# Multifunctional-autoprocessing repeats-in-toxin (MARTX) Toxins of *Vibrios*

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## Abstract

Multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins are a heterogeneous group of toxins found in a number of *Vibrio* species and other Gram-negative bacteria. The toxins are composed of conserved repeat regions and an autoprocessing protease domain that together function as a delivery platform for transfer of cytotoxic and cytopathic domains into target eukaryotic cell cytosol. Within the cells, the effectors can alter biological processes such as signaling or cytoskeletal structure, presumably to the benefit of the bacterium. Ten effector domains are found in the various *Vibrio* MARTX toxins, although any one toxin carries only two to five effector domains. The specific toxin variant expressed by a species can be modified by homologous recombination to acquire or lose effector domains, such that different strains within the same species can express distinct variants of the toxins. This review examines the conserved structural elements of the MARTX toxins and details the different toxin arrangements carried by *Vibrio* species and strains. The catalytic function of domains and how the toxins are linked to pathogenesis of human and animals is described.

# INTRODUCTION

Multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins are large single polypeptide toxins produced by various Gram-negative bacteria that interact with a wide range of insect, mammalian, and aquatic animal hosts. The toxins range in size from 3,500 to 5,300 amino acids and are frequently encoded by the largest open reading frames of a bacterial genome (1). A search of the National Center for Biotechnology Information database revealed that, within the *Vibrio* genus, an *rtxA* gene that could encode a full-length MARTX toxin are present in the genomes of six different species: *Vibrio cholerae, V. vulnificus, V. anguillarum, V. ordalli, V. splendidus, and V. caribbeanicus*. Toxin genes are also present on large plas-mids isolated from *V. vulnificus* and *V. nigripulchritudo*.

Similar to contact-dependent bacterial secretion mechanisms, such as type III secretion (T3S) and type IV secretion (T4S), the ultimate result of delivery of a MARTX toxin to

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target eukaryotic cells is transfer of cytotoxic and cytopathic effectors that disrupt normal eukaryotic cell biology (2, 3), presumably to the benefit of the infectious or commensal organism (Fig. 1). Consistent with the concept that MARTX toxins are an alternate effector delivery platform utilized in bacteria lacking T3S and T4S systems, the common seafood-borne pathogens *V. parahaemolyticus* (4) and *V. alginolyticus* (5) do not have *rtxA* genes, and presumably, instead use their T3S contact-dependent system to deliver effectors to target cells (6). *V. anguillarum* strain 96F interestingly has both a T3S system and encodes a MARTX toxin while other *V. anguillarum* strains have lost many of their T3S genes (7). Thus, it seems probable that trans-location of effectors by vibrios is often important and that either T3S and/or MARTX toxins are used for this function.

# MARTX TOXIN STRUCTURE, SECRETION, AND EFFECTOR TRANSLOCATION

## MARTX Toxin Repeat Structure Is Conserved across the Vibrios

All MARTX toxins have regions at the amino- and carboxyl-termini of strong amino acid sequence similarity that are characterized by extensive glycine rich repeats (1, 8). The amino-terminal conserved region is usually 1,950 to 1,970 amino acids in length and can be separated into distinct regions. (i) The N-terminal extension of ~73 amino acids; (ii) the 19 amino acid A-repeats comprised of 14 arrayed repeats interrupted by a 290 amino acid insertion between repeats A10 and A11; (iii) 38 copies of the 20 amino acid B-repeats; and (iv) the B-repeat/effector junction zone that is a region of high sequence variability between toxins both between species and within the same species (Fig. 2). The carboxyl-terminus conserved region includes two to three additional copies of the B-repeat and 15 copies of a C-repeat (1). The C-repeats are similar to the classical nonapeptide calcium-binding repeat of all repeats-in-toxin (RTX) family proteins (9, 10), except the repeat is 18 amino acids with only half the repeat representing the GD-rich RTX repeat (8). The structural implications of the longer repeat are as yet uncharacterized.

## MARTX Toxin Secretion from the Bacterium

The extreme carboxyl-terminus of a MARTX toxin, like all RTX proteins, presumably is the site of the type I secretion (T1S) signal for export, such that even small deletions that affect the carboxy-terminus can disrupt function of the entire toxin (11). Secretion from the bacterium is mediated by an atypical T1S secretion system comprised of ATPases RtxB and RtxE, a trans-membrane linker RtxD, and the outer membrane porin TolC for direct transfer of the toxin from the cytosol to the extracellular environment (12, 13, 14, 15). The extracellular intermediate of toxin delivery has been verified by detection of toxin in bacterial-free culture supernatant fluids by activity, western blotting, and mass spectrometry (12, 13, 16, 17, 18, 19). The MARTX toxin from *V. cholerae* has also been suggested to be associated with outer membrane vesicles, but this is apparently not the case with *V. vulnificus* MARTX (12, 20).

### Toxin Translocation into Eukaryotic Target Cells

MARTX toxins have been shown to be functional against a wide range of eukaryotic cells types, including erythrocytes, epithelial cells, and phagocytic cells and cells of human, mouse, fish, and eel origins (16, 19, 21, 22, 23, 24, 25, 26, 27). Once secreted from the bacterium, the repeat regions of the toxins are postulated to bind to eukaryotic receptors on target cells where they have been shown to form a pore for trans-location of the central portion of the toxin across the plasma membrane to the eukaryotic cell cytoplasm (26, 28, 29). The portion of the toxin translocated is comprised of domains with low thermostability, suggesting they are easily unfolded for translocation across the plasma membrane (26, 30).

