3-kb homology	1-kb homology	3-kb homology
0	0	0	0	0
50	0	0	4.0 × 10 <sup>3</sup>	1.4 × 10 <sup>5</sup>
500	0	0	1.7 × 10 <sup>4</sup>	4.9 × 10 <sup>5</sup>
1,000	0	0	2.0 × 10 <sup>5</sup>	2.2 × 10 <sup>6</sup>
2,000	0	0	2.1 × 10 <sup>5</sup>	3.8 × 10 <sup>6</sup>
3,000	0	0	2.7 × 10 <sup>5</sup>	7.7 × 10 <sup>6</sup>

<sup>a</sup>*V. parahaemolyticus* CAB2 strain was used in chitin-induced natural transformation with increasing concentrations of tDNA, consisting of either 1-kb or 3-kb homology flanking regions. *V. cholerae* TND 0266 strain was used as a positive control.

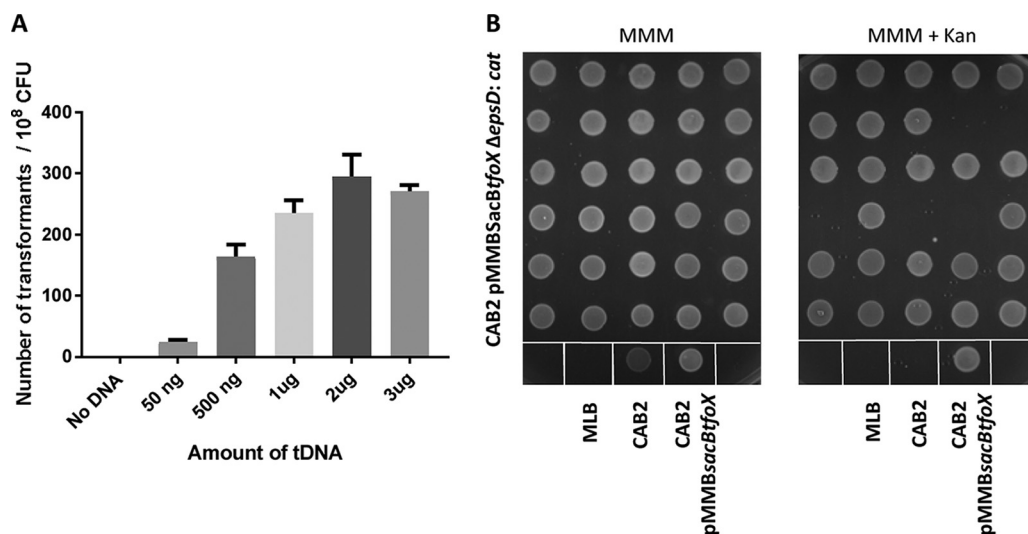
phenicol to select for transformants. Chitin-induced natural transformation resulted in no CAB2 transformants when using increasing concentrations of transforming DNA (tDNA) with either 1-kb ( $\Delta epsD::cat$  1kb HA) or 3-kb ( $\Delta epsD::cat$  3kb HA) homology regions (Table 1).

To confirm that chitin transformation was working in our hands, we used as a positive control, a  $\Delta CTX$  (cholera toxin-deficient) derivative of *V. cholerae* strain E7946 (TND 0266), which was previously shown to be transformable by chitin (19). By following the same protocol as that described above, we replaced the transposase pseudogene VC1807 with a kanamycin resistance cassette and observed 10<sup>5</sup> transformants with 1 kb of flanking DNA (Table 1). Although a previous study reported successful natural transformation of *V. parahaemolyticus* using chitin, we were not able to reproduce these results (16).

