

Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission

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Vibrio cholerae persists in aquatic environments predominantly in a nonculturable state. In this study coccoid, nonculturable *V. cholerae* O1 in biofilms maintained for 495 days in Mathbaria, Bangladesh, pond water became culturable upon animal passage. Culturability, biofilm formation, and the *wbe*, *ctxA*, and *rstR2* genes were monitored by culture, direct fluorescent antibody (DFA), and multiplex PCR. DFA counts were not possible after formation of biofilm. Furthermore, *wbe*, but not *ctxA*, were amplifiable, even after incubation for 54 and 68 days at room temperature ($\approx 25^{\circ}\text{C}$) and 4°C , respectively, when no growth was detectable. Slower biofilm formation and extended culturability were observed for cultures incubated at 4°C , compared with $\approx 25^{\circ}\text{C}$, suggesting biofilm production to be temperature dependent and linked to loss of culturability. Small colonies appearing after incubation in microcosms for 54 and 68 days at 25°C and 4°C , respectively, were *wbe* positive and *ctxA* and *rstR2* negative, indicating loss of bacteriophage CTX Φ . The coccoid *V. cholerae* O1 observed as free cells in microcosms incubated for 495 days could not be cultured, but biofilms in the same microcosms yielded culturable cells. It is concluded that biofilms can act as a reservoir for *V. cholerae* O1 between epidemics because of its long-term viability in biofilms. In contrast to biofilms produced in Mathbaria pond water, *V. cholerae* O1 in biofilms present in cholera stools and incubated under identical conditions as the Mathbaria pond water biofilms could not be cultured after 2 months, indicating that those *V. cholerae* cells freshly discharged into the environment are significantly less robust than cells adapted to environmental conditions.

Bangladesh | bacteriophage CTX Φ | DFA | multiplex-PCR | *ctxA*

Cholera continues to pose a serious health threat globally, notably in those countries where clean drinking water is not available to local populations. *Vibrio cholerae* serogroups O1 and O139 are associated with epidemic and pandemic cholera. Cholera is endemic in the Ganges delta, occurring twice yearly in epidemic form (1). It is also a major health problem for countries of Africa, Latin America, and Asia (2). *V. cholerae* O1 is native to both marine and fresh water environments and exists in association with plankton (3). In general, it can be isolated from only 1% of water samples collected during epidemic periods and rarely, if ever, between epidemics (4). However, fluorescent antibody-based studies show that *V. cholerae* O1 is, nevertheless, present in aquatic environments throughout the year (5). Furthermore, the presence of nonculturable *V. cholerae* O1 is confirmed by molecular methods (6). The question of whether such nonculturable cells in aquatic environments are capable of returning to an actively growing state to initiate cholera epidemics has been debated.

Extensive studies have shown that *V. cholerae* O1 becomes coccoid and enters into a nonculturable state in the environment when conditions are not conducive to active growth (5, 7). Some of the coccoid nonculturable cells retain metabolic activity (5, 8, 9), although little is known about the reservoir of *V. cholerae* between epidemics and the mechanism whereby nonculturable cells become

culturable to initiate seasonal epidemics of cholera. Results of laboratory-based studies suggest biofilms provide a powerful mechanism for the persistence of vibrios in the environment (10). Nonculturable cells undergo reduction in size, becoming coccoid in morphology and unable to grow on bacteriological growth media (7, 11). Although viability (12) and infective potential (13) of nonculturable cells of many species and genera of bacteria have been documented (14), how reversion to culturability occurs remains unclear. Animal passage, employing ligated rabbit ileal loops (RILs), has been found to yield culturable *V. cholerae* O1 (13) and, in a human volunteer study, culturable *V. cholerae* O1 cells were recovered in stool samples 48 h after nonculturable *V. cholerae* O1 were ingested (15).

An epidemiological and ecological survey of *V. cholerae* in the coastal ecosystem of the Bay of Bengal provided firm evidence that nonculturable *V. cholerae* O1 cells are present between epidemics in biofilms in samples collected from bodies of water serving as drinking water source for the Mathbaria area of Bangladesh, where outbreaks of cholera occur annually (16). In the study reported here, biofilms in laboratory microcosms prepared with Mathbaria water (MW) were found to harbor nonculturable coccoid *V. cholerae* O1 cells that became culturable even after 1 year, but only via animal passage. Evidence is provided here that *V. cholerae* O1 persists in the nonculturable state in biofilms in the natural aquatic environment, leading to the conclusion that biofilms play a significant role in the annual cycle of *V. cholerae* O1 in the environment and notably in annual epidemics of cholera in Bangladesh.

Results

Isolation of *V. cholerae* O1 from Environmental Samples. A total of 480 water and plankton samples were analyzed (July 2005 through January 2007) for *V. cholerae* O1 (1, 16). For this study, enriched [6–8 h in alkaline peptone water (APW)] samples were plated on taurocholate/tellurite/gelatin/agar (TTGA) with streptomycin (Sm) (70 $\mu\text{g}/\text{ml}$) and also without Sm (17). *V. cholerae* O1 was isolated from 22 of 480 samples plated on TTGA without Sm, whereas only 11 yielded culturable *V. cholerae* O1 when plated on TTGA containing Sm [supporting information (SI) Table 1]. The frequency of isolation of *V. cholerae* O1 was significantly higher from

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Abbreviations: APW, alkaline peptone water; DFA, direct fluorescent antibody; LA, Luria agar; M-PCR, multiplex-PCR; MW, Mathbaria water; RIL, rabbit ileal loop; Sm, streptomycin; TCBS, thiosulfate/citrate/bile salts/sucrose; TTGA, taurocholate/tellurite/gelatin/agar.

