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Novel Cholix Toxin Variants, ADP-Ribosylating Toxins in *Vibrio cholerae* Non-O1/Non-O139 Strains, and Their Pathogenicity

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Cholix toxin (ChxA) is a recently discovered exotoxin in *Vibrio cholerae* which has been characterized as a third member of the eukaryotic elongation factor 2-specific ADP-ribosyltransferase toxins, in addition to exotoxin A of *Pseudomonas aeruginosa* and diphtheria toxin of *Corynebacterium diphtheriae*. These toxins consist of three characteristic domains for receptor binding, translocation, and catalysis. However, there is little information about the prevalence of *chxA* and its genetic variations and pathogenic mechanisms. In this study, we screened the *chxA* gene in a large number ($n = 765$) of *V. cholerae* strains and observed its presence exclusively in non-O1/non-O139 strains (27.0%; 53 of 196) and not in O1 ($n = 485$) or O139 ($n = 84$). Sequencing of these 53 *chxA* genes generated 29 subtypes which were grouped into three clusters designated *chxA* I, *chxA* II, and *chxA* III. *chxA* I belongs to the prototype, while *chxA* II and *chxA* III are newly discovered variants. ChxA II and ChxA III had unique receptor binding and catalytic domains, respectively, in comparison to ChxA I. Recombinant ChxA I (rChxA I) and rChxA II but not rChxA III showed variable cytotoxic effects on different eukaryotic cells. Although rChxA II was more lethal to mice than rChxA I when injected intravenously, no enterotoxicity of any rChxA was observed in a rabbit ileal loop test. Hepatocytes showed coagulation necrosis in rChxA I- or rChxA II-treated mice, seemingly the major target for ChxA. The present study illustrates the potential of ChxA as an important virulence factor in non-O1/non-O139 *V. cholerae*, which may be associated with extraintestinal infections rather than enterotoxicity.

Vibrio cholerae is a Gram-negative, curved-rod-shaped, motile bacterium with a monotrichous flagellum, and it is transmitted through contaminated water. To date, >200 serogroups of this species have been reported based on the surface O antigen. *V. cholerae* strains which express O1 or O139 surface antigens and possess two major virulent genes, the cholera toxin (CT, encoded by the *ctx* gene) and the toxin-coregulated pilus (TCP, encoded by the *tcpA* gene), are mainly responsible for acute diarrheal disease affecting millions of people every year (1). Strains other than O1 or O139 are commonly referred to as non-O1/non-O139. The non-O1/non-O139 *V. cholerae* generally does not carry *ctx* and *tcpA* genes, but some strains of this group can be potentially virulent in humans, causing sporadic cases of diarrhea and extraintestinal infections (2–4). The toxigenic potential of some of these strains is attributed to the type III secretion system (T3SS), type VI secretion system (T6SS), heat-stable enterotoxin (NAG-ST), hemagglutinin protease (HAP), and zonula occludens toxin (ZOT) (5–10). However, many non-O1/non-O139 strains isolated from patients do not contain any of these potential virulence factors. Some additional genes encoding hemolysin (HLY), repeats in toxin (MARTX), etc., are ubiquitously present in *V. cholerae* strains and are not considered to contribute to diarrhea directly, but they have redundant roles in promoting persistent gut colonization. The accessory toxins, especially HLY and MARTX, act not as direct mediators of adherence but by altering the local host environment and, in part, by enabling *V. cholerae* to survive innate immune clearance (11, 12).

Several pathogenic bacteria (*Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, *Clostridium botulinum*, *Staphylococcus aureus*, *V. cholerae*, *Escherichia coli*, etc.) produce protein toxins, which possess ADP-ribosyltransferase (ADPRT) activity as a key mechanism to modify the properties of host cell proteins to induce disease. Bacterial ADP-ribosylating exotoxins can be differentiated into various families on the basis of their target molecules (eukaryotic elongation factor, GTP binding proteins, actin, etc.) (13). Recently, Jørgensen et al. (14) reported the presence of a eukaryotic elongation factor 2 (eEF2)-specific ADP-ribosylating factor termed cholix toxin (protein designation, ChxA) in non-O1/non-O139 *V. cholerae* strains. The evaluation of the ability of the *chxA* gene to produce an exotoxin was pioneered by the discovery of an open reading frame (ORF) in an environmental non-O1/non-O139 *V. cholerae* strain which showed close sequence identity with the *toxA* gene of *P. aeruginosa* encoding the exotoxin A (ExoA) (15). The mode of action of ChxA in *V. cholerae*, i.e., causing cytotoxicity by targeting eEF2, is similar to that of ExoA from *P. aeruginosa* and diphtheria toxin (DT) from *C. diphtheriae*

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TABLE 1 Abundance of *chxA* (cholix toxin) gene among *Vibrio cholerae* strains used in this study

Serogroup	Country	Source	No. of strains	Presence or absence[a] of *chxA*
O1 ($n = 485$)	India	Clinical	260	−
	India	Environmental	208	−
	Brazil	Clinical	10	−
	Bangladesh	Clinical	4	−
	Peru	Clinical	3	−
O139 ($n = 84$)	India	Clinical	61	−
	Bangladesh	Clinical	23	−
Non-O1/non-O139 ($n = 196$)	India	Clinical	64	−
	India	Clinical	18	+
	India	Environmental	79	−
	India	Environmental	35	+
Total			765	53

[a] −, absence; +, presence.

TABLE 2 Virulence gene profiles of *Vibrio cholerae* strains harboring the *chxA* gene

No. of strains[a]	Presence or absence[b]						
	ctx	*tcpA*	*vcsN2-vcsC2-vopF*	*stn*	*rtx*	*zot*	*hly*
43	−	−	−	−	+	−	+
4	−	−	+	−	+	−	+
3	−	−	+	+	+	−	+
1	−	−	−	+	+	−	+
1	+	+	+	−	+	+	+
1	−	+	−	−	+	−	+
53[c]	1	2	8	4	53	1	53

[a] All strains were of the non-O1/non-O139 serogroup.
[b] −, absence of gene(s); +, presence of gene(s). *ctx*, cholera toxin; *tcpA*, toxin-coregulated pilus; *vcsN2-vcsC2-vopF*, type III secretion system; *stn*, heat-stable enterotoxin; *rtx*, repeats in toxin; *zot*, zonula occludens toxin; *hly*, hemolysin.
[c] Totals of columns are in last row.

(16). However, ChxA and ExoA are more closely related and their domain orientation, receptor recognition, and translocation mechanisms are different from those of DT (17).

The *chxA* gene encodes a 666-amino-acid (aa)-residue protein (70.7 kDa; 634-aa mature protein) including a 32-aa residue leader sequence. ChxA consists of three structural domains with structures similar to that of ExoA: a receptor-binding domain (domain Ia, aa 1 to 264; domain Ib, aa 387 to 423 [unknown function]), the parts of which together form a 13-stranded antiparallel β-jellyroll, a translocation domain (domain II, aa 265 to 386) consisting of a bundle of six α-helices, and a catalytic domain (domain III, aa 424 to 634) with an α/β-fold topology (14). Studies of the toxigenic properties of ChxA are in an earlier stage. There is also little information about the abundance and genetic diversity of the *chxA* gene among *V. cholerae* strains isolated from diarrheal patients and about their pathogenic mechanisms.

The present study has extensively investigated the occurrence and genetic diversity of the *chxA* gene in clinical and environmental *V. cholerae* strains belonging to O1 and O139 as well as non-O1/non-O139 serogroups. In addition, the virulence properties of ChxAs were evaluated by cytotoxicity, rabbit ileal loop (RIL), and mouse lethality assays. Our findings illustrate the potential of ChxA as an important virulence factor among non-O1/non-O139 *V. cholerae* strains. We have observed the high genetic diversity of the *chxA* gene and unveiled the presence of at least three toxinotypes with varied biological activities. To the best of our knowledge, this is the first comprehensive study of ChxA at the genetic and protein levels to understand its role in the pathogenesis of *V. cholerae* infection.