**Natural transformation of *V. parahaemolyticus* by overexpression of *V. cholerae* TfoX improves transformation frequency.** Chitin has been shown to induce natural competence in *V. cholerae* by upregulating expression of the competence master regulator TfoX (15). Additional studies have elucidated the link between chitin sensing and induction of competence in *Vibrio* species via TfoX (15, 20–22). Chitin upregulates TfoX by interacting with TfoS, resulting in exposure of the Shine-Dalgarno sequence of the TfoX mRNA to positively regulate TfoX translation, and this in turn activates the genes necessary for competence (23). Overexpression of TfoX was shown to bypass the chitin requirement for competence, allowing natural transformation in *Vibrio* species (18, 24).

Based on these observations, we next set out to induce natural transformation in *V. parahaemolyticus* by overexpressing the highly conserved *V. cholerae* TfoX, which has 81% similarity and 69% identity to the *V. parahaemolyticus* homologue and was shown to function in other vibrios. The *V. cholerae* TfoX was ectopically expressed in CAB2 using the kanamycin-resistant pMMBtfoX plasmid. This strain was then transformed using the previously described  $\Delta epsD::cat$  1kb HA and  $\Delta epsD::cat$  3kb HA constructs. While transformation deploying the  $\Delta epsD::cat$  1kb HA construct resulted in marginal improvement of transformation frequency (a single transformant for every 10<sup>8</sup> total CFU at 500 ng tDNA), the use of its 3-kb counterpart significantly improved transformation efficiency, with 274 transformants per 10<sup>8</sup> total CFU using 2  $\mu$ g tDNA. Ten transformants were randomly picked, and PCR analysis was carried out to confirm the deletion of the *epsD* gene. PCR amplification using the primers A and B (see Fig. 3A; see also Table S1 in the supplemental material), designed to amplify the region spanning 1 kb upstream to 1 kb downstream of *epsD*, resulted in a 4-kb band in CAB2 (2 kb of *epsD* and 2 kb of flanking region) and in a 2.8-kb band (0.8 kb of *cat* and 2 kb of flanking region of *epsD*) in all transformants tested, thus confirming *epsD* deletion (Fig. 1C, lanes 2 to 11).

Although transformation is complete at this stage, it is necessary to eliminate the *V. cholerae* TfoX plasmid. We next set out to cure the plasmid pMMBtfoX from *V. parahaemolyticus* transformants. Five randomly picked clones from the above-



