Vibrio vulnificus Biotype 3 Multifunctional Autoprocessing RTX Toxin Is an Adenylate Cyclase Toxin Essential for Virulence in Mice

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Vibrio vulnificus is an environmental organism that causes both food-borne and wound infections with high morbidity and mortality in humans. The annual incidence and global distribution of infections associated with this pathogen are increasing with climate change. In the late 1990s, an outbreak of tilapia-associated wound infections in Israel was linked to a previously unrecognized variant of *V. vulnificus* designated biotype 3. The sudden emergence and clonality of the outbreak suggest that this strain may be a true newly emergent pathogen with novel virulence properties compared to those of other *V. vulnificus* strains. In a subcutaneous infection model to mimic wound infection, the multifunctional autoprocessing RTX (MARTX) toxin of biotype 3 strains was shown to be an essential virulence factor contributing to highly inflammatory skin wounds with severe damage affecting every tissue layer. We conducted a sequencing-based analysis of the MARTX toxin and found that biotype 3 MARTX toxin has an effector domain structure distinct from that of either biotype 1 or biotype 2. Of the two new domains identified, a domain similar to *Pseudomonas aeruginosa* ExoY was shown to confer adenylate cyclase activity on the MARTX toxin. This is the first demonstration that the biotype 3 MARTX toxin is essential for virulence and that the ExoY-like MARTX effector domain is a catalytically active adenylate cyclase.

Vibrio vulnificus is a Gram-negative pathogen that inhabits coastal waters throughout the world (1). This pathogen causes severe food-borne and wound infections in humans that can progress to tissue necrosis, septicemia, organ failure, and death, often within 48 h of pathogen exposure (1–3), particularly among individuals who suffer from cirrhosis of the liver and other underlying predisposing conditions (4, 5). Mortality rates can reach as high as 50%, with extreme morbidity among survivors, including amputations, surgical tissue debridement, and long hospital stays (1–3). Although the disease remains rare, the incidence of *V. vulnificus* is rising because of global warming (6, 7) and the geographical area impacted by *V. vulnificus* in the water and food supply is expanding (7–11).

Biotype 1 (BT1) strains are most commonly associated with clinical infections, while BT2 strains cause infections in eels (1, 12, 13). From 1996 to 1999, changes in marketing practices to allow vendors to sell live fish in Israel led to an outbreak of a new lineage of *V. vulnificus*, resulting in a large number of wound infections associated with the handling and preparation of tilapia and carp (14). These BT3 strains continue to be present in fish ponds and the Sea of Galilee and have caused nearly 200 infections with 10% mortality from 1996 to 2008 (15).

The primary virulence factor of *V. vulnificus* BT1 and BT2 strains is the multifunctional autoprocessing RTX (MARTX) toxin (16–18). The secreted MARTX toxins are very large single-polypeptide toxins composed of repeat sequences that form a pore in eukaryotic cell plasma membranes for the translocation of centrally located effector domains (19). Within the cell, the autoproteolytic cysteine protease domain (CPD) is activated by the binding of inositol hexakisphosphate to release individual effector domains into the cytosol (20). Among the BT1 and BT2 *V. vulnificus* isolates studied, at least four different MARTX variants have been annotated that carry distinct repertoires of effector domains and these have been shown to be exchanged frequently in this naturally competent bacterium by horizontal acquisition of DNA, followed by recombination (21–23).

In this study, it is shown that BT3 strains have a unique variant of the *V. vulnificus* MARTX toxin that has been converted to an adenylate cyclase toxin. This toxin is essential for the virulence of BT3 strains in mice infected by the subcutaneous (s.c.) route.

MATERIALS AND METHODS

Bacterial strains, cell lines, and reagents. All of the bacterial strains used in this study are listed in Table 1. Ten-locus multilocus sequence typing (MLST) was used to verify strains as BT3 according to the methods of Bisharat et al. (24) by using the pubmlst.org website developed by Keith Jolley at the University of Oxford (25). A spontaneously rifampin-resistant isolate of BAA87 was selected by serial dilution into Luria-Bertani (LB) broth containing increasing concentrations of rifampin. Bacteria were grown in LB broth and on LB agar supplemented with the antibiotics ampicillin (100 µg/ml), chloramphenicol (10 µg/ml), kanamycin (50 µg/ml), and rifampin (50 µg/ml) as needed.

Chinese hamster ovary (CHO) cells obtained from Rich Longnecker (Northwestern University) were cultured in Life Technologies Ham's F12 nutrient mixture with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin obtained from Gemini Bio-Products (West Sacramento, CA).

All other common chemical reagents were obtained from Fisher or Sigma. Molecular biology enzymes were from New England BioLabs

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TABLE 1 *V. vulnificus* strains used in this study

Strain	Relevant description	Source or reference
CMCP6	BT1, clinical isolate, South Korea, Rif ^r	16
BAA86 (CDC 9505-95)	BT3, human blood, Haifa, Israel, 1995	ATCC ^a
BAA87 (CDC 9530-96)	BT3, human wound, Afula, Israel, 1996	ATCC
11028	BT3, human clinical, Israel, 1996	E. F. Boyd, University of Delaware
2095/00	BT3, environmental isolate, Israel, 2000	A. Depaola, FDA
JK57 (BAA87rif)	Spontaneously rifampin-resistant BAA87	This study
KZ1 (BAA87rtxA1)	BAA87 <i>rtxA1::kan</i> , Kan ^r	This study
KZ4 (BAA87rtxA1/c)	BAA87rtxA1 restored to Kan ^r by recombination with wild-type sequence	This study

^a ATCC, American Type Culture Collection.

(Ipswich, MA), and DNA primers were obtained from Integrated DNA Technologies (Coralville, IA).

***rtxA1* gene sequencing.** The effector domain regions of *rtxA1* genes were amplified, and their nucleotide sequences were determined as previously described (21). The full-length sequence of *rtxA1* from BAA87 was extended from the effector domain region to the 5' and 3' ends of the gene by using primers designed to highly conserved regions of the *rtxA1* genes of BT1 and BT2 strains for amplification into regions of known BAA87 sequence, followed by sequencing of products by primer walking. Coverage was 1× to 6× by this strategy. The accuracy of the sequence across zones of poor coverage was later verified by whole-genome sequencing of BAA87 with the Life Technologies Ion Torrent PGM technology with a 318 chip paired with a 400-bp library. The whole-genome sequence of BAA87 (GenBank accession no. [JDSE000000.1](#)) has been published separately (55).

Generation of BAA87 *rtxA1* mutant and complemented strain. The region of BAA87 *rtxA1* from bp 341 to bp 5446 was amplified by PCR and captured in the plasmid pCR-XL-TOPO (Life Technologies) by TOPO cloning according to the manufacturer's protocol to generate plasmid pJK206. The fragment corresponding to bp 341 to 4974 was transferred into *sacB*-counterselectable plasmid pDS132 (26) with *SacI* and *Sall*. The resulting plasmid, pKZ38, was digested at the unique *FspI* restriction site at bp 2042 into which the 1,170-bp *HincII* kanamycin resistance cassette from pKanπ (27) was inserted so as to create a null mutation in *rtxA1*. The resulting plasmid, pKZ37, has a kanamycin resistance gene under the control of its own promoter with flanking *rtxA1* DNA for recombination. pKZ37 was mated from S17-1λpir to rifampin-resistant BAA87, and merodiploids were selected by gain of chloramphenicol and kanamycin resistance. The colonies were purified and then passaged on LB agar with kanamycin in the absence of chloramphenicol. Recombination events that excised the plasmid from the merodiploid were selected by growth on no-salt LB agar with 6% sucrose and kanamycin. The gain of the kanamycin resistance gene in the correct location on the chromosome of the selected KZ1 isolate (BAA87rtxA1) was confirmed by PCR and sequencing of the PCR product through the *rtxA1::kan* junctions. The strain was then reverted to the wild-type sequence by mating KZ1 with S17-1λpir(pKZ38) and *sacB* counterselection as described above with screening for loss of kanamycin resistance to create strain KZ4 (BAA87rtxA1/c). PCR amplification, followed by sequencing, was done to confirm that the strain was restored to the wild-type sequence. The strains were confirmed to produce a MARTX toxin by gain or loss of *rtxA1*-dependent pore-forming cell lysis.