(This study was performed in partial fulfillment of the requirements of a Ph.D. thesis for Sharda Prasad Awasthi from the Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Vibrio cholerae* strains used in this study were randomly selected from our laboratory collection. A total of 765 *V. cholerae* strains of clinical and environmental origins, including O1 ($n = 485$), O139 ($n = 84$), and non-O1/non-O139 ($n = 196$), were screened for the presence of the *chxA* gene (Table 1). Some of them were originally isolated from diarrheal patients in India, Bangladesh, Brazil, and Peru, and the others were of environmental origin. The identities of the strains were confirmed by molecular methods (18). The strains stored in glycerol stock at −80°C were grown in alkaline peptone water (APW) and subsequently on thiosulfate-citrate-bile salts-sucrose (TCBS) agar at 37°C when needed.

Chemicals and enzymes. Chemicals were purchased from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Tokyo, Japan), or Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and TaKaRa *Ex Taq* were purchased from TaKaRa Bio Inc. (Shiga, Japan). The QIAquick PCR product purification kit was from Qiagen (Hilden, Germany). The pET vector system was purchased from Novagen (New Canaan, CT) and ProTEV protease from Promega (Madison, WI). Luria-Bertani (LB) broth was purchased from Difco Laboratories (Detroit, MI). Tryptic soya agar (TSA) and APW were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), and TCBS agar was from Eiken Chemical Co. Ltd. (Tokyo, Japan). SeaKem Gold agarose was from FMC Bioproducts (Rockland, ME), and SeaKem LE agarose was from Lonza (Rockland, ME). Pulsed-field certified agarose and low-melting agarose were from Bio-Rad Laboratories Inc. (Hercules, CA). Molecular weight markers were purchased from Bio-Rad Laboratories Inc. or Nippon Genetics Co. Ltd. (Tokyo, Japan). Sequencing reagents such as BigDye Terminator, buffers, etc., were purchased from Applied Biosystems Inc. (Foster City, CA), and the Clean Seq kit was from Agencourt Bioscience Corporation, Beckman Coulter (Beverly, MA). Nickel Sepharose was purchased from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, United Kingdom). Minimal essential medium (MEM) was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). MEM-α, Glutamax-I supplement, nonessential amino acids (NEAA), and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA).

Serotyping. Preparation of O antisera and determination of serogroups by slide agglutination were performed as previously described (19). In brief, the serogroup reference strains were cultured overnight in infusion broth, heated at 100°C for 2 h, and washed twice with physiological saline. After centrifugation, pellets were resuspended in 20 ml physiological saline and intravenously administered to rabbits at 4-day intervals. Seven days after the last immunization, the rabbits were anesthetized by intramuscular injection of 45 mg kg^{-1} ketamine (Ketalar; Daiichi Sankyo Co., Ltd., Tokyo, Japan) and 5 mg kg^{-1} xylazine (Selactar; Bayer Healthcare, Leverkusen, Germany), and whole blood was collected from the rabbits. For O antigen determination of target bacterial strains, tube and slide agglutination tests were carried out with a cell suspension heated at 100°C for 1 h.

Colony hybridization. Distribution of the virulence genes (Tables 1 and 2) was examined by colony hybridization assay as described previ-

ously (20). In brief, strains were grown on nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) overlaid on LB agar at 37°C for 4 to 6 h. Then, colonies were lysed, and DNA was denatured *in situ* by the alkaline lysis method followed by UV cross-linking. Specific DNA probes for target genes were prepared by PCR using respective primer sets (see Table S2 in the supplemental material). These probes were labeled with [α-^{32}P]dCTP (PerkinElmer, Wellesley, MA) by a random priming method using the Multiprime DNA labeling system (GE Healthcare Life Sciences). The processed nitrocellulose membranes were hybridized with the target probes under suitable buffer conditions, and radioactivity was visualized by the BAS FLA-3000 system (Fuji Film, Tokyo, Japan). For detection of T3SS genes, three T3SS-specific genes, *vcsN2*, *vcsC2*, and *vopF*, were used as a mixed probe for hybridization. Later on, the presence of each gene was confirmed by PCR.

DNA template preparation. A DNA template of *V. cholerae* strains for PCR was prepared by boiling the overnight cultures (18). In brief, a single yellow colony from the TCBS plate was inoculated into 3 ml LB broth and incubated at 37°C overnight with shaking (180 rpm). The culture was diluted 10 times with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and boiled for 10 min followed by snap cooling on ice. After centrifugation at 8,900 × *g* for 3 min, the supernatant was used as a DNA template and stored at −30°C for future use.

PCR amplification of the *chxA* gene for DNA probe preparation and sequencing. In a 50-μl PCR mixture, 1 μl of boiled DNA template was added to 1.25 U of TaKaRa *Ex Taq* DNA polymerase, its buffer system (TaKaRa Bio Inc.), and the following PCR primers. Based on the analysis of a published *chxA* gene sequence (GenBank accession no. AY876053), a forward primer, *chxA*U (5′-TGTGTGATGATGCTTCTGG-3′), and a reverse primer, *chxA*R1 (5′-TTATTTCAGTTCATCTTTTCGC-3′), were designed and used for PCR. The PCR was carried out in a TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio Inc.) with an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min and a final extension step at 72°C for 7 min. The PCR products were subjected to 1.0% LE agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]). The gels were stained in an ethidium bromide solution (2 μg ml^{-1}) followed by destaining in distilled water, each for 5 to 10 min. Gel images were captured with a Gel-Doc 2000 (Bio-Rad Laboratories Inc.).

A PCR-amplified *chxA* gene (~2.0 kb) was purified using the QIAquick PCR purification kit and then used as a gene probe for hybridization as well as sequencing. Purified DNA (10 ng) was subjected to cycle sequencing using 12 sequencing primers (see Table S2 in the supplemental material) and the BigDye Terminator v 1.1 cycle sequencing kit. The product was then purified using a Clean Seq kit and subjected to sequencing in an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems Inc.). The nucleotide sequences were aligned and analyzed using a Lasergene DNASTAR (Madison, WI) software package.

Genome walking. Genome walking was performed as described by Asakura et al. (21) to sequence the *chxA* gene from strains that were positive by colony hybridization but did not produce amplicons by PCR (*chxA* III) using a *chxA*U/*chxA*R1 primer set. First, an ~1.5-kb *chxA* gene was amplified by a *chxA*U/*chxA*R2 primer set and sequenced (see Table S2 in the supplemental material). The remaining ~0.5-kb sequence was amplified and sequenced by genome walking. In brief, 50 ng genomic DNA was randomly extended for only 1 cycle of 5 min at 94°C, 30 s at 30°C, and 30 s at 72°C, using a 10 μM *chx* random1 primer (see Table S2). Further PCR amplification was performed using a 10 μM *chx* target1 primer (see Table S2) for 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. *Ex Taq* DNA polymerase was used. The amplified fragments were sequenced with 5 μM *chx* seq1 primer (see Table S2) and analyzed by the DNA Lasergene software package.

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed according to the Pulse Net USA protocol (www.cdc.gov/pulsenet/protocols.htm) with slight modifications. Briefly, freshly cultured *V. cholerae* cells were embedded into 0.5% SeaKem Gold agarose followed by *in situ* lysis with 0.5 mg ml^{-1} proteinase K (P8044-5G; Sigma) and 1.0% sarcosine (Sigma) at 54°C for 1 h. Agarose blocks containing genomic DNA were digested with 30 U of NotI restriction endonuclease at 37°C for 3 h. DNA fragments were separated on a CHEF Mapper (Bio-Rad Laboratories Inc.) as described by Yamasaki et al. (22). Gels were stained for 30 min and destained twice for 15 min, and pictures were captured as described before. Lambda ladder (Bio-Rad Laboratories Inc.) was used as a molecular mass standard. PFGE fingerprints were analyzed by Fingerprinting II software (Bio-Rad Laboratories Inc.). The unweighted-pair group method with arithmetic means (UPGMA) was applied during dendrogram analysis following the band-based (Dice coefficient) option.