One domain delivered across the plasma membrane is the cysteine protease domain (CPD) (31), which is activated after translocation to the eukaryotic cell cytoplasm by binding of inositol hexakisphosphate (InsP$_6$) (32). Upon binding InsP$_6$, folding of the CPD is completed and the protease is able to autoprocess the MARTX toxin after leucine residues present in extended unfolded regions found between "effector domains" that confer the cytotoxic functions of the toxin (3, 17, 33). In total, ten different effector domains have been recognized among the various MARTX toxins, although any one toxin carries only two to five effectors (Fig. 3). The domains are assorted across different strains and species and they can be exchanged by homologous recombination. This swapping of *rtxA* effector domain gene sequences results in heterogeneity of the toxins even between different isolates within the same species (11, 34, 35).

## MARTX TOXINS OF *V. CHOLERAE*

### Structure and Function of Most Common MARTX of *V. cholerae*

*V. cholerae* is the pathogen that causes the human diarrheal disease cholera (36). The MARTX toxin of *V. cholerae* El Tor strain N16961 was the first to be discovered and is the representative member of this toxin family (8). This representative toxin, which is produced by most strains of *V. cholerae*, is 4,545 amino acids in length and shows remarkable conservation in sequence (11). An analysis of nearly 70 genome sequences shows that among the El Tor and El Tor-like strains that have caused cholera during the most recent century, the gene has been highly stable with individual isolates showing only one to six SNPs over the course of 100 years resulting in only one to four amino acid changes. This lack of genetic diversity does not reflect specialized conservation of the *rtxA* gene, but rather the stability of the El Tor genomes as a whole (11). The *rtxA* gene in environmental strains also shows strong conservation (only 1% to 2% amino acid differences), with the overall length and organization of the toxin highly conserved (11, 37).

This common variant of *V. cholerae* MARTX toxin carries three effector domains (17, 33). In the first position immediately downstream of the MARTX conserved region is the actin crosslinking domain (ACD), an enzyme related to glutamine sythetases that covalently crosslinks G-actin protomers via introduction of an isopeptide bond between lysine 50 and glutamic acid 270 (16, 38, 39, 40). Similar to the catalytic action of glutamine sythetases, the reaction involves the initial formation of an activated acyl phosphate intermediate formed by the transfer of phosphate from the hydrolysis of ATP to glutamic acid 270

followed by hydrolysis to energize formation of the amide crosslink (41, 42). The crosslinking of actin then causes depolymerization of the actin cytoskeleton both through the removal of G-actin as a substrate for actin assembly and by addition of actin dimers to growing F-actin blocking further filament assembly (40, 41).

The next domain in the *V. cholerae* MARTX is the Rho inactivation domain (RID). This effector is a member of the circular permuted thiol peptidase family, although the RID is predicted to have a distinct arrangement of its catalytic site compared to other proteins in this family (43, 44). After delivery to the cytosol by processing from CPD, the presence of a four helical bundle type membrane localization domain targets the RID to the plasma membrane (45, 46). At the membrane, the cellular protein modified or proteolyzed by the RID is unknown, but the enzymatic modification causes a change in cellular signaling resulting in loss of all active GTP-bound Rho. As Rho is a master regulator of stress fiber assembly, cells treated with RID round due to lack of cytoskeletal assembly (47). Interestingly, although both ACD and RID independently affect polymerized actin, the domains are not synergistic, and thus, neither actin crosslinking nor Rho inactivation is accelerated by the presence of the other domain in the same toxin (26, 44).

The effector located between RID and CPD is the alpha/beta hydrolase (ABH) effector. Sequence analysis indicates this effector is a member of the expansive structural protein family known as the alpha/beta hydrolases that includes proteases, lipases, and esterases (48, 49). This domain has been shown to indirectly activate the small GTPase CDC42. Interestingly, this activity is antagonistic with RID, which inactivates CDC42, such that a toxin with both domains shows little overall change in CDC42 activation. Thus, the biological consequence of the ABH domain remains currently unknown (26).

### Role of *V. cholerae* MARTX in Cholera Pathogenesis

The loss of cell structure due to actin depolymerization by *V. cholerae* has been linked both with loss of tight junction integrity of polarized intestinal epithelial cell and with inhibition of phagocytosis (26, 50). It unclear if affects on epithelial cells are important *in vivo* as only minimal damage occurs to the intestine during cholera disease (51, 52). By contrast, consistent with a primary role for inhibition of phagocytosis during infection, the MARTX toxin of *V. cholerae* is linked to evasion of innate immune defenses during intestinal infection of mice. The toxin works in concert with another secreted toxin, the cytolysin/hemolysin HlyA, such that strains deleted of both *hlyA* and *rtxA* genes fail to colonize the small intestine of mice and are avirulent (52, 53). The contribution of these toxins *in vivo* is, in part, to inhibit clearance of the infecting bacteria from the small intestine by neutrophils, accounting for their requirement for bacterial colonization (54).

Given the role of the MARTX toxin in innate immune evasion rather than diarrheal disease, and its apparent redundancy with HlyA, it is not surprising that some strains would eliminate the toxin entirely. Both classical strains that caused cholera epidemics during the 19th century and the currently circulating altered El Tor strains have either a large deletion or an introduction of an opal stop codon in the *rtxA* gene that renders these strains *rtxA* null mutants (8, 11). These strains also have sequence differences in the cholera toxin B (CtxB) sub-unit of cholera toxin, the primary toxin associated with diarrhea (55). It has been

postulated that CtxB may control immunity during infection just as it does when functioning as a vaccine adjuvant (56). Thus, it is possible that the MARTX likely became redundant in both classical and altered El Tor strains that carry the modified CtxB and it was thus eliminated by spontaneous mutation (8, 11).