Cloning of LF_NVvExoY expression vector and site-directed mutagenesis. The region of BAA87 *rtxA1* from bp 9702 to bp 10791 was amplified by PCR with primers modified for ligation-independent cloning

(LIC) (28). LIC was used to insert the PCR product into plasmid pRT24, a vector modified from the anthrax toxin lethal factor N terminus (LF_N) expression vector pABII (29) by addition of the LIC site (28). The resulting plasmid, pKZ5, was also used as the template for site-directed mutagenesis with Agilent (Santa Clara, CA) cloned *Pfu* polymerase following the manufacturer's protocol. Amplified plasmids were transformed into Agilent XL10-Gold Ultracompetent cells and plated on LB agar with ampicillin. Recovered plasmids were screened for gain of the mutation by sequencing and then transformed to BL21(DE3) for expression of the modified protein.

Expression and purification LF_NVvExoY. BL21(DE3) carrying either plasmid pKZ5 or mutagenized variants was grown in LB with ampicillin (37°C, 250 rpm) to an *A*₆₀₀ of ~0.6, when expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C with shaking. Bacteria were collected by centrifugation, resuspended in lysis buffer (500 mM NaCl, 20 mM Tris [pH 8.0], 5 mM imidazole, 1 mg/ml lysozyme, 1% Triton X-100), and lysed on ice by sonication with a Branson Digital Sonifier with a disruptor horn attachment by eight 30-s pulses at 40% amplitude. The soluble fraction containing LF_NVvExoY was clarified by centrifugation at 27,000 × *g*, filtered across a 0.45-μm syringe filter, and loaded onto a GE Healthcare 1-ml HisTrap HP column with a GE Healthcare ÄKTApurifier fast protein liquid chromatography system. The column was washed with 10 ml of loading buffer (500 mM NaCl, 20 mM Tris [pH 8.0], 5 mM imidazole) and then with a 20-ml buffer gradient of 5 to 250 mM imidazole, wherein the protein eluted at 55 mM. The protein was further purified by size exclusion in 20 mM Tris (pH 8.0)–500 mM NaCl with a Superdex 200 10/30 GL column. The protein was concentrated with Amicon Ultracel 30K and then stored at –20°C in 10% glycerol. The concentration of the protein was determined with a NanoDrop 100, and the purity of 10 μg was assessed by SDS-PAGE, followed by staining with Coomassie blue.

In vitro adenylate cyclase assay. A 100-μl reaction mixture contained 10 μl of 10× reaction buffer (100 mM Tris [pH 8.0], 60 mM MgCl₂, 2 mM CaCl₂, 20 mM dithiothreitol), 30 μg bovine serum albumin (BSA) or a postnuclear CHO cell extract (CE, prepared as described by Xu and Barbieri [30], except with a Sonic microdisruptor to dismembrated the cells), and 1 μmol of LF_NVvExoY, except where noted otherwise. A 0.2 μM concentration of ATP was always added last to prevent ATP rolling, and the reaction was carried out for 30 min at 30°C. The reaction was stopped by adding 186 μl of ethanol and centrifugation at 16,000 × *g* for 5 min. The supernatant was then lyophilized with a Savant SpeedVac DNA 120 until dry. The presence of cyclic AMP (cAMP) in the lyophilized sample was initially assessed by separating the rehydrated reaction mixture on a C₁₈ high-performance liquid chromatography (HPLC) column in 100 mM potassium phosphate, pH 6.0, across an 8-ml gradient of 0 to 10% methanol on the ÄKTApurifier configured for HPLC. The identity of the cAMP peak was confirmed by treatment with phosphodiesterase (PDE; Sigma P9529) for 1 h at 30°C. For quantification, the concentration of cAMP in rehydrated samples was determined by enzyme-linked immunosorbent assay (ELISA) as described below.

cAMP production in CHO cells cocultured with *V. vulnificus*. CHO cells (5 × 10⁵) were seeded into 12-well tissue culture dishes. Two hours prior to bacterial infection, cells were washed three times with phosphate-buffered saline (PBS) and the medium was changed to Ham's F12 plus 10% FBS without antibiotics. *V. vulnificus* grown to mid-log phase in LB was washed in PBS, and the *A*₆₀₀ of all samples was equalized with a Beckman Coulter DU530 spectrophotometer. Bacteria were added to medium over cells at a multiplicity of infection (MOI) of 20, and the plates were centrifuged at 500 × *g* for 5 min. The plates were then incubated at 37°C with 5% CO₂ for 90 min. Cells were washed in PBS and then lysed in the wells with 600 μl of PBS with 1% Triton X-100 at 37°C for 10 min, and the lysate was stored at –20°C. The concentration of cAMP in 100 μl of lysate was determined as described below. Cell viability throughout the assay was verified by phase microscopy for the presence of cells and in some experiments by measuring the release of lactate dehydrogenase

(LDH) into the medium as an indicator of cell lysis with the Promega CytoTox96 kit according to the manufacturer's instructions. To generate a positive control for all cAMP assays, 10 μ M forskolin was added to an extra well 30 min prior to the end of the incubation period and assayed for maximum cAMP production in parallel with the other samples.

cAMP ELISA. cAMP concentrations from *in vitro* assays or cell lysates were measured with the Enzo Life Sciences cAMP Complete ELISA by following the manufacturer's protocol, except for the use of the alternative lysis buffer indicated above for lysate assays. For some *in vitro* assays, lysates were diluted in the lysis buffer to reduce the cAMP concentration to the detectable range. Plates were read on a Molecular Devices Spectra-Max M5 plate reader, and concentrations were determined on the basis of a standard curve prepared on the same assay plate.

Mouse survival assay. All mouse infections were conducted in accordance with protocols approved by the Northwestern University Institutional Animal Care and Use Committee. Five- to 6-week-old female C57BL/6 mice for assays were obtained from Harlan Laboratories. *V. vulnificus* was grown to mid-log phase and washed in PBS, and the A_{600} was equalized. A 50- μ l volume of PBS containing *V. vulnificus* was injected s.c. under the dorsal skin of mice anesthetized with 100 μ l of a cocktail of 10 μ g/ml ketamine and 2 μ g/ml xylazine in PBS. After 8 h of infection, mouse survival was observed every 2 h. Severely moribund mice were humanely euthanized by cervical dislocation under anesthesia and counted as nonsurvivors. Surviving mice showing no symptoms of infection were euthanized at the termination of the experiment.