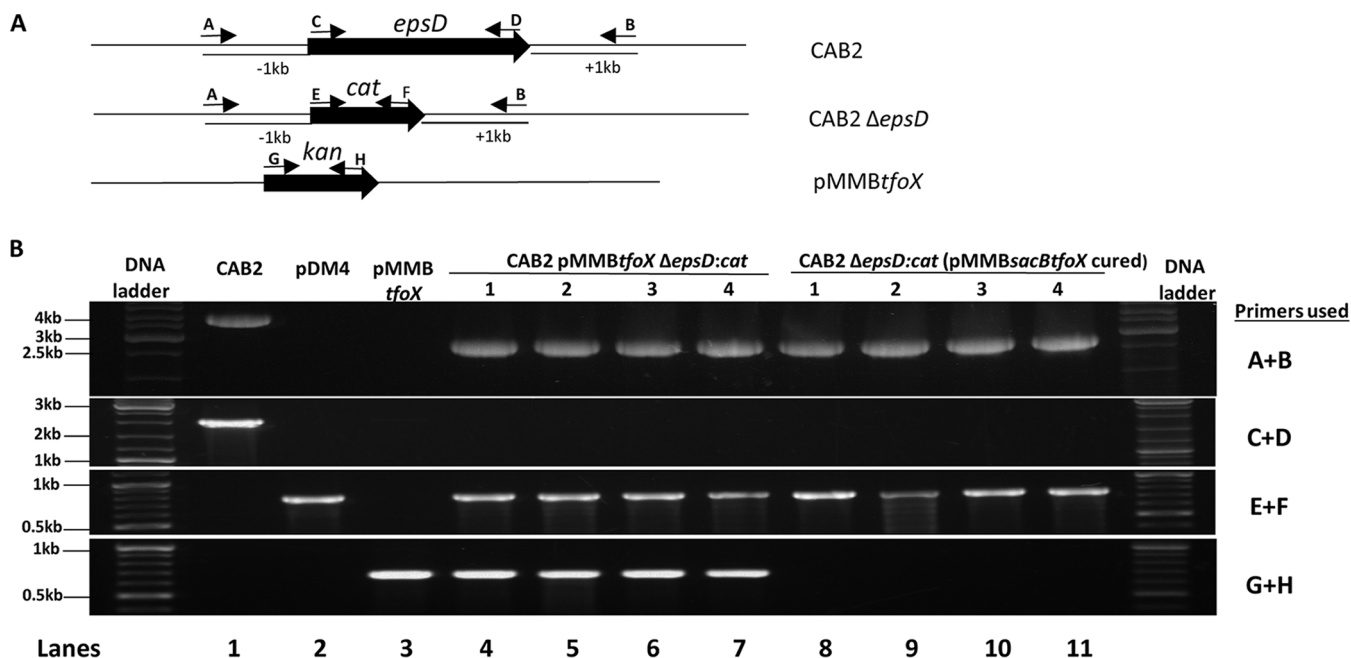
**FIG 2** Addition of *sacB* gene to pMMBtfoX plasmid helps in curing the plasmid from the transformants. (A) *V. parahaemolyticus* CAB2 strain expressing *V. cholerae tfoX* gene via pMMBsacBtfoX was used in transformation with various concentrations of transforming DNA (tDNA). (B) Thirty randomly chosen CAB2 pMMBsacBtfoX ΔepsD::cat transformants were patched onto MMM plates with kanamycin (right) and MMM plain plates (left) after growth on MMM plates containing sucrose to cure the plasmid pMMBsacBtfoX. MLB is the medium used for growth. CAB2 and CAB2pMMBsacBtfoX serve as negative and positive controls for growth on MMM<sup>+</sup> kanamycin plates, respectively.

confirmed ΔepsD::cat transformants were subcultured 10 times in MLB medium (Luria-Bertani [LB] medium supplemented with 3% NaCl) without kanamycin (pMMBtfoX-borne antibiotic resistance marker) to cure the *V. parahaemolyticus* transformants of the plasmid pMMBtfoX, followed by plating onto MMM plates to obtain single colonies. Thirty of these single colonies (CAB2 pMMBtfoX ΔepsD::cat) were randomly picked and patched onto MMM plates with and without kanamycin to check for plasmid curing. Unfortunately, all of the 30 tested colonies grew on both MMM and MMM-plus-kanamycin plates, demonstrating that none of the clones were cured of the plasmid (Fig. 1D). Therefore, although ectopic expression of *V. cholerae* TfoX allows for natural transformation of *V. parahaemolyticus* strains, this *Vibrio* species appears to be resistant to curing of the TfoX plasmid.

**Addition of *sacB* gene to pMMBtfoX plasmid allows for curing of *V. parahaemolyticus* transformants.** SacB-mediated sucrose sensitivity is a popular method for counterselection in genetic editing of bacterial genomes (25). The *Bacillus subtilis sacB* gene encoding levansucrase is not toxic to its native Gram-positive bacteria; however, when expressed in Gram-negative bacteria it is lethal in the presence of sucrose (26). The mechanism of this toxicity is not well understood but is thought to be due to accumulation of the high-molecular-weight fructose polymers synthesized by the levansucrase in the periplasm of Gram-negative bacteria (27).