Cloning of *chxA* gene and expression of rChxA. *chxA* genes representing the three toxinotypes, *chxA* I (strain C9), *chxA* II (strain Vc106), and *chxA* III (strain Vc36), were amplified by PCR. The amplified DNA fragments were cloned downstream of the His_6 tag sequence of the pET28a+ vector (Novagen). Just upstream of the *chxA* DNA fragment, a TEV protease recognition sequence was incorporated. This recombinant plasmid was transformed into *E. coli* BL21(DE3). After induction with isopropyl β-D-1-thiogalactopyranoside (IPTG), each construct yielded a considerable amount of fusion protein (His_6-TEV-cholix). These fusion proteins were purified with a Ni-Sepharose column. The purified fusion proteins were digested with ProTEV protease to remove His_6-TEV from recombinant cholix toxin (rChxA). The digested product was checked by SDS-PAGE and again passed through the Ni-Sepharose column to obtain the purified rChxA without a His_6 tag.

Antiserum against ChxA. Antiserum against purified rChxA was basically prepared as described previously (23). In brief, 100 μg of purified rChxA I in phosphate-buffered saline (PBS) (pH 7.0) was emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, MI). The emulsion was administered intramuscularly to an adult New Zealand White rabbit (2 kg). Subsequently, two booster doses of 100 μg toxin were given in similar fashion at 30 and 42 days after first immunization. The serum antibody titer was regularly monitored by a Ouchterlony gel diffusion assay as described previously (23). Up to a dilution of 1:16 of the antisera, a precipitin line against 1 μg of homologous toxin could be observed. At the end, the rabbits were anesthetized by intramuscular injection of 45 mg kg^{-1} ketamine (Ketalar; Daiichi Sankyo Co., Ltd.) and 5 mg kg^{-1} xylazine (Selactar; Bayer Healthcare), whole blood was collected from the rabbits, and the serum was separated and stored at −80°C for further use.

Cytotoxicity assay. Eukaryotic cells were routinely cultured in their respective media: HeLa and Vero in MEM with 5% FBS, Int-407 and Hep-2 in MEM with 10% FBS, Caco-2 in MEM with 10% FBS and NEAA, CHO in MEMα with 10% FBS, Y-1 in Ham's F-12 with 5% FBS, and NIH-3T3 in Dulbecco MEM (DMEM) with 10% FBS. The cells were incubated with 0.25% trypsin in 1 mM EDTA at 37°C for 5 min, and the concentration of cells was determined using a hemocytometer. The cells (100 μl of 10^5 cells ml^{-1}) were cocultured with various amounts (0.05 to 1 μg) of purified rChxA in the presence or absence of antiserum against rChxA I in a 96-well culture plate (Orange Scientific, Braine-l'Alleud, Belgium) at 37°C in a water-jacketed incubator with 5.0% carbon dioxide in air for 24 to 48 h, and cytotoxic effects were observed under a Leica DMI6000 B microscope (Leica Microsystems, Mannheim, Germany).

Competition assay. To examine the binding competition between ChxA toxinotypes, a competition assay using HeLa cells was performed. In brief, HeLa cells were cultured, harvested, and seeded to 96-well culture plates as described previously and incubated to form a monolayer. Then, cells were washed with PBS (pH 7.0), and rChxA I (200 ng or 1 μg well^{-1}) was added in the presence or absence of excess (up to 20 μg well^{-1}) rChxA II or rChxA III. Cytotoxicity was observed under a microscope after 48 h of incubation.

Rabbit ileal loop assay. A rabbit ileal loop (RIL) assay was carried out as described earlier (24). Adult 2-kg New Zealand White rabbits were used in this assay. The rabbits were anesthetized by intramuscular injection of

45 mg kg^{-1} ketamine (Ketalar; Daiichi Sankyo Co., Ltd.) and 5 mg kg^{-1} xylazine (Selactar; Bayer Healthcare). Laparotomy was performed on anesthetized animals from the lower liver margin and 8 loops (8 cm long) with a 3-cm inter loop were ligated. The first loop was injected with CT (3 μg) and the last loop with PBS (pH 7.0) as positive and negative controls, respectively. The intermediate loops were inoculated with rChxA I, rChxA II, or rChxA III at varied concentrations (10, 50, 100, or 500 μg loop^{-1}). Loops were placed back in the peritoneal cavity. The animals were euthanized by injection of 200 mg kg^{-1} pentobarbital (Nembutal; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) 16 h after inoculation, and the loops were examined for fluid accumulation.

Mouse lethality assay. Lethality assays were performed as described by Yutsudo et al. (25) with some modifications. rChxA I and rChxA II were used for analysis of mouse lethality. rChxA III was not analyzed for mouse lethality, as it failed to cause cytotoxicity or enterotoxicity. For each assay, 7 male ICR mice with an average body weight of approximately 20 g (Nihon Clea Co., Japan) were intravenously injected with 1, 5, 10, or 25 μg mouse^{-1} (0.05, 0.25, 0.5, or 1.25 mg kg^{-1}) of either rChxA I or rChxA II. Mice were observed for the specific time interval, and the number of mice that died within this time interval was recorded. The major organs (heart, kidney, lung, liver, and spleen) were removed, and histopathological analysis was performed. PBS (pH 7.0) was used as a carrier control. All animal experiments were performed according to the Guidelines for Animal Experimentation of Osaka Prefecture University and approved by the Animal Experiment Committee of Osaka Prefecture University.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the *chxA* genes have been deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers AB754424 to AB754476 (53 entries).

RESULTS

Prevalence of *chxA* among *Vibrio cholerae* strains. A total of 765 *V. cholerae* strains, including O1 ($n = 485$), O139 ($n = 84$), and non-O1/non-O139 ($n = 196$) were screened by colony hybridization for the presence of the *chxA* gene. Among them, 53 (27%) non-O1/non-O139 strains from diverse serogroups harbored the *chxA* gene. All O1 and O139 strains tested were negative for this gene (Table 1; see Table S1 in the supplemental material). The 53 *chxA* gene sequences were grouped into three clusters designated *chxA* I ($n = 33$), *chxA* II ($n = 16$), and *chxA* III ($n = 4$) (Fig. 1). The 33 isolates from *chxA* I belonged to 14 different serogroups, whereas the 16 isolates from *chxA* II were from 11 distinct serogroups. All 4 isolates from *chxA* III belonged to the O64 serogroup (see Table S1). The *V. cholerae* isolates positive for the *chxA* gene were from both clinical (22%; 18 of 82) and environmental (30%; 35 of 114) origins. Among 18 clinical strains, 16 *chxA* genes belonged to *chxA* I, 2 to *chxA* II, and none to *chxA* III. In strains of environmental origin ($n = 35$) 17, 14, and 4 *chxA* genes belonged to *chxA* I, *chxA* II, and *chxA* III, respectively.

Furthermore, the *chxA* gene-positive strains ($n = 53$) were screened for the presence of other reported virulence genes (*ctx* [CT], *tcpA* [TCP], *vcsN2*, *vcsC2*, and *vopF* [T3SS], *stn* [NAG-ST], *zot* [ZOT], *rtx* [MARTX], *hly* [HLY]) in *V. cholerae*. All the strains were positive for *rtx* and *hly* genes. The majority of *chxA* gene-positive strains (43 of 53 non-O1/non-O139) did not have other tested virulence genes. Of the remaining 10 *chxA* gene-positive non-O1/non-O139 strains, one strain from the O141 serogroup was positive for *ctx*, *tcpA*, *vcsN2/vcsC2/vopF* (T3SS), and *zot*, three were positive for *vcsN2/vcsC2/vopF* (T3SS) and *stn*, four carried only *vcsN2/vcsC2/vopF* (T3SS), one had only *stn*, and one possessed *tcpA* (Table 2).