### Rare *V. cholerae* MARTX Toxin Variants among Environmental Isolates

Environmental strains of *V. cholerae* are not commonly associated with disease but can cause sporadic outbreaks or food-borne infection (57, 58, 59, 60, 61). Although less conserved at the amino acid level than the El Tor strains, the MARTX toxins of the environmental strains with genomes tightly linked with the *V. cholerae* human isolates do predominantly retain the ACD-RID-ABH effector domain structure (11). However, among the extensive database of sequenced environmental isolates are strains that have a distinct effector domain structure. The strain RC385 isolated from Chesapeake Bay has a unique domain structure that includes an adenylate cyclase (AC) similar to the T3S effector ExoY from *Pseudomonas aeruginosa* (11, 62, 63). This AC domain is flanked by duplicated copies of a novel domain of unknown function that has been designated Domain X (DmX) (63). This domain is present also in one of four MARTX toxins of the insect pathogen *Photorhabdus asymbiotica* and in *V. vulnificus* biotype 3 strains (see below) (11, 63, 64).

The non-O1, non-O139 strain HE-09 isolated from the environment in Haiti (11, 37) and strain PS-15 isolated from Puget Sound in Washington State (65) also have unique toxin structures. For both strains, the toxins have in the first position, DUF1, a domain of unknown function that has no homology to any non-MARTX proteins. This domain is present in the first position of a number of other MARTX toxins including those from *V. vulnificus* (34, 35) and the nematode symbionts *Xenorhabdus bovienii* and *Xenorhabdus nematophila* (29, 66). In the second position is a RID domain similar to the common arrangement of *V. cholerae* MARTX toxins. The two strains vary however in the third position. For PS-15, the domain of unknown function in the third position (known as DUF5) shares limited homology with the C1 and C2 domains of *Pasteurella multocida* toxin (PMT) and has been partially characterized in *V. vulnificus* as a cytotoxin (see below). In HE-09, the third domain is a novel domain denoted as VIP2, as it is similar to the catalytic domain of ADP-ribosyltransferase toxins that ADP-ribosylate G-actin, including *Clostridium botulinum* C2 toxin (67), *Aeromonas hydrophila* VgrG1 (68), and *Bacillus cereus* VIP2 (69).

Thus, with the increasing monitoring of *V. cholerae* in the environment by genome sequencing, there is the potential greater diversity of this toxin will be revealed, suggesting *V. cholerae* is altering its MARTX toxin as part of its niche adaptation. However, both clinical and environmental isolates that are closely linked to human pathogenesis all share a highly conserved three domain (ACD-RID-ABH) structure that does not vary, suggesting that this particular arrangement of the toxin is potentially linked to its role in human infection.

# MARTX TOXINS OF *V. VULNIFICUS*

## Extensive Diversity among *V. vulnificus* MARTX Toxins

*V. vulnificus* is a pathogen that causes wound and food-borne intestinal diseases, both of which can spread from the site of initial infection to cause sepsis with high mortality rates (70). In contrast to the conservation of the MARTX toxin domain structure among most of the *V. cholerae* isolates, the *V. vulnificus* MARTX toxins are strikingly diverse (Fig. 3) (34, 35, 63). Interestingly, variation does not seem to track with multi-locus sequence typing-based evolutionary trees, indicating that variation arises by homologous recombination between strains rather that clonal expansion of strains after the *rtxA1* gene has recombined (34, 35).

Among the biotype 1 *V. vulnificus* isolates most commonly associated with clinical infections, there are two prominent MARTX toxin arrangements. The first is the five effector arrangement represented by sequenced clinical isolates, CMCP6 (71) and YJ016 (72), and also identified in sequenced environmental isolate, JY1305 (73). This variant has been independently termed MARTX/C (35) or type I (34). These strains have DUF1 in the first position, RID in the second position, ABH in the third position. The domain in the fourth position has homology to a portion of the *Makes caterpillars floppy* (Mcf) toxins of *Photorhabdus luminescens* (74, 75). This domain has recently been shown to be an autoprocessing cysteine protease associated with a cytopathic cell rounding (76).

The DUF5 effector domain in the fifth position has homology to PMT as noted above, but this does not inform about its function, since the aligned domain in PMT has not been characterized. Indeed, expression of DUF5 in both mammalian cell and yeast is cytotoxic, although expression of the aligned domain from PMT does not apparently affect the cells (77). The cellular target of DUF5 is currently unknown but is likely to be a membrane protein. DUF5, similar to *V. cholerae* RID, has a functional four helical bundle type membrane localization domain and DUF5 has been found to tightly localize to cellular junctions (45, 46). Surprisingly, within the conserved membrane localization domain for *V. vulnificus* RID, many strains have a point mutation in a critical residue that affects protein localization; therefore, RID from *V. vulnificus* is not predicted to localize to eukaryotic cell membranes and it is unclear if it will be functional after translocation to cells (46).

The second prominent MARTX toxin arrangement of *V. vulnificus* biotype 1 strains of the clinical clade is thought to have arisen by homologous recombination in the *rtxA1* gene, apparently with *rtxA* DNA acquired from *V. anguillarum*. Independently, the same arrangement arose in biotype 1 strains within the environmental clade by recombination with *rtxA* DNA, possibly from a *V. vulnificus* plasmid. Both recombination events resulted in *rtxA1* genes that encode for MARTX toxins with only four effectors and missing the DUF5 effector domain (35). This variant is termed MARTX/M (35) or type II (34) and is encoded by many clinical isolates including sequenced strains MO6–24/O, ATCC 27562, and B2 (34, 35, 78, 79, 80).