Wound pathology. Five mice per group were inoculated s.c. with 10^6 CFU of *V. vulnificus* and euthanized after 12 h. Skin wounds were excised with the fur intact, and the tissue was fixed in 10% neutral buffered formalin overnight and then embedded in paraffin. Sections were stained with hematoxylin and eosin. Slides were viewed by a trained skin pathologist (R.M.L.) on a Zeiss Axioplan2 microscope for pathology, which was consistent across all five animals, and representative images were taken at magnifications of $\times 400$ and $\times 630$.

Nucleotide sequence and MLST data accession numbers. MLST data from this study have been deposited with pubmlst.org/vvulnificus as follows: BAA87, id285; 11028, id288; 2095/00, id289. All of the *rtxA1* nucleotide sequences obtained in this study have been deposited in GenBank as follows: full-length BAA87 *rtxA1*, accession number [KJ131555](#); partial *rtxA1* sequences of BAA87, 11028, 2095/00, and BAA86, accession numbers [KJ143989](#) to [KJ143992](#), respectively.

RESULTS

Sequencing of the *rtxA1* genes of various BT3 *V. vulnificus* strains. BT3 *V. vulnificus* BAA87 isolated from a patient wound during the original epidemic in 1996 was obtained. The sequence of the *rtxA1* gene, which encodes the MARTX toxin, was determined by sequencing of PCR products generated with primers designed to *rtxA1* genes from other strains. The gene was determined to be 15,627 nucleotides (nt) long and to encode a deduced protein of 5,208 amino acids (aa). The accuracy of our BAA87 sequence was later verified, as it matched the *rtxA1* gene from whole-genome sequencing of BT3 isolate VVyb1, which was from tilapia fish (31), except that BAA87 has a confirmed 15-nt deletion of a sequence that is precisely duplicated in VVyb1 (Table 2).

Surprisingly, neither the BAA87 nor the VVyb1 *rtxA1* sequence matched a previously annotated *rtxA1* sequence from BT3 clinical isolate 11028 (GenInfo identifier 283558386), a sequence of 14,112 nt with a deduced product of 4,703 aa (22). Thus, we obtained three additional BT3 isolates to determine which sequence is representative of the BT3 *rtxA1* gene. To confirm the identities of the strains, we first conducted a 10-locus MLST analysis based on the methods of Bisharat et al. (24). The sequence typing (ST) profile of clinical isolate ATCC BAA86 was previously published

TABLE 2 Nucleotide polymorphisms in BT3 *rtxA1* genes compared to BAA87

Strain	Nucleotide polymorphism	Amino acid change in MARTX
ATCC BAA86 ^a	G10916C, T12332C	S $\rightarrow$ T, V $\rightarrow$ A
BT3 clinical isolates		
11028 ^a	C6214T, C9273T, C11062G, T11841C	P $\rightarrow$ S, SYN, L $\rightarrow$ V, SYN
2095/00 ^a	C2937T, 18 other mutations	N $\rightarrow$ stop (CAG $\rightarrow$ TAG)
VVyb1 ^b	15-nt duplication inserted at nt 3114	LSVTA inserted

^a Only the effector domain region (*rtxA1* nt 5678 to 12746) was sequenced.

^b Compared to *rtxA1* gene from published whole-genome sequence (31).

and deposited at www.pubmlst.org/vvulnificus (25), and this published ST profile, number 8, was used as the reference. The ST profile of BAA87 was a 100% match for that of BAA86, as was the profile of isolate 11028, which was obtained from E. Fidelma Boyd (University of Delaware), and that of 2095/00 (32), which was obtained from the FDA. Thus, all of the strains in this study were confirmed by MLST to be BT3 strains.

We next sequenced the 7,068-nt region of the *rtxA1* gene corresponding to the effector domain region of each strain. The nucleotide sequences in the effector domain regions of BAA86 and 11028 were identical in length to that of BAA87 but with two and four single nucleotide polymorphisms, respectively (Table 2). Within the 7,068 nt sequenced from strain 2095/00, there were 19 single nucleotide polymorphisms. Most notably, one of these changes introduced a stop codon, indicating that this is a natural *rtxA1* null strain.

Overall, it is concluded that, with the exception of spontaneously *rtxA1* null strain 2095/00, all BT3 strains, including the 11028 isolate we obtained, have *rtxA1* genes that are nearly identical in sequence and would produce a new variant of the MARTX toxin that has the same domain structure.

The BT3 MARTX toxin is a novel variant. Annotation of the deduced amino acid sequence of the BT3 MARTX toxin showed that the predicted arrangement of effector domains differs from that of previously annotated BT1 and BT2 strain variants (Fig. 1A and B). Similar to the variants produced by commonly studied BT1 strains CMCP6 and MO6-24/O (also known as type I and II MARTX toxins), the BT3 strain variants have a domain of unknown function in position 1 (DUF1), a Rho inactivation domain (RID) in position 2, and a domain with homology to the alpha-beta hydrolase (ABH) family of enzymes in position 3 (Fig. 1A) (21, 22).

Two new effector domains not previously detailed as being present in the *V. vulnificus* MARTX toxins are in the next two positions. The domain in the fourth position is most similar to the type III secretion effector ExoY adenylate cyclase from *Pseudomonas aeruginosa*. Sequence comparison studies have also identified this domain in putative MARTX toxins from environmental *V. cholerae* isolate RC385 and coral *V. caribbeanicus* isolate BAA-2122 and from the plasmid-borne MARTX toxin produced by the shrimp pathogen *V. nigripulchritudo* (Fig. 1C) (19, 33, 34), although no functional studies have established that this domain confers adenylate cyclase activity on these putative toxins. Gene annotation, done for this study, of the *rtxA1* gene from recently sequenced BT1 *V. vulnificus* environmental isolate E64MW (23) also revealed that this strain carries an ExoY-like adenylate cyclase

FIG 1 The BT3 MARTX toxin is unique among MARTX toxins. Linear schematics of MARTX toxins are depicted to scale with the conserved regions shown with repeats indicated as hatched gray boxes and the autoprocessing CPD in light blue. The effector domain arrangement is different for each MARTX toxin. (A) Deduced structures of all BT3 strains, represented by BAA87, compared to those of BT1 CMCP6 and MO6-24/O, which are the closest in overall structure. (B) BT2 strains have a distinct structure that bears some similarity to BT1 E64MW, which was found here to also have acquired an ACD and an ExoY-like domain. (C) Other species noted as having genes for MARTX toxins with an ExoY-like domain are shown; some also have DmX. Effector domain designations are DUF1, RID, ABH family protein, makes caterpillars floppy homology (Mcf), DUF5, ExoY-like adenylate cyclase (ExoY), DmX, and the ACD. More detailed descriptions of the effector domains can be found in a recent review article (19).

effector domain, as well as an actin cross-linking domain (ACD), a domain shared with the BT2 MARTX toxin (Fig. 1B), although again no functional studies have shown that this putative MARTX toxin has adenylate cyclase activity.

The effector domain in the fifth position is a DUF. As “DUF in the fifth position” (DUF5) is already used to designate the *Pasteurella*-like effector domain of the CMCP6 MARTX toxin (35), we refer to this domain as domain X (DmX). Sequence analysis revealed that this effector domain is present also in the putative MARTX toxin from *V. cholerae* environmental isolate RC385, where it is duplicated and present on either side of the ExoY-like domain and in the plasmid-borne MARTX toxin of *V. nigrispulchritudo* in the first position (Fig. 1C) (19, 33, 34). No studies of the functional activity of this domain have been conducted.