Diversity in the cholix toxin gene sequence. The *chxA* genes from all the positive strains ($n = 53$) were sequenced, aligned, and compared with a published prototype sequence of the *chxA* gene (GenBank accession no. AY876053). The length of the *chxA* gene (without leader sequence) in the test strains varied from 1,887 bp (628 aa) to 1,905 bp (634 aa). Deletion, insertion, and/or substitution of nucleotides affected amino acid substitutions. Phylogenetic analysis based on the *chxA* gene sequence differentiated 53 *chxA* genes into 29 subtypes and further grouped them into three major clusters designated *chxA* I, *chxA* II, and *chxA* III (Fig. 1). Sequence types belonging to *chxA* I prevailed in the majority of *chxA* gene-positive *V. cholerae* strains (33 of 53; ~63%). The prototype *chxA* gene sequence belonged to this cluster. About one-third (16 of 53; ~30%) of the *chxA* sequences belonged to the *chxA* II cluster. The *chxA* sequences belonging to the *chxA* III cluster were less abundant (4 of 53; ~7.5%) among the tested strains. Despite the presence of differences in sequences, the GC content of the *chxA* genes (43% to 44%) was almost conserved (Fig. 1 and Table 1).

The *chxA* gene sequences obtained in this study as well as the prototype sequence were translated using the Editseq program from DNASTAR, and deduced amino acid (aa) sequences were

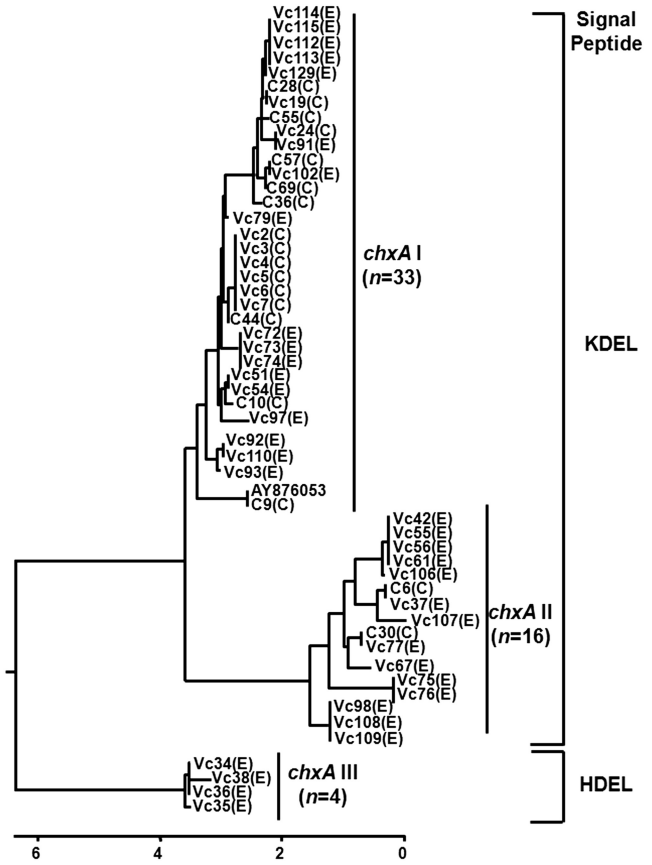

FIG 1 Dendrogram depicting the diversity of the *chxA* gene from *V. cholerae* non-O1/non-O139 strains. Overall, 29 different *chxA* subtypes were observed among 53 sequences. These strains were grouped into three sequence clusters: *chxA* I ($n = 33$), *chxA* II ($n = 16$), and *chxA* III ($n = 4$). The *chxA* I sequences are close to the published prototype sequence (accession no. AY876053). Letters in parentheses show the origin of the isolate: C and E, clinical and environmental, respectively. At protein level, the signal peptide in ChxA I and II is KDEL whereas ChxA III has HDEL.

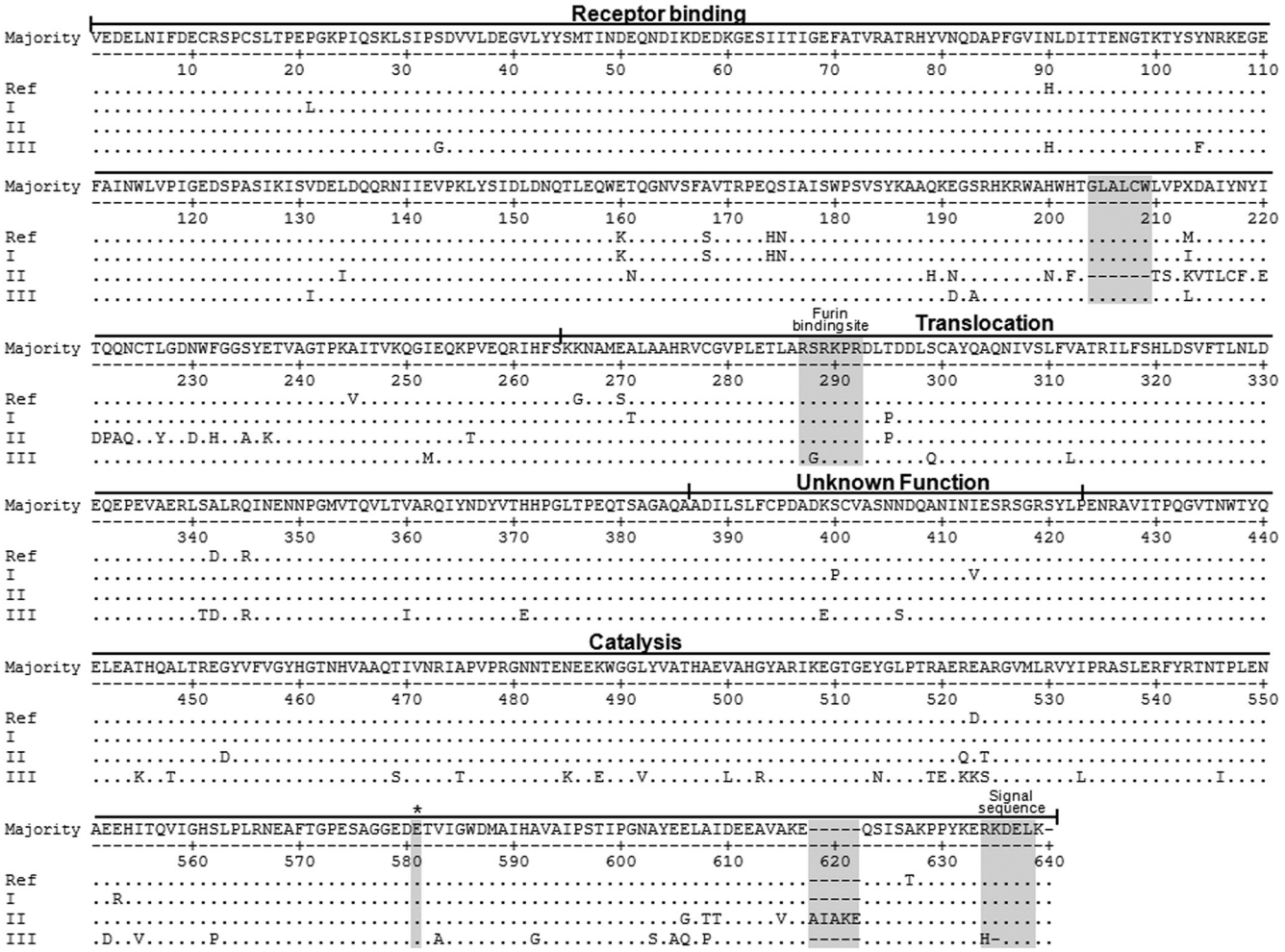

FIG 2 Alignment of the amino acid sequences representing the three clusters of ChxA (ChxA I, II and III) from *V. cholerae* strains. Only one ChxA sequence from each cluster, along with the published prototype sequence (AY876053), is shown here for simplicity. The ChxA I sequences are identical or similar to prototype ChxA. The ChxA II sequences clearly define the amino acid changes in the receptor binding domain (RBD) and ChxA III in the catalytic domain (CD). All the sequences of ChxA II possess an identical deletion of 6 amino acids (GLALCW) in the RBD, and 6 sequences have a 5-amino-acid (AIAKE) insertion in CD. ChxA I and II possess KDEL whereas ChxA III possesses an HDEL signal peptide at the C-terminal end. Serine-to-glycine substitution can be observed at the P5 position of the furin binding site in ChxA III. *, conserved amino acid residue critical for catalytic activity. Matching amino acids are indicated by dots and deleted ones by dashes.

analyzed to understand the diversity of ChxA at the amino acid sequence level. ChxA I sequences had up to 97.6% similarity among themselves, and ChxA sequence from one strain (C9; see Table S1 in the supplemental material) showed 100% similarity to the prototype sequence. The amino acid sequences of ChxA II had 91.8% to 93.7% similarity whereas the ChxA III sequences had 91.2% to 92.0% similarity to the prototype sequence (Fig. 1). ChxA III possessed a different signal peptide, i.e., an HDEL in place of KDEL, compared to all other sequences of ChxA I and ChxA II (Fig. 1 and 2).