We therefore attempted to use the *Bacillus subtilis sacB* gene as a counterselection method to remove pMMBtfoX from *V. parahaemolyticus*. The SacB counterselection marker was incorporated into pMMBtfoX to create pMMBsacBtfoX. This plasmid was used to ectopically express *V. cholerae* TfoX for natural transformation of *V. parahaemolyticus* CAB2 along with the tDNA ΔepsD::cat 3kb HA construct. pMMBsacBtfoX-mediated transformation was comparable to that of pMMBtfoX, with a similar number of transformants that increased with increasing amounts of tDNA up to 1 μg (Fig. 2A). Five randomly chosen transformants (CAB2 pMMBsacBtfoX ΔepsD::cat) were then plated in the presence of sucrose for curing of the plasmid. To confirm loss of the plasmid, single colonies were patched onto MMM plates with or without kanamycin plates (plasmid-borne antibiotic resistance marker). Up to 16% (5 out of 30 colonies patched) of the clones were unable to grow on kanamycin plates, suggesting loss of plasmid (Fig. 2B).

To confirm the genetic composition of the naturally transformed and cured *V. parahaemolyticus*, we analyzed transformants using PCR. Using kanamycin-sensitive



**FIG 3** PCR analysis confirms the replacement of *epsD* gene with *cat* gene in the transformants and the absence of kanamycin resistance cassette in the cured transformants. (A) Schematic of the *epsD* locus in CAB2 and CAB2  $\Delta$ *epsD* strains and the location of primers used for PCR amplification. (B, top row) Primers A and B, designed to amplify the region spanning 1 kb upstream to 1 kb downstream of *epsD*, were used, which resulted in a 4-kb band in CAB2 (lane 1; 2-kb *epsD* gene plus 2-kb flanking region) and 2.8-kb band (0.8-kb *cat* plus 2-kb flanking *epsD*) in all transformants (both uncured CAB2 pMMBtfoX  $\Delta$ *epsD*::*cat* strains 1 to 4 [lanes 4 to 7] and cured CAB2  $\Delta$ *epsD*::*cat* strains 1 to 4 [lanes 8 to 11]). Gene-specific primers C and D were used to amplify the *epsD* gene in the second row, confirming the deletion of the *epsD* gene in all transformants (both uncured CAB2 pMMBtfoX  $\Delta$ *epsD*::*cat* strains 1 to 4 and cured CAB2  $\Delta$ *epsD*::*cat* strains 1 to 4). The third row, using primers E and F, shows the replacement of the *epsD* gene with the *cat* gene in all transformants (both uncured CAB2 pMMBtfoX  $\Delta$ *epsD*::*cat* strains 1 to 4 and cured CAB2  $\Delta$ *epsD*::*cat* strains 1 to 4). (Bottom) Using primers G and H confirms the absence of Kan cassette in the cured CAB2  $\Delta$ *epsD*::*cat* strains 1 to 4 compared to its amplification in the uncured CAB2 pMMBtfoX  $\Delta$ *epsD*::*cat* strains 1 to 4. CAB2, pDM4, and pMMBtfoX were used as positive controls for the *epsD* gene, and *cat* and Kan cassettes, respectively.

clones along with uncured transformants (kanamycin resistant), we analyzed the presence or absence of *epsD*, *cat*, and the Kan cassette. Using PCR amplification with primers A and B, we observed a 2.8-kb band (0.8-kb *cat* and 2-kb flanking region of *epsD*) in all transformants compared to the 4-kb band (2-kb *epsD* and 2-kb flanking) in CAB2 and confirmed the deletion of the *epsD* gene in all transformants (Fig. 3A and B, top row of gels, lanes 4 to 11). Absence of amplification of *epsD* using the primers C and D further demonstrated the successful deletion of this gene (Fig. 3A and B, second row, lanes 4 to 11). The replacement of *epsD* with *cat* was observed in all of the transformants using primers E and F (Fig. 3A and B, third row, lanes 4 to 11). Finally, the absence of amplification of the kanamycin cassette using the primers G and H in the kanamycin-sensitive transformants confirmed curing of pMMBsacBtfoX from these clones (Fig. 3A, bottom row, lanes 8 to 11).