BT3 *V. vulnificus* coculture increased intracellular cAMP concentrations in CHO cells. A notable feature of the unique sequence of the BT3 MARTX toxin is the gain of a domain with 26% amino acid sequence identity and 42% similarity to *P. aeruginosa* ExoY (PaExoY) (30) and 19 to 20% identity and 35 to 36% similarity to the adenylate cyclase domains of the *Bordetella pertussis* adenylate cyclase toxin (BpACT) and the *Bacillus anthracis* edema factor (EF) (36). A domain analysis revealed strong conservation in the regions corresponding to the active site defined by the crystal structures of the adenylate cyclase domains of BpACT and EF (Fig. 2).

When CHO cells were cocultured with BT3 strains BAA86, 11028, and BAA87rif at an MOI of 20 for 90 min, their intracellular concentration of cAMP increased by >30-fold (Fig. 3A and B). In contrast, the spontaneous *rtxA1* null strain 2095/00 induced only a 3- to 10-fold increase in cAMP production in CHO cells during coculture, suggesting a link to the *rtxA1* gene (Fig. 3A and B). The loss of cAMP was not due to a growth defect in 2095/00, as all of our BT3 isolates grew at similar rates in Luria broth, although all grew more slowly than the representative BT1 strain CMCP6 (Fig. 3C), consistent with previous studies that BT3

strains have a lower growth rate in all media (37). Thus, it is most likely that the lack of induction of cAMP in CHO cells by 2095/00 is due to its naturally occurring null mutation in *rtxA1*.

To affirmatively link the adenylate cyclase activity to the *rtxA1* gene, an isogenic null mutation in the BAA87rif *rtxA1* gene was generated by recombining the kanamycin resistance cassette *nptII* into the 5' end of the *rtxA1* gene in a way that completely disrupted the transcription and translation of the downstream *rtxA1* gene. The KZ1 mutant strain (BAA87rif *rtxA1*) was unable to induce cAMP on coculture with CHO cells (Fig. 3B). In order to show that the loss of function was not due to spontaneous mutation at another locus during genetic manipulation, we used genetic recombination to remove the kanamycin resistance cassette from KZ1 and restore the wild-type sequence. The restored KZ4 strain regained the ability to stimulate cAMP production (Fig. 3B). Overall, these data demonstrate that the BT3 *rtxA1*-encoded MARTX toxin induces cAMP production in cells.

The BT3 MARTX toxin also causes CHO cell lysis. A phenotype closely associated with *V. vulnificus* BT1 MARTX toxin is the ability to induce lysis of cells in culture. Upon coculture at an MOI of 20 for 90 min as conducted for the cAMP assays, lysis of cells was either undetectable or minimal (<5%) and cells generally appeared normal, although some cell rounding and distention was observed (Fig. 4A and B). This low level of lysis increased over the next 90 min such that after 3 h, 90 to 100% of the CHO cells incubated with BT3 strain BAA87rif were lysed and the cells were visually necrosed. The disruption of *rtxA1* resulted in an 87% decrease in LDH release at 180 min, indicating that lysis at an MOI of 20 was due primarily to the MARTX toxin. However, the detection of 6 to 15% LDH release and the appearance of necrotic cells demonstrate that other secreted cytotoxins also contribute. At a higher MOI of 100, the amount of cell lysis at 90 min varied up to ~25%, with 80 to 100% lysis occurring by 180 min with all of the strains, including the *rtxA1* mutant. These data demonstrate that BT3 MARTX toxin can induce cell lysis, although cytotoxicity is not

FIG 2 Alignment of VvExoY with adenylate cyclase toxins and effectors. (A) Schematic diagrams of the *V. vulnificus* MARTX toxin VvExoY effector domain (corresponding to its putative cleavage sites) and full-length secreted bacterial proteins with adenylate cyclase activity *P. aeruginosa* ExoY (PaExoY), *B. pertussis* adenylate cyclase toxin (BpACT), and *B. anthracis* EF. On the basis of comparative analysis of the crystal structures of BpACT and EF bound to CaM (36), the catalytic domain is composed of C_B and the C_{A1} and C_{A2} segments, which fold to form the C_A subdomain. The binding site for ATP is formed at the interface between the two subdomains and contacting switch B (SwB). The boxed residues are known to be essential for cyclase activity. Poorly conserved switch A (SwA) contacts the C terminus of CaM in both EF and BpACT. (B) ClustalW alignment generated in MacVector shows conservation in the catalytic regions of VvExoY aa 3255 to 3547, PaExoY aa 34 to 307, BpACT aa 9 to 314, and EF aa 299 to 594. The C-terminal tail portion of EF, which is known to contact the N terminus of CaM (N-CaM), is shown in diagrams, but this part is not conserved in ExoY proteins and is poorly conserved in BpACT. GD, Gly-Asp-rich repeats; PA, protective antigen.

due solely to the MARTX toxin, as has been shown also for BT1 and BT2 (16–18, 38, 39).

Purified recombinant *V. vulnificus* ExoY (VvExoY) has adenylate cyclase activity. As the BT3 MARTX toxin is associated with perturbation of the cell at an MOI of 20, one mechanism for the increase in cAMP caused by the BT3 MARTX toxin could be induction of endogenous host adenylate cyclases activated by pore formation. To further demonstrate that the ExoY-like effector domain is the adenylate cyclase, the sequence of the gene for the ExoY-like effector domain was amplified and cloned fused to the anthrax toxin lethal factor N terminus (LF_N). MARTX toxin effectors are highly unstable in solution because of a natural tendency to unfold and difficulties identifying the termini of the domains within the larger holotoxins. However, we have found through studies of the ACD (40) and RID (41) that fusion proteins can be sufficiently stable for limited biochemical and cell biological analyses.

In an *in vitro* reaction, LF_NVvExoY catalyzed the conversion of ATP to cAMP. When the terminated reaction was separated on a C₁₈ reverse-phase column, a new peak corresponding to the expected retention time of cAMP was observed. This peak was eliminated when the reaction was conducted in the presence of PDE to degrade the phosphodiester bond in cAMP (Fig. 5A).

The amount of cAMP produced during the *in vitro* reaction was quantified by ELISA, which showed that cAMP production occurred in a dose-dependent manner. The overall activity was, however, about 100-fold less efficient than the basal level of activity of the nearest protein homolog, PaExoY (Fig. 5B). Yet, purified LF_NVvExoY containing single amino acid substitutions in four conserved residues required for catalysis by PaExoY (42) (Fig. 2) did not catalyze cAMP production, confirming that the ExoY-like effector domain is a class II adenylate cyclase (Fig. 5C).

The adenylate cyclase BpACT and EF are activated by calmod-

FIG 3 Coculture of *V. vulnificus* BT3 increases the intracellular cAMP concentration in CHO cells. (A, B) The strains indicated were cocultured with CHO cells at an MOI of 20 for 90 min. The intracellular concentration of cAMP in the clarified lysate was determined by ELISA. (C) Growth kinetics of the BT3 strains indicated and BT1 CMCP6 measured in Luria broth with shaking at 37°C. Experiments with three biological replicates are representative of two independent experiments performed with similar results. Statistical significance was determined first by analysis of variance as $P < 0.05$, and the specific P values indicated were obtained for compared samples with the Student t test. OD, optical density.

ulin (CaM) through contact with the catalytic domain switch A region and a helical domain to the C-terminal side of the catalytic domain (36, 43, 44) (Fig. 2). However, the closest homolog of VvExoY is PaExoY, and this protein is activated not by CaM but by an unknown eukaryotic protein (30). However, in a parallel experiment, CE, which activated PaExoY, did not stimulate the activity of LF_N VvExoY (Fig. 5B) and, indeed, partially inhibited its activity. It is unknown if the VvExoY effector domain is constitutively active or if it requires an activator that is distinct from the unknown activator used by PaExoY.