The leader sequence of ChxA (32-aa polypeptide) is encoded by a 96-nucleotide region just upstream of the *chxA* sequence in the prototype sequence. Analysis of the leader sequence revealed that it was conserved in all the *chxA* genes from *chxA* I ($n = 33$) and 11 of 16 genes from *chxA* II. The leader gene sequence from 5 of 16 *chxA* II variants and all sequences of *chxA* III ($n = 4$) had insertions of nucleotide repeats between nucleotide positions 12 and 13 compared to the prototype sequence (Fig. 3). The 4 and 1 *chxA* II leader gene sequences had insertions of single or 3 copies of the tandem octamer (ACTTGACA) repeats, respectively, between positions 12 and 13. All *chxA* III leader sequences also had insertions of single copies of nearly identical octamers (ACTTGATA) between the same positions. This insertion of nucleotides resulted in incorporation of a termination codon (TGA) at 16 to 18 nucleotide positions (Fig. 3). However, the bead enzyme-linked immunosorbent assay (ELISA) developed in our laboratory for detection of ChxA indicated that all these strains express ChxA extracellularly (unpublished data), indicating that the putative initiation codon might be different.

Diversity at the RBD. The receptor-binding domain (RBD; 264 aa residues in the prototype sequence) of ChxA I was conserved in length (264 aa), and there were 3 to 4 amino acid substitutions at several positions (Fig. 2; see Table S1 in the supplemental material) in comparison to the prototype sequence. In the case of the RBD of ChxA II, there was a common deletion of 6 amino acids (^{204}GLALCW^{209}) in comparison to that of ChxA I or ChxA

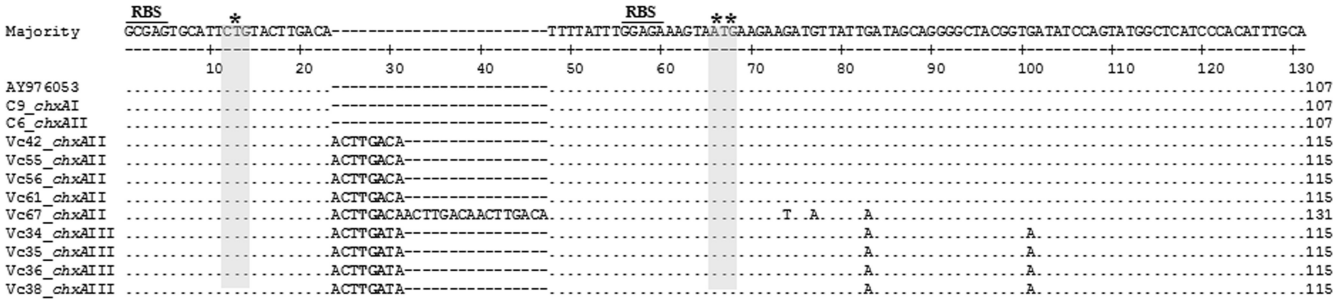

FIG 3 *chxA* leader gene sequence alignment for the strains with nucleotide repeat insertion. One *chxA* leader gene sequence from *chxA* I and *chxA* II, along with the published prototype sequence (AY876053), is shown here for comparison. All the *chxA* I leader gene sequences are conserved. The *chxA* II leader sequences from 5 strains have insertions of single or 3 copies of octamer repeats, and all 4 *chxA* III also have insertions of single copies of nearly identical octamers. The bold characters show the termination codon; *, initiation codon in the prototype sequence; **, proposed initiation codon with probable ribosome binding site (RBS). Matching amino acids are indicated by dots and deleted ones by dashes.

III. Therefore, the length of the RBD in ChxA II was shortened to 258 amino acids. Moreover, there were also 28 to 32 amino acid substitutions at several positions (Fig. 2; see Table S1 in the supplemental material). The RBD of ChxA III was of a length identical to that of ChxA I (264 aa) but substitutions of 12 amino acids at varied positions were observed compared to the prototype sequence. Overall, the RBD of ChxA II was highly divergent in comparison to that of ChxA I or ChxA III. In some strains, the total number of amino acid replacements was the same (see Table S1) but the positions of substituted amino acids were either the same or different (details not shown).

Diversity at the UFD. The length of the unknown function domain (UFD; 37 aa residues in the prototype sequence) of ChxA was conserved in all three toxinotypes, and the sequence diversity was also less. For example, the amino acid sequence of the UFD was conserved in 11 of 16 ChxA II variants and there was only one amino acid substitution in each of the remaining 5 compared to the prototype sequence (see Table S1 in the supplemental material). Among the 33 sequences belonging to ChxA I, 4 did not have any change, and the rest had 1 or 2 amino acid substitutions in comparison to the prototype sequence. In ChxA III, the UFD had 2 amino acid substitutions with respect to the prototype sequence (see Table S1).

Diversity at the TD. The translocation domain (TD; 122 aa residues in the prototype sequence) in all sequences of ChxA comprised 122 amino acids, and there were 5 to 8 amino acid substitutions at different positions compared to the prototype sequence (see Table S1 in the supplemental material). Among 33 ChxA I sequences, 21, 11, and 1 had 5, 6, and 7 amino acid substitutions, respectively, in the TD. All ChxA II ($n = 16$) sequences differed by 5 amino acid substitutions in the TD except for 2 ChxA sequences which had 7 amino acid substitutions. In the case of ChxA III, a total of 8 amino acid substitutions were observed in the TD (see Table S1). In comparison to ChxA I and ChxA II, all the ChxA III ($n = 4$) sequences had serine (polar amino acid) to glycine (nonpolar amino acid) substitutions at the P5 position of the furin binding site, which is located inside the furin binding pocket (P1 to P6 and P1′ to P2′) (Fig. 2).

Diversity at the CD. Among the observed *V. cholerae* strains possessing the *chxA* gene, the catalytic domain (CD; 211 aa residues in the prototype sequence) of ChxA I was conserved in length (211 aa) while ChxA II and ChxA III had insertions and deletions of some amino acids in this domain. Out of 33 ChxA I sequences, 13, 11, and 1 carried 3, 1, and 2 amino acid substitutions in the CD, respectively, at various positions with respect to the prototype sequence (see Table S1 in the supplemental material). ChxA II ($n = 16$) could be divided into two categories based on the diversity at CD: the first category had 211 amino acids ($n = 10$), like ChxA I, while the second category had 216 amino acids ($n = 6$). Among the first category of ChxA II there were up to 3 amino acid replacements at various positions of the CD. On the other hand, the second category of ChxA II carried an identical insertion of 5 amino acids (AIAKE) between ^{617}E and ^{618}Q and additionally 8 amino acid replacements at other positions with respect to the CD of the prototype sequence (Fig. 2; see Table S1). ChxA III (210 aa) had substantial modifications in the CD, with 29 to 32 amino acid changes along with an identical deletion of a lysine (^{629}K) compared to the prototype sequence (Fig. 2; see Table S1). This deletion of lysine and a prior replacement of a histidine altered the signal peptide of ChxA III into HDEL, while ChxA I and ChxA II had KDEL as a signal peptide (Fig. 1 and 2). The five amino acids (H^{460}, Y^{493}, Y^{504}, E^{574}, and E^{581}) which have been reported to play a vital role in the catalysis in all ADP-ribosyltransferases (14) were conserved among all the ChxA sequences, irrespective of their diversity (Fig. 2).