Other variants of the MARTX toxin have arisen among clinical and environmental biotype 1 isolates and these include a mosaic of effector domains, including those found in CMCP6

and M06–24/O, but also toxins with actin crosslinking activity due to the ACD (35), predicted actin ADP-ribosylating toxins due to the VIP2 domain, and predicted AC toxins due to the acquisition of the ExoY-like AC domain (63). The promiscuous nature of the recombination is evident in environmental isolate E64MW, which has complete AC and VIP2 domains, but also incomplete remnants of ABH and MCF domains from the progenitor toxins (Fig. 3) (63, 73).

## Cell Biological Effects of Intoxication by *V. vulnificus* MARTX

The most prominent activity of both MARTX/C-type (or type I) and MARTX/M-type (or type II) biotype 1 MARTX toxins is the ability to induce cytolysis of a broad range of cell types including erythrocytes, epithelial cells, and macrophages (21, 22, 24, 27, 81). The lysis had been proposed to occur by calcium-dependent necrosis in epithelial cells dependent on the carboxy-terminal repeat region plus the CPD (18). However, the relevance of this finding to programmed necrotic epithelial cell death is as yet unclear as CPD proteins alone are known to induce cell lysis when transfected (31), but not when presented from the bacterium (44). In addition, more recent studies show that calcium in the media is required for secretion of the toxin from the bacterium, not for necrosis, and that cell lysis depends on both the amino and carboxy repeat regions of the toxin (28). Moreover, protein corresponding to the C-terminus does not induce necrosis when added to cell culture media (82).

At lower multiplicity of infection or short incubation times such that epithelial cells are not lysed, *V. vulnificus* with the common MARTX/C (type I) and MARTX/M (type II) arrangements have been linked to activities other than cytolysis. These include induction of caspase 3-dependent apoptosis (83), cytoskeleton disassembly (84), and activation of Rac2 and Nox for increased generation of reactive oxygen species (ROS) (85). When the bacteria are added to cultured macrophages, the action of the MARTX toxin of *V. vulnificus* inhibits bacterial killing (27). At low multiplicity of infection, macrophages are also known to undergo NLRP3-dependent inflammasome activation to cleave caspase-1 and to release the proinflammatory cytokine IL-1β (86). How each of these activities are linked to the various domains of the large toxin has not, as yet, been determined.

## Role of Biotype 1 *V. vulnificus* MARTX Toxin during Mouse Infection

The MARTX toxin of *V. vulnificus* has been shown to be essential for pathogenesis of several mouse models. In models of septicemia where bacteria are delivered intraperitoneally, mutants disrupted in the gene *rtxA1* show a 250- to 450-fold increase in $LD_{50}$ in ironoverloaded mice (21, 22) and a 100- to 150-fold increase in normal mice (24, 27). These data show that the MARTX toxin is important for septicemic disease.

During subcutaneous infection to mimic wound infection, *rtxA1* exerted a 72-fold effect on virulence in normal mice, but only a 13-fold defect in virulence in neutropenic mice. In addition to decreased virulence, *rtxA1* mutants are defective for colonization of the wound site, and thereby, do not successfully spread from the initial site of infection and are cleared. All together, these data support a role for the MARTX toxin in innate immune evasion and

suggest that the MARTX toxin has a shared role with other secreted factors during wound infection (27).

For food-borne infection, the MARTX toxin has been shown to have a more significant impact on infection than either septicemic or wound-induced models. Initial studies with MARTX/M (or type II) variant strain M06–24/O indicated that *rtxA1* mutants are ~100-fold less virulent than wild type in intragastrically inoculated infant mice (24), and these data are similar to findings in intragastrically inoculated adult mice where an *rtxA1* mutant in M06–24/O is 180-fold less virulent (35). Yet, a similar mutant in the MARTX/C-type (or type I) MARTX variant strain CMCP6 exerted a 2,600-fold effect on virulence. Conversion of the MARTX/C-type toxin in CMCP6 to the MARTX/M type toxin by recombination decreased virulence by 54-fold indicating that, for the food-borne route of infection, the MARTX/ C-type toxin is more potent than the MARTX/M-type toxin (35). These data indicate that there may be selection in the environment for decreased virulence of *V. vulnificus* during intestinal infection of aquatic animals, perhaps to avoid killing fish or other animals that become colonized with *V. vulnificus*.

During intestinal infection, similar to wound infection, the toxin also has a role in promoting growth of the bacterium within the small intestine such that mice infected with mutants without the toxin have increased survival (81). However, apparently in contrast to wound infection where other secreted factors contribute to tissue destruction (27), necrosis of the intestinal villi is directly caused by the MARTX toxin with a secondary additive effect with the secreted cytolysin/hemolysin VvhA. The combination of cytotoxins destroys the intestinal epithelium, providing a route for bacterial dissemination to the bloodstream and other organs (81). The more significant role of the MARTX toxin in this process makes it the most important virulence factor contributing to *V. vulnificus* food-borne disease and accounts for the more significant impact of the toxin during $LD_{50}$ studies of CMCP6 MARTX on intestinal infection (35) compared to wound and septicemic models (22, 27).