BT3 MARTX toxin is essential for virulence and tissue damage at the wound site. Both CMCP6- and MO6-24/O-type MARTX toxins of BT1 strains and the actin-cross-linking MARTX toxins of BT2 strains have been shown to be important for virulence with mice as a model system (16–18, 38, 39, 45, 46). As the BT3 MARTX toxin is unique, it was necessary to determine if the toxin is still an important virulence factor. As BT3 strains in the outbreak in Israel are all linked to wounds, we used s.c. infection of 5- to 6-week-old inbred C57BL/6 mice as a model for wound-induced disease. Mice injected with iron dextran complex to increase serum iron levels rapidly succumbed to infection with as few as 100 CFU (Fig. 6A). However, BT3 infections are not strongly correlated with underlying infection or high iron content, as 45% of patients have no predisposing conditions (15). In mice not treated with iron dextran, mice also succumbed to infection with BAA87rif in a dose-dependent manner (Fig. 6B). By comparison, at a dose that was lethal to 10/10 mice, the BAA87rtxA1 mutant was significantly less virulent, resulting in the deaths of only 2/10 mice. Genetic exchange of the kanamycin resistance cassette for the wild-type sequence restored virulence to BAA87rtxA1, demonstrating that the virulence defect was directly associated with the *rtxA1* gene disruption and not due to unlinked mutations acquired during genetic manipulation (Fig. 6C).

Histological evaluation of skin revealed a thin epidermis overlying a dermis with a marked inflammatory infiltrate (Fig. 7B and C). The fabric of the dermis appeared fractured throughout and lacked the customary fibrillar organization seen in mock-treated animals and in mice treated with the BAA87rtxA1 mutant (Fig. 7A and D). The dermis of BAA87-infected mice contained greater numbers of inflammatory cells than the dermis of mutant-in-

fected mice. Most striking in the BAA87-infected mice was the appearance of the s.c. fat and the panniculus carnosus of the skin. The s.c. fat layer was markedly thicker and filled primarily with polymorphonuclear lymphocytes (PMNs). In addition, small, rod-like bacteria were noted within the s.c. fat. A brisk, dense infiltrate was observed at the junction of the s.c. fat and the panniculus carnosus. At high magnification, this infiltrate consisted mainly of PMNs. In contrast, the s.c. fat layer of mice infected with BAA87rtxA1 contained fewer infiltrating cells, although more than noted in the mock-treated animal. Also, by 12 h postinoculation, no bacteria were noted in the mutant-infected animal, consistent with resolution of the skin wound infection. Overall, the severity of the pathology of wounds of BAA87-infected mice was dependent on *rtxA1* and the wounds were highly inflamed, with severe damage that affected every layer of the skin tissue.

DISCUSSION

BT3 *V. vulnificus* is associated with an outbreak of infections in Israel, and these infections are apparently currently limited to Israel. These strains have been considered hybrids of two lineages of BT1 strains, the E strains enriched for environmental isolates (also known as lineage 2 and clade 1) and the C strains enriched for clinical isolates (also known as lineage 1 and clade 2) (24, 47, 48). In this study, we considered if these strains might be unique in their pathogenesis, as well as their genetics. As the *V. vulnificus* MARTX toxins are known to be variable in effector domain composition (21, 22), it was considered that any difference in pathogenesis determined could be linked to a unique toxin composition.

Sequencing revealed that all BT3 strains have nearly identical *rtxA1* gene sequences. All of the BT3 strains examined are predicted to have the same unique arrangement of five effector domains. We cannot account for the variation compared to the previously published sequence of 11028 (22), as the 11028 isolate we obtained matched the other BT3 *rtxA1* sequences examined. Our sequencing in the 5' arm of BAA87 *rtxA1* also differed from the *rtxA1* gene of VVyb1 derived by whole-genome sequencing (31), as the latter had a 15-nt exact duplication increasing the length of the deduced protein by 5 aa. The lack of this duplication in BAA87 was confirmed, although it is unknown if this difference is due to

FIG 4 BT3 *rtxA1*-dependent and -independent CHO cell lysis. (A) Supernatant fluids of CHO cells incubated with *V. vulnificus* as indicated for the times indicated were assayed for LDH release as an indicator of cell lysis. (B) Prior to collection of supernatant fluids, digital phase-contrast images of cells were taken with a Nikon TS100 at a magnification of $\times 200$. The experiment shown was conducted with three biological replicates and is representative of at least two independent experiments performed with similar results. The differences between the *rtxA1* mutant and (i) BAA87rif and (ii) the complemented mutant at an MOI of 100 were determined for each time point and found by analysis of variance to be significant ($P < 0.0001$) at 90 min (dagger) but not significant (ns) at 180 min. All other differences were not significant or were within the range of variability observed across repeated assays.

an error in the automated assembly of VVyb1 or if this represents a true difference between the two sequences.

The effector domain repertoire of the BT3 MARTX toxin was distinct from all other previously annotated MARTX toxins, carrying two effector domains not previously known to be present in *V. vulnificus* MARTX toxins. DmX in the fifth position remains a DUF. This domain joins DUF1, RID, and ABH, which are shared

FIG 5 The ExoY-like domain of BT3 MARTX toxin has *in vitro* adenylate cyclase activity. Purified recombinant VvExoY fused to anthrax toxin lethal factor to enhance stability (LF_NVvExoY) or purified *P. aeruginosa* ExoY (PaExoY) as a positive control (gift from W. J. Tang, University of Chicago) was used in an *in vitro* assay of ATP conversion to cAMP. (A) The product of the completed reaction of 1 μ mol of LF_NVvExoY in the presence (solid line) or absence (dashed line) of PDE was separated by C₁₈ reverse-phase chromatography. The expected peaks are marked. (B) Final reactions conducted in the presence of either 30 μ g BSA or CE were diluted in buffer for quantification by ELISA. Values were corrected against a mock control containing either BSA or CE to negate potential effects due to cellular adenylate cyclases present in the extract. (C) Reaction mixtures with 1 μ mol of LF_NVvExoY enzyme in the presence of BSA (no CE) were not diluted to allow direct comparison with low-activity mutants. Statistical significance was determined first by analysis of variance as $P < 0.05$, and the specific P values indicated for the samples compared were obtained with the Student t test. Daggers in panel B indicate statistically significant differences from BSA controls and in panel C from the LF_NVvExoY control set at a maximum value of 200 for all samples. All results shown are means and standard deviations of three biological replicates in experiments conducted at least twice to ensure reproducibility.

with the BT1 strains and whose biological function also are not well characterized.