PFGE analysis. The clonality of the *V. cholerae* strains harboring the *chxA* gene was examined by PFGE after digestion with NotI (see Fig. S1 in the supplemental material). The PFGE patterns exhibited the distribution of 14 to 23 bands over a size range of 20 to 500 kbp. A high level of genetic diversity was observed among the strains, although some of the strains showed nearly identical PFGE patterns (see Fig. S1). Dendrogram analysis revealed that at an 85% cutoff value, the 53 *chxA*-positive strains could be classified into 41 pulsotypes. No clear-cut correlation was observed between *chxA* sequence diversity, serogroup, and clonality.

Cytotoxicity assay. After assessing the diversity of ChxA, three strains were selected as representatives of ChxA I (C9), ChxA II (Vc106), and ChxA III (Vc36). The ChxA sequence of strain C9 was 100% identical to the prototype sequence, whereas strain Vc106, representing ChxA II, possessed a high level of amino acid changes (37 aa changes) in the RBD of ChxA in comparison to that of C9 (Fig. 4; see Table S1 in the supplemental material). The Vc36 strain representing ChxA III had a highly diverse CD of ChxA with an altered furin-binding site (Fig. 2). Each *chxA* gene was PCR amplified, cloned, and expressed in *E. coli* using the pET vector system as described in Materials and Methods. The rChxA pro-

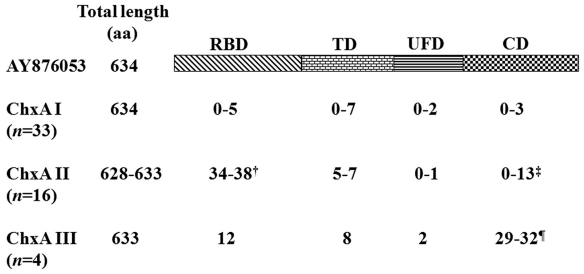

FIG 4 Number of amino acid changes at each domain of the three ChxA toxinotypes in comparison to the prototype sequence (AY876053). The length of ChxA peptide is 634 amino acids for ChxA I and 633 for ChxA III. The peptide length of ChxA II varies from 628 to 633 amino acids. The receptor binding domain (RBD) of ChxA II with 34 to 38 amino acid changes and the catalytic domain (CD) of ChxA III with 29 to 32 amino acid changes are the most diverse domains. TD, translocation domain; UFD, unknown function domain. †, common deletion of 6 amino acids in all strains; ‡, insertion of 5 amino acids in 6 strains; ¶, deletion of 1 amino acid in all strains.

teins were purified and used for a cytotoxicity assay against multiple eukaryotic cells. Among the 8 cell lines listed in Table 3, rChxA I (C9) showed strong cytotoxicity against HeLa and Y1 cells (death within 24 to 48 h), but rChxA II (Vc106) failed to show any such effect on the same cell lines (Table 3). In contrast, when CHO or Caco-2 cells were cocultured with these toxins there was no apparent cell death at the early stage (24 to 48 h). However, microscopic observation revealed that both rChxA I and rChxA II inhibited (at similar sensitivity levels) the cell growth (Table 3). When NIH-3T3, Vero, Int-407, and HEp-2 cells were cultured with rChxA I and rChxA II, both toxinotypes showed strong cytotoxicity. This cytotoxicity by rChxA I and rChxA II could be neutralized by ChxA I antisera (data not shown). In contrast to the cytotoxicity patterns of rChxA I and rChxA II, rChxA III (Vc36) failed to cause cytotoxicity in any of the tested cell lines. A competition assay using HeLa cells between rChxA I and rChxA II/rChxA III revealed that rChxA III could partially suppress rChxA I-induced cytotoxicity, whereas no suppression of the rChxA I cytotoxicity was observed when cells were cocultured with rChxA I in the presence of rChxA II (data not shown).

Rabbit ileal loop assay. Inoculation of 10 and 100 μg loop^{-1} of rChxA I (C9), rChxA II (Vc106), or rChxA III (Vc36) into the rabbit ileum did not cause any fluid accumulation irrespective of the ChxA type (data not shown). In a subsequent experiment, a higher dose (500 μg loop^{-1}) of rChxA I, rChxA II, and rChxA III was used, which also could not stimulate any fluid accumulation (data not shown).

TABLE 3 Summary of cytotoxicity assays of various cell lines

Cell line	Origin	Source	Result[a]		
			ChxA I	ChxA II	ChxA III
HeLa	Human	Cervical cancer	O	X	X
Y-1	Mouse	Adrenocortical tumor	O	X	X
Int-407	Human	Embryonic intestine	O	O	X
Hep-2	Human	Epidermoid cancer	O	O	X
NIH-3T3	Mouse	Embryonic fibroblast	O	O	X
Vero	Monkey	Kidney epithelium	O	O	X
Caco-2	Human	Colorectal adenocarcinoma	Δ	Δ	X
CHO	Hamster	Ovary	Δ	Δ	X

[a] O, cytotoxic; X, non-cytotoxic; Δ, cell growth retardation.

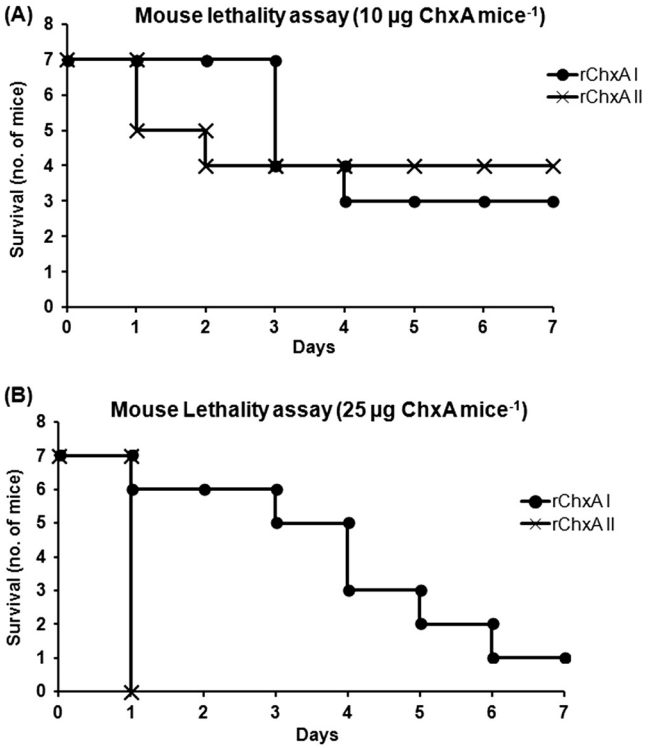

FIG 5 Mouse lethality assay with rChxA I and rChxA II. Specific-pathogen-free male ICR mice ($n = 7$, each group) with an average body weight of 20 g were intravenously injected with rChxA I and rChxA II. The survival was observed for 1 week. Survival curves with 10 μg mouse^{-1} (500 μg kg^{-1}) rChxA (A) and 25 μg mouse^{-1} (1.25 mg kg^{-1}) rChxA (B) are shown here.

Mouse lethality assay. Intravenous injection of rChxA I (C9) and rChxA II (Vc106) administered to mice at a dose of 1 or 5 μg mouse^{-1} (50 or 250 μg kg^{-1}) had no lethal effect, and all mice ($n = 7$ for each toxinotype) survived during 10 days of observation. Injection of a dose of 10 μg mouse^{-1} (500 μg kg^{-1}) of rChxA I and rChxA II led to the deaths of 4 and 3 mice, respectively, of 7 during the 1-week observation period (Fig. 5). rChxA I at a dose of 25 μg mouse^{-1} (1.25 mg kg^{-1}) caused the deaths of 6 mice at regular time intervals from days 1 to 7, whereas rChxA II at a similar dose killed all mice ($n = 7$; mortality rate, 100%) within 24 h of the injection (Fig. 5). The five major organs, *viz.*, heart, kidney, lung, liver, and spleen, were examined for histopathology. Visual inspection of these organs clearly showed severe damage to the liver but no other organs when injected with either rChxA I or rChxA II (data not shown). Histopathology also revealed severe coagulation necrosis of the hepatocytes in mice injected with either toxinotype, suggesting that the liver could be the major target of ChxA-mediated cytotoxicity (Fig. 6). Interstitial edema with mild inflammation was also observed around the arterioles in the lungs (Fig. 6) of mice injected with either rChxA I or rChxA II. However, none of the other organs, including heart, kidney, or spleen, were damaged. The mouse lethality for rChxA III was not evaluated, as the representative rChxA III (Vc36) failed to cause any cytotoxicity or enterotoxicity.