**Validation of *epsD* deletion strains using MS.** Since *V. parahaemolyticus epsD* encodes secretin, a major component of the T2SS, we predicted that its loss would have an effect on the type 2 secretome (28). To test this hypothesis, we performed mass spectrometry (MS) analysis of the secretome isolated from the CAB2 and newly created CAB2 $\Delta$ *epsD* strains. We observed that deletion of *epsD* impaired secretion of many known T2SS proteins, such as lipases, serine proteases, toxins, and enzymes involved in carbohydrate utilization (29, 30). A selected list of proteins found in the CAB2 but not found in the CAB2 $\Delta$ *epsD* secretome is presented in Table 2. One such protein, annotated as putative trypsin (VP1642), is a homologue of *V. cholerae* VesB, recently identified as a T2SS-secreted protein (30).

Counterintuitively, further comparison of CAB2 and CAB2 $\Delta$ *epsD* secretomes revealed that some proteins were secreted at higher levels in the mutant secretome, and other proteins were found to be secreted by the mutant but not by CAB2 (Table S2).

**TABLE 2** Absence of T2SS-secreted proteins from CAB2 $\Delta$ *epsD* secretome<sup>a</sup>

Accession no. <sup>b</sup>	Name	Gene
28901322	Protease II	VPA1467
28810041	Secreted RNase, putative	VPA1639
28900714	Lipase	VPA0859
28806633	Trypsin, putative	VP1642
28806328	Collagenase	VP1340
28897534	Chitoporin	VP0760
28900569	Collagenase	VPA0714
28806169	Lactonizing lipase	VP1181
28806248	Outer membrane phospholipase, chain A	VP1260
28899947	Spindolin-like protein	VPA0092
28808615	Serine proteinase, putative	VPA0449
28900610	Metalloproteinase	VPA0755
28901386	Serine protease	VPA1531
28898960	Bacteriocin production protein	VP2186

<sup>a</sup>List of T2SS proteins identified by mass spectrometry that are present in the CAB2 secretome but absent from the CAB2 $\Delta$ *epsD* secretome.

<sup>b</sup>Accession numbers were obtained from the NCBI protein database for *V. parahaemolyticus* RIMD 2210633.

Interestingly, some of the proteins with increased levels or that were exclusively present in the CAB2 $\Delta$ *epsD* secretome (e.g., superoxide dismutase and catalases) are known to be involved in combating oxidative stress (Table S2). In *V. cholerae*, the equivalent *epsD* deletion mutant was also reported to have increased secretion of proteins involved in oxidative stress, and such effect was attributed to the leaky nature of the cell envelope due to T2SS disruption (29, 30). Altogether, our findings indicate the *epsD* deletion in *V. parahaemolyticus* results in an altered secretome, indicative of a disabled T2SS.

*V. parahaemolyticus*, a pathogenic marine bacterium, while not amenable to chitin-induced transformation, did allow for natural transformation by obviating the chitin requirement with TfoX overexpression. Since a PCR construct can be directly used for homologous recombination through natural transformation, the multistep process involved in conventional protocols, i.e., generation and propagation of a suicide vector containing the homologous construct followed by conjugation, can be bypassed to achieve diverse genetic modifications in *V. parahaemolyticus* in a timely manner. For example, it takes 2 to 3 weeks to generate a gene deletion in *V. parahaemolyticus* by conventional methods but only 1 week by the natural transformation method described here.