In this study, we show that the new domain in the fourth position is an adenylate cyclase—the first demonstration that this effector domain is a catalytically active cyclase enzyme. The acqui-

FIG 6 BAA87 is virulent in mice inoculated s.c. dependent upon *rtxA1*. Survival curves after s.c. infection from iron dextran-treated (A) and untreated (B) mice (five per group) infected with increasing numbers of CFU of BAA87. (C) Pooled data from two experiments with 5 mice per group infected with 10⁵ CFU of BAA87 and the mutant strains indicated. The *rtxA1* mutant in panel C is significantly different from both BAA87 and the complemented strain, as determined by the Mantel-Cox log rank test.

sition of this domain resulted in *V. vulnificus* BT3-dependent cAMP production upon coculture with CHO cells. This gain of function was due to the MARTX toxin, since loss of the *rtxA1* gene either by a naturally occurring null mutation in 2095/00 or by insertional mutagenesis in BAA87 eliminated the ability of *V. vulnificus* to induce cAMP production.

This activity was linked directly to the ExoY-like domain, which was able to convert ATP to cAMP *in vitro*. The activity depended on residues critical for catalysis by other adenylate cyclase toxins, confirming that this enzyme is a class II adenylate cyclase. Compared to the activity of purified PaExoY, it was less efficient and was not induced by the unknown activator present in CHO cell extract that was able to induce the activity of PaExoY. Variable activity of class II adenylate cyclases has been previously

FIG 7 Pathology of wounds in BAA87- and BAA87rtxA1-infected mice. Mice used for pathology determination were either mock inoculated by injection with PBS (A) or inoculated s.c. with 10⁶ CFU of the *V. vulnificus* strain indicated (B to D). Mice were euthanized after 12 h, and wounds were prepared for histopathological examination. Photographs are from representative mice imaged at a magnification of $\times 400$ (large panels) or $\times 630$ (insets and right portion of panel B). Epidermis (E), s.c. fat (S), and muscle (M) tissues are indicated, as are *V. vulnificus* BAA87 rod-shaped bacteria (Vv) and infiltrating PMNs.

noted, as the adenylate cyclase activity of EF is ~ 10 - to 100-fold lower than that of BpACT, which also functions optimally at lower Ca²⁺ and CaM concentrations (49). It is unclear if the observed activity of VvExoY indicates a weak constitutively active enzyme or if the *in vitro* conditions identified for this initial report are simply not yet optimized. A problem with studying MARTX effectors is that the “ends” of the structurally stable domain can be difficult to predict because of long unstructured regions found between effector domains. This can lead to the inclusion of either too much or too little of the unstructured linker, affecting the stability of recombinant proteins. In addition, purified MARTX effectors tend, in general, to behave poorly in solution, as they seem to be evolutionarily selected to be unstable, likely because of a need to unfold easily for type I secretion out of the bacterium and again for translocation into the eukaryotic cell cytosol. ACD and CPD are both known to require cofactors (50, 51), and for the CPD, it has been demonstrated that the recombinant protein does not become stably folded *in vitro* until it associates with its activator (52). Thus, it is possible that VvExoY does require a cofactor that would increase both its *in vitro* stability and its activity. This activator will likely be different from that for PaExoY, as the same extract that stimulated PaExoY did not stimulate VvExoY and indeed seemed to inhibit it. Another distinct difference between the two proteins is that PaExoY has been shown to also be efficient in the conversion of GTP to cGMP (53). We found that under the reaction conditions employed here, VvExoY did not function as a guanylate cyclase either during coculture with CHO cells or *in vitro* (data not shown). However, as these are negative data, we

cannot exclude the possibility that, under optimized conditions or with an appropriate activator, improved production of both cAMP and cGMP would be observed. Under the conditions detailed here, the enzyme functioned solely as an adenylate cyclase.

This gain of adenylate cyclase activity by the MARTX toxin will likely be shared with other species, as effector domains with homology to VvExoY are found in *Vibrio* MARTX toxins from *V. cholerae*, *V. nigripulchritudo*, and *V. caribbeanicus* (19). As the MARTX toxin effector domains seem to be freely exchangeable in the environment among the natural competent bacteria, it seems reasonable that this domain will appear via whole-genome sequencing in the MARTX toxins of even more species.

Since the effector domain repertoire of the BT3 MARTX toxin was distinct, it was determined if the toxin would be a virulence factor, as has been shown for BT1 and BT2 strains. It is shown here that the BT3 *rtxA1* gene is essential for wound-induced infection in mice inoculated s.c. The wounds showed significant epithelial damage and inflammation. In the absence of *rtxA1*, 2/10 mice still succumbed to infection and showed some inflammation, although the overall pathology was much less severe. These results are consistent with other studies of BT1 *V. vulnificus* wound infection in mice, where the cytolysin encoded by the gene *vvhA* is also linked to virulence, and the potential remains that other secreted proteases and elastase contribute to these infections as well (17).

In a final set of experiments, we attempted to compare the pathology of the wounds induced by BT1 strain CMCP6 and the pathology of the wounds induced by BT3 strain BAA87 to determine if the conversion from a BT1-type MARTX toxin to the adenylate cyclase toxin would affect qualitative aspects of the wound. However, the observed differences in inflammatory infiltration and the degree of damage could not be assigned specifically to the BT3 *rtxA1* gene (our unpublished results). These findings indicated that it is the sum of all of the variations in the overall genome context, in addition to changes in the MARTX content, that caused the difference in disease progression between BT1 CMCP6 and BT3 BAA87. This important finding demonstrates that *V. vulnificus* infection is highly variable and strain specific and can involve changes in many genetic components that dictate virulence.

This vast heterogeneity of strains may pose a problem, as seen in this outbreak in Israel, for there are potentially regional differences in the nature of the infections, and thus, pathogenic findings based on studies of a single strain may not be globally applicable. This may also pose challenges for the development of therapeutic agents. For example, it has recently been determined that an antibody raised against the carboxyl-terminal fragment of the MARTX toxin from BT1 strain MO6-24/O can be used in mice as a passive treatment for infections with that same strain of *V. vulnificus* (54). However, if a patient has a strain with MARTX toxin epitopes different from those of the strain used to generate the antibody, the therapy may be limited in its application. These regional differences in strain and the resulting therapeutic barriers have the potential to then be exported as food markets expand globally, just as the incidence of *V. vulnificus* is also expanding globally because of climate change.