## Biotype 2 MARTX Toxins and their Role in Infection

*V. vulnificus* biotype 2 strains are associated with vibriosis in fish and sporadic infections in humans (87). Two prevalent serovars have been described with both serovar A and serovar E able to cause disease in eels, sea bass, tilapia, and trout, although strains of serovar E are more virulent and able to cause disease in both sea bass and trout at lower inocula levels. Colonization studies indicate that serovar A strains predominantly colonize the anus and mouth, while serovar E strains colonize the gills (88, 89).

All biotype 2 strains, including the representative strain CECT4999, carry a plasmid that encodes an entire *rtx* locus (90, 91). This plasmid is essential for virulence of eels due not to the *rtxA* gene, but to a different RTX-family toxin encoded by *vep07*, which enhances bacterial survival in eel serum (91). The reason for the lack of essentiality of the plasmid-encoded MARTX toxin is that the *rtxA* gene on the plasmid is a duplicate of a toxin encoded on the chromosome (34). All biotype 2 chromosomal- and plasmid-encoded MARTX toxins share the same domain structure, known as MARTX type III (34, 91). They all have an ACD in the first position, suggesting these toxins have actin crosslinking activity similar to *V. cholerae* and the rare variants of the biotype 1 toxins that have the same overall structure

(35). The ACD is followed by an MCF domain, an ABH domain, and then a second copy of MCF (91). The implication of the duplication of MCF is unknown as the function of this domain has not, as yet, been elucidated (92).

Deletion of either the plasmid or the chromosomal *rtxA* gene copy has minimal impact on virulence of either mice or eels. However, loss of both copies rendered the bacterium essentially avirulent for eels both by intraperitoneal infection or by immersion inoculation (23). The same strain showed a 100-fold decreased virulence in mice (23) similar to the impact of the *rtxA1* toxin genes of *V. vulnificus* biotype 1 strains during in-traperitoneal infection of noniron overloaded mice (24, 27). Thus, the function of this toxin is absolutely essential for virulence of eels and likely for sporadic infection of humans. However, a strain with deletions in both copies of *rtxA* was able to colonize the gills of eels, invade to the bloodstream, and colonize other organs, in some cases better than wild type bacteria. Yet, the eels do not succumb to infection. The difference in infection appears to be that the toxins enhance survival in eel blood, kidneys, and liver, indicating that growth of bacteria in these organs and presence of the toxin itself may be directly linked to animal death from septic shock (23). Further, the *rtxA*-encoded toxins appear to be essential for protection from phagocytic cells and from predation by amoeba that colonize the gills of eels (23). How both copies of the genes and whether there are individual differences in the contribution of the toxins to virulence remains to be investigated.

### Biotype 3 MARTX Toxins and their Role in Infection

Biotype 3 strains are thought to have emerged from biotype 1 and are hybrids of the two major clades of the biotype 1 strains (93, 94). The clonal biotype 3 strain is notable as it was responsible for a major outbreak of *V. vulnificus* wound infections linked to tilapia ponds and aquaculture during the late 1990s (95). Although the outbreak was abated by changes in fish marketing practices, infections with *V. vulnificus* biotype 3 still occur in Israel associated with tilapia and wild carp (96). Two genomes of biotype 3 strains are available and analysis of these reveals that they have a unique structure of the MARTX toxin (63, 97, 98). These toxins, similar to the common biotype 1 arrangements, have DUF1-RID-ABH in the first three positions. However, the last two positions have been swapped for the Exo Y-like AC domain and DmX (63). In the strain ATCC BAA87, isolated from a wound during the original Israel outbreak, the *rtxA1* gene that encodes the MARTX toxin was found to be essential for wound-induced virulence in mice. In tissue culture, the acquisition of the ExoY-like AC domain was correlated with an *rtxA1*-dependent acquisition of the adenylate cyclase activity and this activity was directly due to newly acquired AC domain (63). How gain of the AC domain contributed to the virulence of this strain is not yet known, but it is intriguing to consider that this exchange contributed to the local emergence of this pathogen in Israel.

Indeed, as a global concept, given the extensive variation of the *V. vulnificus* MARTX toxins, it will be interesting to consider how this extensive swapping of domains between strains and species could contribute in the future to emergence of sudden outbreaks and epidemics.

## MARTX TOXINS OF *V. ANGUILLARUM* AND *V. ORDALLI*

*V. anguillarum* (occasionally known as *Listonella anguillarum*) and the closely related species *V. ordalli* (formerly *V. anguillarum* biotype 2) are marine pathogens that cause hemorrhagic septicemia in cultured and wild fish, mollusks, and crustaceans (99, 100).

The representative isolate *V. anguillarum* M93 (serotype J-O-1) originally isolated from an ayu fish in Japan (101) produces a MARTX toxin that has been shown to be the major virulence factor for vibriosis. Using juvenile Atlantic salmon as a model, an isogenic *rtxA* mutant is avirulent at a dose that causes 100% fatality from the wild type strain. The toxin produced by M93 is hemolytic and induces rounding of salmon kidney cells (19). As encoded in M93, the MARTX toxin has three domains: RID, ABH, and MCF with the presence of a RID domain consistent with studies demonstrating *V. cholerae* RID induces rounding of cells (19, 47).

The MARTX toxin of the *V. anguillarum* O2β serotype strain RV22 isolated from a turbot fish in Spain (102) is identical to the toxin of M93 except for three conservative amino acid changes arising from only four single nucleotide polymorphisms across the 13,200 nt gene (7). Thus, despite having been isolated from drastically different geographic zones, the toxins are virtually unchanged.