In summary, we have successfully used natural transformation in *V. parahaemolyticus* to create genetically modified mutants. This approach will likely be applicable to many other *Vibrio* species and be beneficial to the *Vibrio* community as a whole.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *V. parahaemolyticus* CAB2 strain used in this study was made by deleting the transcriptional factor ExsA from the POR1 strain (RIMD 2210633  $\Delta$ *tdhAS* strain), generously provided by Tetsuya Iida and Takeshi Honda (31). The clinical isolate RIMD 2210633 contains thermostable direct hemolysins (TDHs) and two type 3 secretion systems, T3SS1 and T3SS2. The CAB2 strain contains deletions in TDH genes and in the transcriptional regulator gene *exsA*, resulting in an inactive T3SS1 (7). CAB2 strain was grown in MLB medium (Luria-Bertani [LB] medium supplemented with 3% NaCl) at 30°C. *V. cholerae* strain TND 0266 is a cholera toxin-deficient mutant of an *V. cholerae* strain (E7946, an El Tor *V. cholerae* isolate) and was grown in LB medium at 30°C (19). When necessary, the medium was supplemented with 25  $\mu$ g/ml chloramphenicol or 250  $\mu$ g/ml kanamycin.

**Generation of PCR constructs.** The PCR constructs were generated by splicing-by-overlap extension (SOE) PCR (32). For the generation of  $\Delta$ *epsD*::*cat* 1kb HA constructs, the nucleotide sequences 1 kb upstream and downstream of the gene *epsD* (VP0133; GenBank sequence accession number [NC\\_004603](#)) were amplified with Phusion polymerase (ThermoFisher Scientific) using the primer pairs EpsD1kb\_UpF1-EpsD\_UpR1 and EpsD\_DownF1-EpsD1kb\_DownR1, respectively. The *cat* cassette was amplified from pDM4, a Cm<sup>r</sup> Ori6RK suicide plasmid with the primers CatF and CatR. Gel-purified PCR products of 1 kb upstream, *cat*, and 1 kb downstream were then mixed in equal ratios and used as the template for an SOE PCR with the primers 1kb\_UpF1 and 1kb\_DownR1 to generate the construct  $\Delta$ *epsD*::*cat* 1kb HA. Similarly, the  $\Delta$ *epsD*::*cat* 3kb HA construct was generated using the 3-kb upstream and downstream se-

quences of *epsD* with the primer pairs EpsD3kb\_UpF1-EpsD\_UpR1 and EpsD\_DownF1-EpsD3kb\_DownR1, respectively. All of the primers used in this study are listed in Table S1 in the supplemental material.

**Chitin-induced natural transformation.** Natural transformation through chitin was performed as described previously (19). Briefly, *Vibrio* cultures were inoculated into 1 ml defined artificial seawater (DASW; 7 g/liter Instant Ocean; Aquarium Systems) containing 80 mg of autoclaved chitin (AAJ6120622; Fisher) at an optical density at 600 nm ( $OD_{600}$ ) of 0.1 ( $\sim 10^8$  CFU) and allowed to grow statically at 30°C for 16 to 24 h. Transforming DNA was then added by exchanging the supernatant with fresh DASW containing tDNA without disturbing the settled chitin and allowed to incubate for an additional 16 to 24 h at 30°C. One milliliter of MLB was then added, and transformants were allowed to outgrow at 30°C with shaking for 1 to 2 h. Transformation mixtures were then plated on MMM plates with or without chloramphenicol (25  $\mu$ g/ml) to assess transformation efficiency.

**Natural transformation by overexpression of *V. cholerae* TfoX.** TfoX-induced natural transformation was performed as described previously (18). Briefly, *V. parahaemolyticus* CAB2 strains harboring pMMB-tfoX were obtained via conjugation with S17-1 ( *$\lambda$ pir*) *Escherichia coli* containing the plasmid pMMBtfoX, and transconjugants were selected for on MMM agar containing 250  $\mu$ g/ml kanamycin. CAB2 pMMBtfoX strains were induced with 100  $\mu$ M isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) overnight in MLB plus kanamycin (250  $\mu$ g/ml) at 30°C. The cultures were then diluted 100-fold in 2 $\times$  Instant Ocean plus 100  $\mu$ M IPTG (28 g/liter of Instant Ocean), and tDNA was added. Reaction mixtures were gently mixed and incubated at 30°C statically for 4 to 6 h. One milliliter of MLB was added, transformants were allowed to outgrow at 30°C with shaking for 1 to 2 h, and then they were plated on MMM plates with and without chloramphenicol to determine transformation efficiency.