DISCUSSION

Although *V. cholerae* serogroups O1 and O139, which produce CT, are clinically the most important, non-O1/non-O139 *V. chol-*

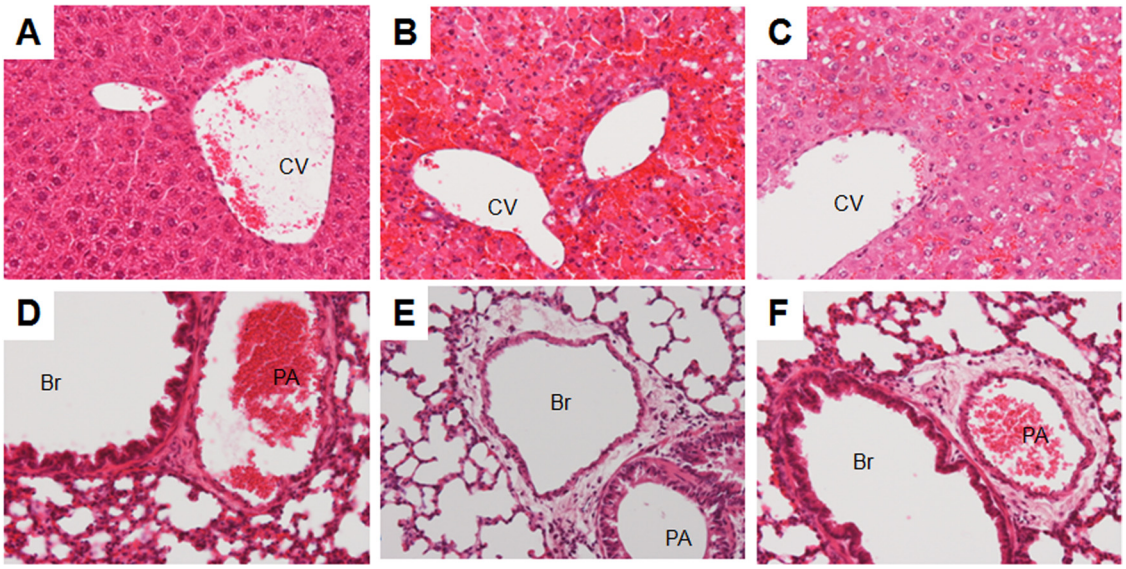

FIG 6 Hematoxylin-and-eosin-stained sections of mouse liver (A, B, and C) and lung (D, E, and F). (A and D) Control group. (B and E) rChxA I-treated group. (C and F) rChxA II-treated group. Severe coagulation necrosis of the hepatocytes and interstitial edema with mild inflammation around the arteriole in lungs can be observed in both the groups treated with either rChxA I or rChxA II. CV, central vein; Br, bronchiole; PA, pulmonary arteriole. Magnification, ×200.

erae strains, which normally do not produce CT, are also isolated from hospitalized patients with severe diarrhea (2). There are also reports of the occurrence of toxigenic non-O1/non-O139 *V. cholerae* in the environment. Several virulence factors have been identified in non-O1/non-O139 *V. cholerae*, such as T3SS, NAG-ST, HLY, etc. (5, 7, 11). However, pathogenic mechanisms of non-O1/non-O139 *V. cholerae* in diarrheal diseases as well as extraintestinal infection are still unclear. Recent identification of a *chxA* gene, encoding ChxA, has added a new member to the group of potentially virulent genes among *V. cholerae* strains (14).

The present study has revealed the presence of the *chxA* gene among a large number (53 of 196, 27%) of non-O1/non-O139 *V. cholerae* strains of clinical and environmental origin (Table 1). These 53 isolates belonged to 27 diverse serogroups (see Table S1 in the supplemental material). By sequencing the entire *chxA* genes of 53 isolates, we further identified 2 variants of the *chxA* gene, designated *chxA* II and *chxA* III, besides the prototype *chxA* gene sequence, representing the *chxA* I toxinotype, reported by Jørgensen et al. (14). We have also observed high sequence diversity in *chxA* sequences (29 subtypes among 53 sequences). The deduced amino acid sequences revealed that the RBD of ChxA II and the CD of ChxA III (Fig. 1 and 2) were significantly diverse compared to that of the ChxA I or prototype sequence. This diversity in the RBD and CD may facilitate the targeting of various hosts by *chxA*-positive *V. cholerae* strains. The identification of at least three different toxinotypes (*chxA* I, *chxA* II, and *chxA* III) of this exotoxin with extensive genetic diversity gives us new insights into ChxA-mediated *V. cholerae* pathogenicity.

As mentioned in Results, 5 of 16 *chxA* II and all 4 *chxA* III leader gene sequences had insertions of nucleotide repeats which resulted in the incorporation of the termination codon in the *chxA* gene sequence (Fig. 3). This insertion should lead to nonexpression of this toxin by these strains, but the bead ELISA for ChxA revealed that all these isolates express ChxA (unpublished data). On analyzing the DNA sequence carefully, we found that a possible initiation codon (ATG) and probable ribosome binding site (RBS) were present in the leader sequences of all the *chxA* genes (Fig. 3). Thus, we propose that, at least for these 9 *chxA* genes, the ATG could be used as an initiation codon rather than CTG as documented by previous authors for the prototype sequence. This change of the initiation codon resulted in a decrease in the leader sequence polypeptide length from 32 to 22 amino acids. We also assume that the proposed ATG may act as an initiation codon for all the *chxA* gene sequences.

The occurrence of the *chxA* gene among *V. cholerae* strains is mostly independent of the presence of other major virulence genes, e.g., those related to CT, TCP, T3SS, NAG-ST, etc. (Table 2; see Table S1 in the supplemental material). The presence of the *chxA* gene among a large number of genetically and serotypically divergent non-O1/non-O139 strains isolated from patients as well as the environment and the absence of any other important virulence genes (Tables 1 and 2; see Table S1) illustrate the potential of ChxA as an important virulence factor in non-O1/non-O139 *V. cholerae*. Among non-O1/non-O139 *V. cholerae* strains, the O141 serogroup is predominantly reported as a causative agent in sporadic cases of diarrhea. The majority of O141 isolates are reported to have *ctx* and *tcpA* genes (26, 27). In this study, a *V. cholerae* isolate possessing multiple virulence factors, *viz.*, *ctx*, *vcsN2/vcsC2/vopF* (T3SS), and *chxA*, was found to be from the O141 serogroup (see Table S1). Although the virulence profile analysis of the *chxA* gene-positive *V. cholerae* strains revealed that the existence of *chxA* is independent of other virulence factors, it also coexists with factors like *ctx*, *vcsN2/vcsC2/vopF* (T3SS), *stn*, etc. (Table 2), which are associated with enterotoxicity. The present study clearly demonstrates a strong cytotoxic effect of rChxA I on HeLa and Y1 cells but no effect of rChxA II (Table 3). Interestingly, both of the toxinotypes have similar growth retardation effects on CHO and Caco-2 cells (Table 3). On the other hand, both of the toxinotypes showed cytotoxicity on Vero, Int-407, Hep-2, and NIH-3T3 cells. rChxA III failed to cause cytotoxicity to any cell lines used in this