By contrast, the O1 strain 775 isolated from Coho salmon in Washington state (7, 103) and the isolate NB10 from rainbow trout in Sweden (104) (GenBank LK021130.1) have a different MARTX toxin organization, although the amino acid sequences are identical despite the disparate geographical locations. This toxin has a remnant of a RID, but the toxin has undergone a recombination to acquire VIP2 and AC domains in place of the ABH and MCF domains. The toxin undergoes a further change in strain 96F (isolated from striped bass in Chesapeake Bay) where the AC domain is retained in the last position, but acquires ACD and MCF in the first and second positions (7, 105). Finally, the closely related species *V. ordalli* 33509 isolated from Coho salmon in the Puget Sound has another distinct arrangement consisting solely of VIP2 and AC domains (7, 106). How all this heterogeneity accounts for colonization of different species, different organs within species, or clinical outcome of disease is currently not known but merits further investigation given the economic importance of this disease to the aquaculture industry (99).

## PLASMID-ASSOCIATED MARTX TOXIN OF *V. NIGRIPULCHRITUDOM*

The shrimp pathogen, *V. nigripulchritudo*, has gene sequences for a MARTX toxin that has four domains comprised of DmX, DUF1, RID, and AC domains. The *rtxA* toxin gene and the necessary maturation and secretion genes are carried on a large 247-kilobase plasmid that also carries sequences for a siderophore and a metalloprotease. This plasmid has been identified in highly pathogenic and moderately pathogenic strains, but has a modified structure in nonpathogenic strains including absence of the *rtx* locus. During infection studies, the plasmid is essential for virulence in shrimp injected intramuscularly. Future studies will be necessary to determine if the requirement for the plasmid in pathogenesis is due to the gene for the MARTX toxin directly or due to other genes (107).

## MARTX TOXIN VARIATION IN *V. SPLENDIDUS*

*V. splendidus* is a heterogeneous bacterium found in seawater. For studies of genetic diversity and evolution, many strains have been subjected to whole genome sequencing and five of these have assembled contiguous sequences that carry genes for full-length MARTX toxins. Strains 12F01 and 12B01 are closely related by *hsp60* sequencing and pulse-field gel electrophoresis genotyping (108), and they encode an identical MARTX toxin. Other isolates, FF-6, and possibly, FF-500, also encode an identical toxin (109). In the first position of this toxin is a domain of unknown function from MARTX toxins of the insect pathogens, *P. luminescens* and *P. asymbiotica* (PasyHD), that has otherwise not been identified among the *Vibrio* genus (1). This domain includes a subdomain that in the *Bordetella pertussis* type III secretion effector BteA has been shown to direct the effector to ezrin-rich lipid rafts (110). Other domains of the *V. splendidus* toxin are the ABH, MCF, and DUF5.

Two other *V. splendidus* genomes, 5S-101 and ZF-90, encode a different toxin in which the PasyHD domain has been swapped with an ACD, but otherwise is highly conserved (111). No investigations with these toxins have been performed; therefore, the role of these toxins in the lifestyle of *V. splendidus* is currently unknown.

## MARTX TOXIN OF MARINE SPONGE-ASSOCIATED *V. CARIBBEANICUS*

The final *Vibrio* species that genome analysis reveals carries a gene for a MARTX toxin is *V. caribbeanicus*. This is a recently recognized *Vibrio* species isolated from a marine sponge in the Caribbean Sea off the coast of Curaçao (112). The type strain N384(T) (ATCC BAA-2122) has been sequenced and the genome deposited as *V. caribbenthicus* (GenBank AEIU00000000). Sequence analysis indicates this strain produces a MARTX toxin with RID, ABH, and AC domains, but no other information is known about this species or how the toxin might be advantageous to its interaction with the sponge.

## SUMMARY

Overall, the vibrios are a rich source of MARTX toxins that are likely to have a range of functional activities. In addition to variation across the effector domains, it is likely that sequence variations within the repeat regions will contribute to host selection or tissue distribution within a host. It is interesting to speculate that each *Vibrio* isolate is exquisitely selected to colonize or infect its specific host and has achieved this by evolving its MARTX toxin to carry a unique combination of conserved region modifications for receptor recognition coupled to an optimal effector domain repertoire.

In some cases, it is possible that a species is still sampling the optimal configuration to occupy a certain niche, accounting for the many different variants found within some species such as *V. vulnificus*. By contrast, the conservation of the toxin arrangement, for example in *V. cholerae*, would suggest that these strains are already optimally suited to their particular niche, and thus, a specific arrangement has already been evolu-tionarily selected. In the future, additional studies regarding how the conserved regions contribute to toxin specificity needs to be addressed along with how different effector domain arrangements

contribute to host-bacterial interactions. Thus far, investigations have focused on connection of the holotoxins to pathogenesis with limited consideration for the function of any one domain or how domain changes can alter the patho-genesis program. In addition, for other *Vibrio* species, future studies could determine if the MARTX toxin can contribute to the development of a commensal relationship or if they are solely related to induction of disease in a variety of animal hosts.