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REFERENCES

- Jones MK, Oliver JD. 2009. *Vibrio vulnificus*: disease and pathogenesis. Infect. Immun. 77:1723–1733. <http://dx.doi.org/10.1128/IAI.01046-08>.
- Oliver JD. 2005. Wound infections caused by *Vibrio vulnificus* and other marine bacteria. Epidemiol. Infect. 133:383–391. <http://dx.doi.org/10.1017/S0950268805003894>.
- Horseman MA, Surani S. 2011. A comprehensive review of *Vibrio vulnificus*: an important cause of severe sepsis and skin and soft-tissue infection. Int. J. Infect. Dis. 15:e157–166. <http://dx.doi.org/10.1016/j.ijid.2010.11.003>.
- Menon MP, Yu PA, Iwamoto M, Painter J. 2014. Pre-existing medical conditions associated with *Vibrio vulnificus* septicemia. Epidemiol. Infect. 142:878–881. <http://dx.doi.org/10.1017/S0950268813001593>.
- Dechet AM, Yu PA, Koram N, Painter J. 2008. Nonfoodborne *Vibrio* infections: an important cause of morbidity and mortality in the United States, 1997–2006. Clin. Infect. Dis. 46:970–976. <http://dx.doi.org/10.1086/529148>.
- Baker-Austin C, Trinanes JA, Taylor NGH, Hartnell R, Siitonen A, Martinez-Urtaza. 2013. Emerging *Vibrio* risk at high latitudes in response to ocean warming. Nat. Clim. Chang. 3:73–77. (Letter.) <http://dx.doi.org/10.1038/nclimate1628>.
- Paz S, Bisharat N, Paz E, Kidar O, Cohen D. 2007. Climate change and the emergence of *Vibrio vulnificus* disease in Israel. Environ. Res. 103:390–396. <http://dx.doi.org/10.1016/j.envres.2006.07.002>.
- Dalsgaard A, Frimodt-Møller N, Bruun B, Hoi L, Larsen JL. 1996. Clinical manifestations and molecular epidemiology of *Vibrio vulnificus* infections in Denmark. Eur. J. Clin. Microbiol. Infect. Dis. 15:227–232. <http://dx.doi.org/10.1007/BF01591359>.
- Frank C, Littman M, Alpers K, Hallauer J. 2006. *Vibrio vulnificus* wound infections after contact with the Baltic Sea, Germany. Euro Surveill. 11: E060817.1.
- Veenstra J, Rietra PJ, Coster JM, Slaats E, Dirks-Go S. 1994. Seasonal variations in the occurrence of *Vibrio vulnificus* along the Dutch coast. Epidemiol. Infect. 112:285–290. <http://dx.doi.org/10.1017/S0950268800057691>.
- Torres L, Escobar S, Lopez AI, Marco ML, Pobo V. 2002. Wound infection due to *Vibrio vulnificus* in Spain. Eur. J. Clin. Microbiol. Infect. Dis. 21:537–538. <http://dx.doi.org/10.1007/s10096-002-0767-4>.
- Amaro C, Biosca EG. 1996. *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. Appl. Environ. Microbiol. 62:1454–1457.
- Tison DL, Nishibuchi M, Greenwood JD, Seidler RJ. 1982. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl. Environ. Microbiol. 44:640–646.
- Bisharat N, Agmon V, Finkelstein R, Raz R, Ben-Dror G, Lerner L, Soboh S, Colodner R, Cameron DN, Wykstra DL, Swerdlow DL, Farmer JJ, III. 1999. Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. Israel *Vibrio* Study Group. Lancet 354:1421–1424. [http://dx.doi.org/10.1016/S0140-6736\(99\)02471-X](http://dx.doi.org/10.1016/S0140-6736(99)02471-X).
- Zaidenstein R, Sadik C, Lerner L, Valinsky L, Kopelowitz J, Yishai R, Agmon V, Parsons M, Bopp C, Weinberger M. 2008. Clinical characteristics and molecular subtyping of *Vibrio vulnificus* illnesses, Israel. Emerg. Infect. Dis. 14:1875–1882. <http://dx.doi.org/10.3201/eid1412.080499>.
- Jeong HG, Satchell KJ. 2012. Additive function of *Vibrio vulnificus* MARTX_{Vv} and VvhA cytolysins promotes rapid growth and epithelial tissue necrosis during intestinal infection. PLoS Pathog. 8:e1002581. <http://dx.doi.org/10.1371/journal.ppat.1002581>.
- Lo HR, Lin JH, Chen YH, Chen CL, Shao CP, Lai YC, Hor LI. 2011. RTX toxin enhances the survival of *Vibrio vulnificus* during infection by protecting the organism from phagocytosis. J. Infect. Dis. 203:1866–1874. <http://dx.doi.org/10.1093/infdis/jir070>.