study (Table 3). The hallmark catalytic residues (H^{460}, Y^{493}, Y^{504}, E^{574}, and E^{581}) for ADPRT activity (14) are conserved among all the ChxA sequences observed in our study. Therefore, the variations in cytotoxicity on HeLa and Y1 cells by rChxA I and rChxA II may be due to the large amino acid differences in the RBD that reflect on their receptor recognition. To confirm this, we performed a competition assay by using HeLa cells between different ChxA toxinotypes. rChxA I-induced cytotoxicity was suppressed by rChxA III but not by rChxA II, indicating that ChxA I and ChxA III possibly share a receptor on HeLa cells whereas ChxA II may not bind to the same receptor (data not shown). The high-resolution crystal structures of ChxA I at 2.1 Å have been previously described (Protein Data Bank [PDB]: 2Q5T) (14). *In silico* structural analyses of ChxA II and ChxA III with ChxA I suggest that the CDs of all three ChxA toxinotypes are nearly identical, with no significant differences. Similarly, the RBDs of three ChxA toxinotypes are nearly identical; however, ChxA II has an altered β-jellyroll chain in the particular region (corresponding to amino acid residues H^{200} to T^{238} and N^{200} to T^{238} in ChxA I and ChxA II, respectively [Fig. 2]), where the primary structure is also diverse between these toxinotypes, which resulted in altered conformation of this domain. Thus, the diversity of the RBD sequence, competition assay data, and structural analysis of ChxA toxinotypes further support our assumption that varied cytotoxicity of ChxA II may be due to diversity of the RBD. However, the growth retardation effect of both rChxA I and rChxA II on CHO and Caco-2 cells and cytotoxicity on other cell lines illustrates the probable presence of an alternative mechanism to target host cells or the possession of varied receptors by these cell lines. These variable effects suggest that there may be more than one biological activity of ChxA, a possibility which demands further detailed investigations. The *in silico* analysis also revealed that although the CD of ChxA III is highly diverse, structurally ChxA I and ChxA III are nearly identical, with no significant differences. Moreover, the failure of ChxA III to cause cytotoxicity may not be related to its binding ability or catalytic activity because rChxA III could inhibit rChxA I-induced cytotoxicity (data not shown).

Furins are expressed in all tissues/cell lines and process latent precursor proteins into their biologically active products (28). The ExoA and DT are activated within host cells by furin cleavage (29, 30). The ChxA proteins also possess the furin cleavage site for cellular activation (14). The furin cleavage site is a 20-aa motif running from positions P14 to P6′ with a core region of 8 amino acids (P6 to P2′) which form the furin binding pocket and determine the binding strength (31). ChxA III has an altered furin binding site with a serine-to-glycine substitution at the P5 position, which is inside the furin binding pocket (P1 to P6 and P1′ to P2′). This mutation at the furin binding site hampers the proteolysis of rChxA III by furin *in vitro* (data not shown) and thus may repress cellular activation and subsequently translocation. This could explain the reason for the failure of ChxA III to induce cytotoxicity in the tested cell lines (Table 3).

The RIL assay is one of the most extensively used assays for the analysis of the enterotoxic potency of a particular toxin or pathogen (32–34). It was, however, observed in this study that rChxA factors failed to cause any fluid accumulation in this assay. Nevertheless, we cannot rule out the possibility that ChxA could mediate enterotoxicity in humans, as some of the *chxA*-positive strains used in this study are of clinical origin. ExoA is reported as an important virulence factor in *P. aeruginosa* infections like septicemia, lung, renal, and liver infections (35–38). The contribution of ChxA as a virulence factor to organ dysfunction has not yet been studied. The present study revealed that intravenous injection of rChxA into mice could result in their deaths (Fig. 5), probably via damage to the critical organs. Indeed, the occurrence of severe coagulation necrosis of the hepatocytes after injection of rChxA into mice (Fig. 6) suggests that the liver could be the primary target of ChxA, although interstitial edema and mild inflammation were also observed around the arterioles in the lungs of mice injected with either rChxA I or rChxA II (Fig. 6). The data obtained through the mouse lethality assay suggest that ChxA II could be more lethal than ChxA I (Fig. 5). Thus, taking together the results of the RIL and mouse lethality assays, it may be concluded that ChxA could be an important virulence factor of non-O1/non-O139 *V. cholerae*, which may be associated with extraintestinal infections rather than enterotoxicity. This hypothesis may be also supported by our recent isolation of a *chxA*-positive non-O1/non-O139 *V. cholerae* strain in a patient reported to have septicemia, disseminated intravascular coagulation, and multiple organ failure (unpublished data). Interestingly, this *V. cholerae* strain possesses only *chxA* as a virulence factor, and its involvement in septicemia demands detailed investigation. Thus, we can say that the infections caused by *V. cholerae* strains possessing *chxA* may be linked to systemic infections.

The present study shows the presence of a C-terminal KDEL endoplasmic reticulum (ER) retention sequence in all sequences of ChxA I and ChxA II (Fig. 1 and 2). The presence of the tetrapeptide KDEL may facilitate its comparatively low K_m (3-fold lower than that of ExoA, with REDL as a C-terminal retention sequence) and higher affinity for the substrate (14). In the case of ExoA, replacement of REDL with KDEL can increase its cytotoxicity (39, 40). In that sense, ChxA can be an alternative candidate as a therapeutic immunotoxin for tumor suppression, which is expected to be more effective than ExoA. Sarnovsky et al. (41) have already constructed immunotoxin from ChxA as a possible therapy for tumor suppression, which may be of potential utility after a patient has developed neutralizing antibodies against ExoA-based immunotoxin. Interestingly, we have observed the presence of HDEL instead of KDEL among *V. cholerae* strains possessing ChxA III (Fig. 1 and 2). The receptor for KDEL is present mainly in animal cells, whereas the HDEL receptor is found in *Saccharomyces cerevisiae* and plants (42–44). Moreover, a receptor present in human cells has been shown to bind both the KDEL and HDEL sequences *in vitro* (45, 46). Therefore, ChxA may have functional capabilities to target diverse host systems. Like ExoA, all the ChxA sequences carry the C-terminal lysine residue (Fig. 2), and its removal from the C-terminal (K/H)DELK pentapeptide may be a prerequisite for the binding of ChxA to the receptor in the ER of host cells that ChxA invades (47).

Despite the existence of high genetic diversity among the subtypes of the *chxA* gene (Fig. 2; see Table S1 in the supplemental material), the GC content is almost conserved (43% to 44%) and is very close to that of the *V. cholerae* N16961 genome (47%) but far from that of the *toxA* gene (~70%; GenBank accession no. AE004091) of *P. aeruginosa*. The GC content of the DT gene (42.5%; GenBank accession no. NC_002935) is close to that of the *chxA* gene, but a very low sequence identity and the reverse orientation of functional domains in comparison to ChxA rule out the possibility of horizontal transfer of the *chxA* gene from *C. diphtheriae*. The flanking sequence analyses of the *chxA* gene do not

suggest the presence of any transposon- or phage-related genes and thus do not provide any known clue to its horizontal transmission (15, data not shown). Flanking sequence analysis also revealed that the insertion site of the *chxA* gene is the same for all the *chxA*-positive *V. cholerae* strains analyzed in this study (data not shown). Therefore, the presence of the *chxA* gene among a wide variety of genotypes (or serogroups) of *V. cholerae* (see Fig. S1 in the supplemental material) may be through vertical transmission or genetic exchange by homologous recombination from a close neighbor of the *Vibrionaceae* or just through capture as naked DNA in the environment by a chitin-induced DNA uptake process (48–50).

There are still a lot of undefined research queries about ChxA, including but not limited to the following. (i) What is the significance of ChxA in *V. cholerae* infection, whether systemic or intestinal? (ii) What are the other biological activities the toxin possesses in addition to cytotoxicity? (iii) What is the actual mode(s) of transmission of the *chxA* gene? (iv) What are the expression levels of ChxA in different *chxA*-positive *V. cholerae* strains?

In conclusion, *chxA* is prevalent among a large proportion of non-O1/non-O139 *V. cholerae* strains from diverse serogroups and possesses significant genetic diversity. This study reports for the first time the existence of at least three *chxA* toxinotypes (*chxA* I, *chxA* II, and *chxA* III). Among them, ChxA II and ChxA III possess a highly diverse RBD and CD, respectively, which may be attributable to their varied virulence patterns. Although none of the ChxA toxinotypes can cause enterotoxicity in rabbits, ChxA I and ChxA II can cause extensive damage to the internal organs of mice, especially the liver; this liver damage is lethal. This study suggests that ChxA I and ChxA II may be associated with extraintestinal infections rather than enterotoxicity at least in the animal model. Screening of a large number of *V. cholerae* strains isolated from patients with systemic infections and diarrhea for the presence of *chxA* would provide a better understanding of the importance of ChxA in its pathogenicity.

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