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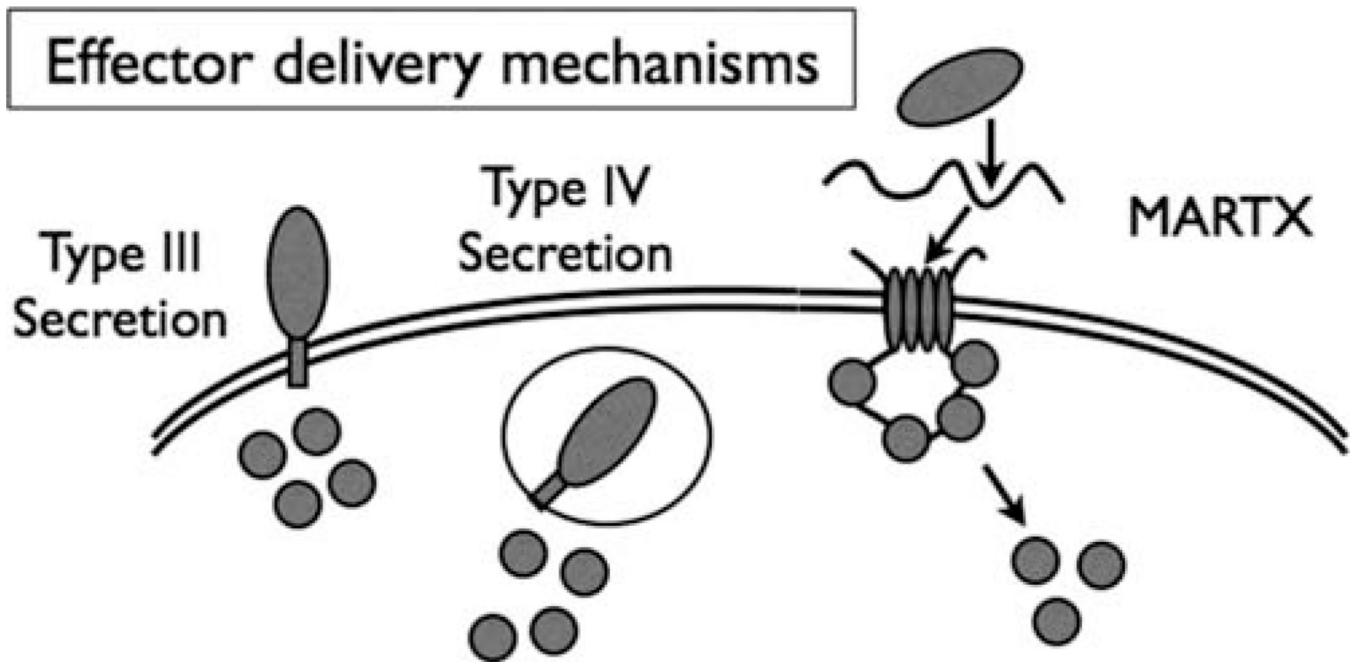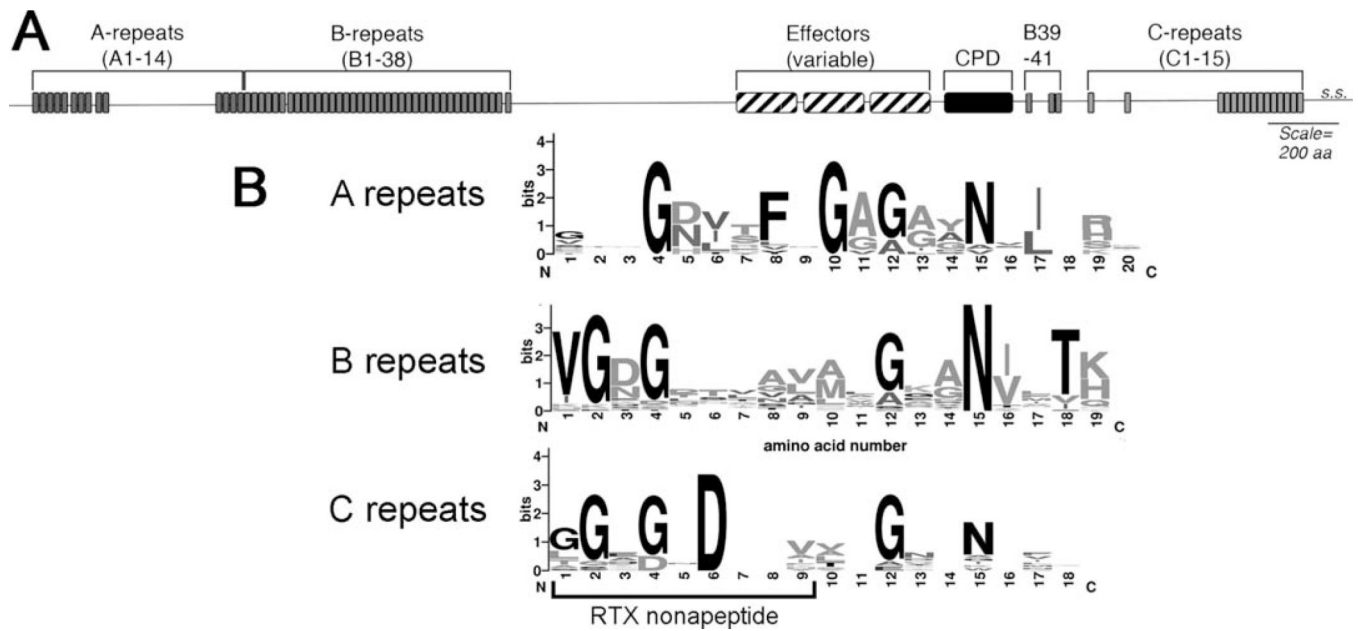
**FIGURE 1.**
The multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins are a form of effector delivery similar in concept to contact-dependent type III secretion and type IV secretion. The major difference is that the toxin is secreted from the bacterium by type I secretion and then the large single polypeptide toxin delivers effectors directly across the plasma membrane with delivery occurring by polypeptide autoprocessing. Similar to type III and type IV secretion, the effector themselves confer cytopathic and cytotoxic activities that then alter host-cell biology to the benefit of the bacterium. doi:10.1128/microbiolspec.VE-0002-2014.f1