18. Lee CT, Pajuelo D, Llorens A, Chen YH, Leiro JM, Padros F, Hor LI, Amaro C. 2013. MARTX of *Vibrio vulnificus* biotype 2 is a virulence and survival factor. *Environ. Microbiol.* 15:419–432. <http://dx.doi.org/10.1111/j.1462-2920.2012.02854.x>.
19. Satchell KJ. 2011. Structure and function of MARTX toxins and other large repetitive RTX proteins. *Annu. Rev. Microbiol.* 65:71–90. <http://dx.doi.org/10.1146/annurev-micro-090110-102943>.
20. Egerer M, Satchell KJ. 2010. Inositol hexakisphosphate-induced autoproducting of large bacterial protein toxins. *PLoS Pathog.* 6:e1000942. <http://dx.doi.org/10.1371/journal.ppat.1000942>.
21. Kwak JS, Jeong HG, Satchell KJ. 2011. *Vibrio vulnificus* *rtxA1* gene recombination generates toxin variants with altered potency during intestinal infection. *Proc. Natl. Acad. Sci. U. S. A.* 108:1645–1650. <http://dx.doi.org/10.1073/pnas.1014339108>.
22. Roig FJ, Gonzalez-Candelas F, Amaro C. 2011. Domain organization and evolution of multifunctional autoproducting repeats-in-toxin (MARTX) toxin in *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 77:657–668. <http://dx.doi.org/10.1128/AEM.01806-10>.
23. Morrison SS, Williams T, Cain A, Froelich B, Taylor C, Baker-Austin C, Verner-Jeffreys D, Hartnell R, Oliver JD, Gibas CJ. 2012. Pyrosequencing-based comparative genome analysis of *Vibrio vulnificus* environmental isolates. *PLoS One* 7:e37553. <http://dx.doi.org/10.1371/journal.pone.0037553>.
24. Bisharat N, Cohen DI, Harding RM, Falush D, Crook DW, Peto T, Maiden MC. 2005. Hybrid *Vibrio vulnificus*. *Emerg. Infect. Dis.* 11:30–35. <http://dx.doi.org/10.3201/eid1101.040440>.
25. Jolley KA, Chan MS, Maiden MC. 2004. mlstdbNet—distributed multi-locus sequence typing (MLST) databases. *BMC Bioinformatics* 5:86. <http://dx.doi.org/10.1186/1471-2105-5-86>.
26. Philippe N, Alcaraz JP, Coursange E, Geiselmann J, Schneider D. 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. *Plasmid* 51:246–255. <http://dx.doi.org/10.1016/j.plasmid.2004.02.003>.
27. Oka A, Sugisaki H, Takanami M. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. *J. Mol. Biol.* 147:217–226. [http://dx.doi.org/10.1016/0022-2836\(81\)90438-1](http://dx.doi.org/10.1016/0022-2836(81)90438-1).
28. Stols L, Gu M, Dieckman L, Raffin R, Collart FR, Donnelly MI. 2002. A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. *Protein Expr. Purif.* 25:8–15. <http://dx.doi.org/10.1006/prep.2001.1603>.
29. Spyres LM, Qa'Dan M, Meader A, Tomasek JJ, Howard EW, Ballard JD. 2001. Cytosolic delivery and characterization of the TcdB glucosylating domain by using a heterologous protein fusion. *Infect. Immun.* 69:599–601. <http://dx.doi.org/10.1128/IAI.69.1.599-601.2001>.
30. Yahr TL, Vallis AJ, Hancock MK, Barbieri JT, Frank DW. 1998. ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proc. Natl. Acad. Sci. U. S. A.* 95:13899–13904. <http://dx.doi.org/10.1073/pnas.95.23.13899>.
31. Danin-Poleg Y, Elgavish S, Raz N, Efimov V, Kashi Y. 2013. Genome sequence of the pathogenic bacterium *Vibrio vulnificus* biotype 3. *Genome Announc.* 1:e0013613. <http://dx.doi.org/10.1128/genomeA.00136-13>.
32. Chatzidaki-Livanis M, Hubbard MA, Gordon K, Harwood VJ, Wright AC. 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 72:6136–6141. <http://dx.doi.org/10.1128/AEM.00341-06>.
33. Dolores J, Satchell KJ. 2013. Analysis of *Vibrio cholerae* genome sequences reveals unique *rtxA* variants in environmental strains and an *rtxA*-null mutation in recent Altered El Tor isolates. *mBio* 4:e00624–00612. <http://dx.doi.org/10.1128/mBio.00624-12>.
34. Le Roux F, Labreuche Y, Davis BM, Iqbal N, Mangenot S, Goarant C, Mazel D, Waldor MK. 2011. Virulence of an emerging pathogenic lineage of *Vibrio nigrripulchritudo* is dependent on two plasmids. *Environ. Microbiol.* 13:296–306. <http://dx.doi.org/10.1111/j.1462-2920.2010.02329.x>.
35. Geissler B, Tungekar R, Satchell KJ. 2010. Identification of a conserved membrane localization domain within numerous large bacterial protein toxins. *Proc. Natl. Acad. Sci. U. S. A.* 107:5581–5586. <http://dx.doi.org/10.1073/pnas.0908700107>.
36. Guo Q, Shen Y, Lee YS, Gibbs CS, Mrksich M, Tang WJ. 2005. Structural basis for the interaction of Bordetella pertussis adenylate cyclase toxin with calmodulin. *EMBO J.* 24:3190–3201. <http://dx.doi.org/10.1038/sj.emboj.7600800>.
37. Chase E, Harwood VJ. 2011. Comparison of the effects of environmental parameters on growth rates of *Vibrio vulnificus* biotypes I, II, and III by culture and quantitative PCR analysis. *Appl. Environ. Microbiol.* 77:4200–4207. <http://dx.doi.org/10.1128/AEM.00135-11>.
38. Lee JH, Kim MW, Kim BS, Kim SM, Lee BC, Kim TS, Choi SH. 2007. Identification and characterization of the *Vibrio vulnificus* *rtxA* essential for cytotoxicity *in vitro* and virulence in mice. *J. Microbiol.* 45:146–152.
39. Kim YR, Lee SE, Kook H, Yeom JA, Na HS, Kim SY, Chung SS, Choy HE, Rhee JH. 2008. *Vibrio vulnificus* RTX toxin kills host cells only after contact of the bacteria with host cells. *Cell. Microbiol.* 10:848–862. <http://dx.doi.org/10.1111/j.1462-5822.2007.01088.x>.
40. Cordero CL, Kudryashov DS, Reisler E, Satchell KJ. 2006. The actin cross-linking domain of the *Vibrio cholerae* RTX toxin directly catalyzes the covalent cross-linking of actin. *J. Biol. Chem.* 281:32366–32374. <http://dx.doi.org/10.1074/jbc.M605275200>.
41. Ahrens S, Geissler B, Satchell KJ. 2013. Identification of a His-Asp-Cys catalytic triad essential for function of the Rho inactivation domain (RID) of *Vibrio cholerae* MARTX toxin. *J. Biol. Chem.* 288:1397–1408. <http://dx.doi.org/10.1074/jbc.M112.396309>.
42. Hritonenko V, Mun JJ, Tam C, Simon NC, Barbieri JT, Evans DJ, Fleiszig SM. 2011. Adenylate cyclase activity of *Pseudomonas aeruginosa* ExoY can mediate bleb-niche formation in epithelial cells and contributes to virulence. *Microb. Pathog.* 51:305–312. <http://dx.doi.org/10.1016/j.micpath.2011.08.001>.
43. Confer DL, Eaton JW. 1982. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science* 217:948–950. <http://dx.doi.org/10.1126/science.6287574>.
44. Leppla SH. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. U. S. A.* 79:3162–3166. <http://dx.doi.org/10.1073/pnas.79.10.3162>.
45. Liu M, Alice AF, Naka H, Crosa JH. 2007. The HlyU protein is a positive regulator of *rtxA1*, a gene responsible for cytotoxicity and virulence in the human pathogen *Vibrio vulnificus*. *Infect. Immun.* 75:3282–3289. <http://dx.doi.org/10.1128/IAI.00045-07>.
46. Gulig PA, Tucker MS, Thiaville PC, Joseph JL, Brown RN. 2009. USER friendly cloning coupled with chitin-based natural transformation enables rapid mutagenesis of *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 75:4936–4949. <http://dx.doi.org/10.1128/AEM.02564-08>.
47. Rosche TM, Yano Y, Oliver JD. 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiol. Immunol.* 49:381–389. <http://dx.doi.org/10.1111/j.1348-0421.2005.tb03731.x>.
48. Cohen AL, Oliver JD, DePaola A, Feil EJ, Boyd EF. 2007. Emergence of a virulent clade of *Vibrio vulnificus* and correlation with the presence of a 33-kilobase genomic island. *Appl. Environ. Microbiol.* 73:5553–5565. <http://dx.doi.org/10.1128/AEM.00635-07>.
49. Shen Y, Lee YS, Soelaiman S, Bergson P, Lu D, Chen A, Beckingham K, Grabarek Z, Mrksich M, Tang WJ. 2002. Physiological calcium concentrations regulate calmodulin binding and catalysis of adenylate cyclase exotoxins. *EMBO J.* 21:6721–6732. <http://dx.doi.org/10.1093/emboj/cdf681>.
50. Kudryashov DS, Cordero CL, Reisler E, Satchell KJ. 2008. Characterization of the enzymatic activity of the actin cross-linking domain from the *Vibrio cholerae* MARTX_{vc} toxin. *J. Biol. Chem.* 283:445–452. <http://dx.doi.org/10.1074/jbc.M703910200>.
51. Prochazkova K, Satchell KJ. 2008. Structure-function analysis of inositol hexakisphosphate-induced autoproducting of the *Vibrio cholerae* multifunctional autoproducting RTX toxin. *J. Biol. Chem.* 283:23656–23664. <http://dx.doi.org/10.1074/jbc.M803334200>.
52. Prochazkova K, Shuvalova LA, Minasov G, Voburka Z, Anderson WF, Satchell KJ. 2009. Structural and molecular mechanism for autoproducting of MARTX toxin of *Vibrio cholerae* at multiple sites. *J. Biol. Chem.* 284:26557–26568. <http://dx.doi.org/10.1074/jbc.M109.025510>.
53. Ochoa CD, Alexeyev M, Pastukh V, Balczon R, Stevens T. 2012. *Pseudomonas aeruginosa* exotoxin Y is a promiscuous cyclase that increases endothelial tau phosphorylation and permeability. *J. Biol. Chem.* 287:25407–25418. <http://dx.doi.org/10.1074/jbc.M111.301440>.
54. Lee TH, Kim MH, Lee CS, Lee JH, Rhee JH, Chung KM. 2014. Protection against *Vibrio vulnificus* infection by active and passive immunization with the C-terminal region of the RtxA1/MARTX_{vc} protein. *Vaccine* 32:271–276. <http://dx.doi.org/10.1016/j.vaccine.2013.11.019>.
55. Phillips KE, Schipma MJ, Satchell KJF. 2014. Draft genome sequence of Israeli outbreak-associated *Vibrio vulnificus* biotype 3 clinical isolate BAA87. *Genome Announc.* 2:e00032-14. <http://dx.doi.org/10.1128/genomeA.00032-14>.