**SacB cloning into pMMBtfoX and curing of pMMBsacBtfoX from the transformants.** The *sacB* gene along with its endogenous promoter was amplified from the pDM4 plasmid using the primers SacBF and SacBR and cloned into pMMBtfoX between the restriction sites BamHI and PstI. The resulting pMMBsacBtfoX was transferred into CAB2 via conjugation with S17-1 ( *$\lambda$ pir*) *Escherichia coli*. Transconjugants were selected for on MMM agar containing 250  $\mu$ g/ml kanamycin (pMMBsacBtfoX-borne plasmid resistance marker). Curing of pMMBsacBtfoX from *Vibrio* strains was achieved by plating the bacterium onto MMM agar plates containing 15% (wt/vol) sucrose once to obtain isolated colonies. These single colonies were subsequently plated on MMM plates with or without kanamycin to check for kanamycin sensitivity, i.e., confirmation of curing of plasmid.

**Secretome analysis by mass spectrometry.** The secretomes from *V. parahaemolyticus* strains were isolated from 50 ml of *Vibrio* cultures grown in MLB. Briefly, 50 ml MLB was inoculated with overnight-grown *V. parahaemolyticus* culture to an  $OD_{600}$  of 0.18/ml and allowed to grow for 3 h at 30°C with shaking. The cultures were then centrifuged at 4,000 rpm for 20 min at 4°C to pellet the bacteria, and the supernatant was passed through a 0.22- $\mu$ m syringe filter. Secreted proteins were precipitated from this cell-free supernatant by the addition of trichloroacetic acid (TCA) at 8% (vol/vol) and incubation at 4°C overnight. Precipitated proteins were pelleted by centrifugation for 50 min at 16,000  $\times$  *g* at 4°C using a Beckman-Coulter centrifuge. The protein precipitate was washed twice in 4 ml of chilled acetone, air dried, and resuspended in 1 ml of cold 10 mM Tris, pH 8.0, buffer. A second round of TCA precipitation was carried out at 4°C for 1 h. The final protein precipitate was once again washed twice with 400  $\mu$ l chilled acetone, air dried, resuspended in 20  $\mu$ l of SDS sample buffer, and then analyzed by mass spectrometry.

Sample preparation for mass spectrometry analysis included the excision of proteins from polyacrylamide gels via SDS-PAGE. Proteins were reduced and alkylated using dithiothreitol (DTT) and iodoacetamide, respectively. In-gel overnight digestion of proteins with trypsin followed, and the samples were desalted via solid-phase extraction (SPE) prior to analysis. Liquid chromatography-tandem MS (LC-MS/MS) experiments were performed on a Thermo Scientific EASY-nLC 1200 liquid chromatography system coupled to a Thermo Scientific Orbitrap Fusion Lumos mass spectrometer. MS1 spectra were acquired in the Orbitrap mass analyzer with a resolution of 120,000. Subsequent peptide fragmentation took place via high-energy collision-induced dissociation (HCD), generating MS2 spectra which were acquired in the ion trap. Resulting MS/MS spectral data were searched using Proteome Discoverer 2.1 software (Thermo Scientific) against entries included in the *V. parahaemolyticus* RIMD 2210633 protein database (NCBI). Carbamidomethylation of cysteine residues (+57.021 Da) was set as a static modification, while oxidation of methionine (+15.995 Da) and acetylation of peptide N termini (+42.011 Da) were set as dynamic modifications. The precursor ion tolerance was set to 10 ppm, and the product ion tolerance was set to 0.6 Da for all searches. Peptide spectral matches were adjusted to a 1% false discovery rate.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00032-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.1 MB.

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