**FIGURE 2.**
(A) The general structure of a multifunctional-autoprocessing repeats-in-toxin (MARTX) toxin showing the number and position of various repeat sequences, auto-processing cysteine protease domain (CPD), and variable region containing the effector domains. The secretion signal (s.s.) is shown at the extreme C-terminus. (B) Graphical representation of the different repeat sequences generated by Weblogo 2.8.2 [(113); weblogo.berkeley.edu]. The sequences used were repeat sequences from *Vibrio vulnificus* CMCP6 identified based on the alignment of sequence to the repeat annotation of *V. cholerae* (1). The portion of the C-repeat that aligns to the calcium-binding beta roll nonapeptide repeat of other RTX family proteins is indicated. Note conservation of a G-7x–G-4x–N repeat in all of the repeats. doi: 10.1128/microbiolspec.VE-0002-2014.f2
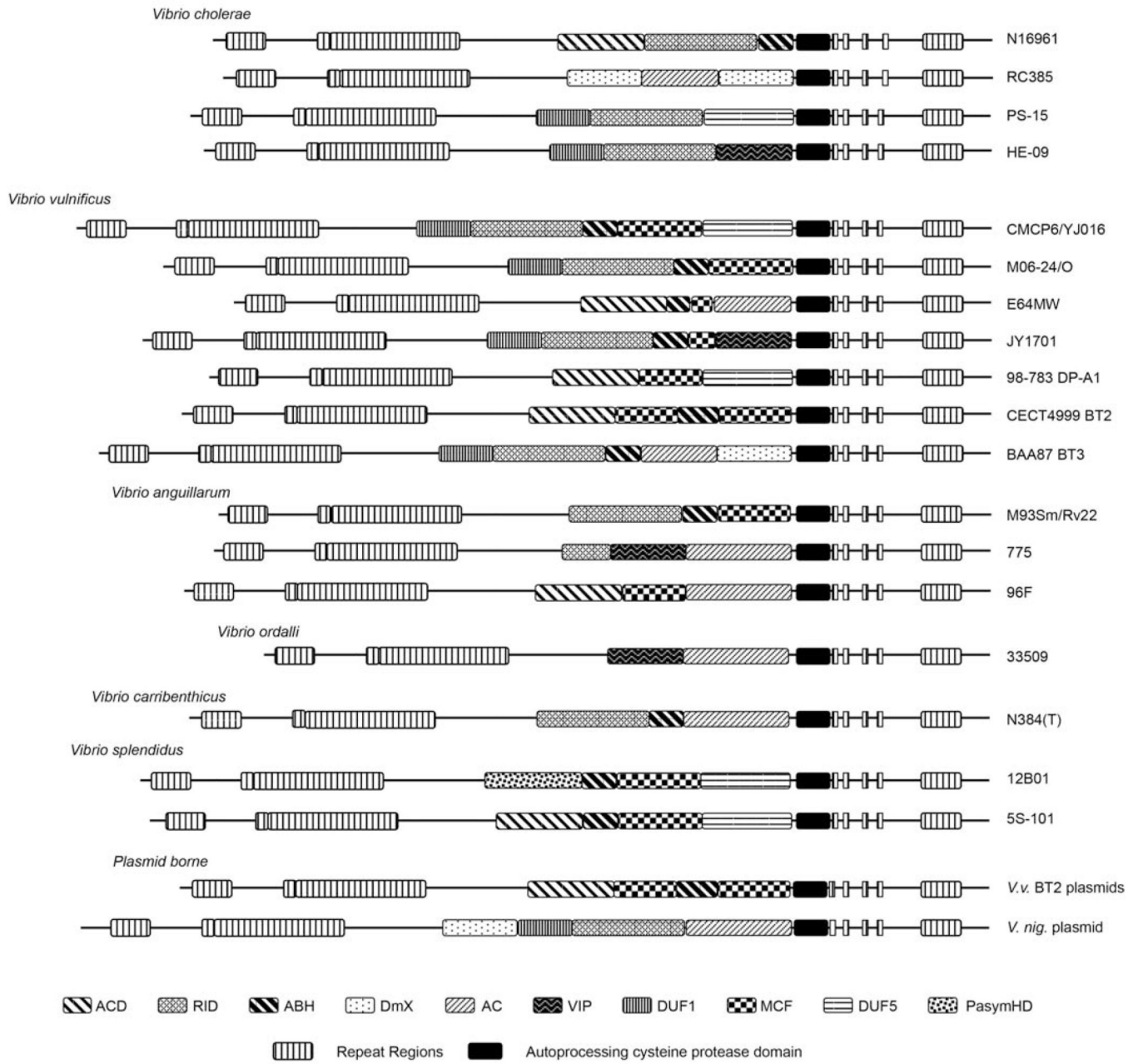
**FIGURE 3.**

Schematic representation of all multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins described in text. Toxins are identified by species at left with representative strain isolate designation at the right. BT2 and BT3 refer to *Vibrio vulnificus* biotype 2 and 3, respectively, and Vnig_pA refers to the large pA1066 plasmid of *V. nigripulchritudo*. Legend for different domains is at the bottom. All amino acid sequences are from published papers referenced in the text and the sequences were downloaded from the NCBI website (www.ncbi.nlm.nih.gov). All effector domain arrangements are as annotated previously (1, 11, 29, 34, 35, 63) except for new sequences done specifically for this article, including *V.*

*vulnificus* JY1701, *V. anguillarum* 775 and 96F, *V. ordalli* 33509, and *V. splendidus* 5S-101.
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