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Mathbaria site 2, which is connected to a flowing river, and Bakerganj site 5, also a river site, compared with sites 3, 4, and 8, which are ponds (SI Table 1). Also, the only *V. cholerae* O1 isolated by exploiting Sm resistance phenotype occurred in May 2005 at Mathbaria site 2, a pond connected to a flowing river that, with or without antibiotic in the isolation medium, had yielded *V. cholerae* O1 in an immediate past sampling round in April 2005 (SI Table 1). All strains isolated on TTGA without antibiotic were resistant to Sm and also to trimethoprim, sulfamethoxazole, and furazolidone. The difference in efficiency of the two methods (with and without Sm) in isolating *V. cholerae* O1 from water samples, 2% of samples plated on TTGA with Sm and 5% of samples plated on TTGA without Sm, was statistically significant (paired *t* test; $P = 0.002$).

Water collected from Mathbaria site 2, which contained *V. cholerae* O1, as demonstrated by both enrichment culture and direct fluorescent antibody (DFA), was used for the laboratory microcosm experiments. DFA staining of the water revealed clusters of biofilm fragments containing *V. cholerae* O1 (1). Multiplex-PCR (M-PCR) using a DNA template prepared from water of the same microcosm amplified primers for the genes *wbe* and *ctxA* (SI Table 2), confirming that MW is, in fact, an environmental reservoir for potentially toxigenic *V. cholerae* O1.

***V. cholerae* O1 Cells in Pond Water Microcosms.** Autoclaved MW microcosms inoculated with *V. cholerae* O1 yielded initial counts of 10^7 cfu/ml when plated on TTGA and Luria agar (LA), whereas counts on thiosulfate/citrate/bile salts/sucrose (TCBS) agar were 10^5 cfu/ml (SI Table 2). Initial DFA counts (day 1) were 8.0×10^8 cells per ml. M-PCR with template DNA prepared from the two microcosms on day 1 amplified the primers for *wbe* and *ctxA* (SI Table 2). Plate counts of MW microcosms maintained at room temperature (MW-RT) varied significantly from those maintained at 4°C (MW-4C). Both microcosms showed a gradual reduction in plate counts on TTGA and LA with passage of time. However, an even faster decline in plate counts on TCBS agar was observed, with no counts after 15 days. Conversely, counts for both MW-RT and MW-4C microcosms varied significantly on TTGA and LA at day 15, and subsequently, with respect to time of incubation, continued to decline; eventually no *V. cholerae* O1 counts were obtained after 40 and 85 days, respectively (SI Table 2). As the monitoring for culturable and nonculturable *V. cholerae* O1 in the microcosms continued over several weeks after nonculturability, i.e., as the decline in number of culturable cells occurred, it was observed that those fewer and fewer colonies appearing on TTGA and LA, and notably at 40 days, were tiny (<1.0 mm), grew very slowly, and did not grow on TCBS.

Interestingly, DNA templates supported amplification of primers for genes *wbe* and *ctxA* by M-PCR in cells incubated up to 40 days (SI Table 2). After 68 days, MW-4C yielded only a few colonies on TTGA and LA, and the DNA template from the same microcosm amplified primers for *wbe* but not *ctxA* (SI Table 2). At this stage of extended incubation, some colonies among many tiny colonies on TTGA and LA were confirmed as *V. cholerae* O1 (1, 16). However, the tiny colonies did not appear on TTGA and LA at day 85 and thereafter, even when concentrates of larger volumes (>10 ml) from microcosms were enriched and plated. Furthermore, template DNA from these preparations did not amplify the genes *rstR2*, *wbe*, and *ctxA*.

Unlike plate counts, which declined sharply with time of incubation, the DFA counts did not change for both the MW-RT and MW-4C microcosms, until day 15. As shown in SI Table 2, the counts for MW-4C microcosm further remained unchanged until day 26, whereas those for MW-RT were not countable because the cells had aggregated within biofilm (Fig. 1). After incubation for 2 days, the MW-RT microcosm revealed clusters of cells assembled in a coordinated pattern (Fig. 1A), with a large number of cells in clusters on day 7 (Fig. 1B), finally transforming to much larger biofilms after 26 days (Fig. 1C). With time, the curved rods of *V.*

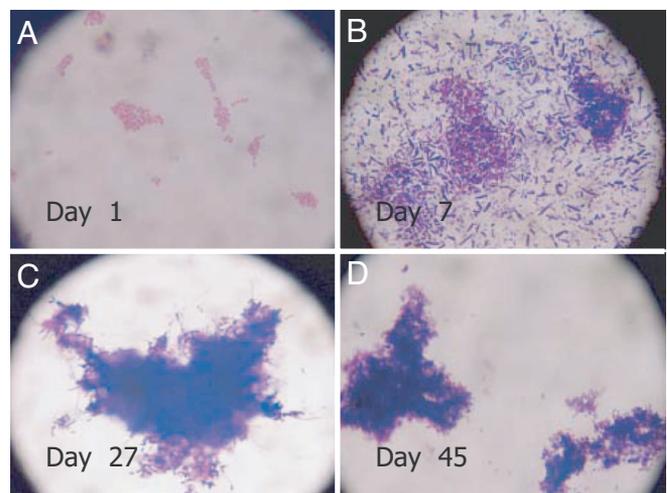


Fig. 1. Micrographs showing *V. cholerae* O1 in MW microcosms at different stages of incubation at room temperature (RT) (MW-RT). Cells were collected from microcosms, and smears were prepared on clean glass slides, air-dried, stained with crystal violet, washed, and visualized by using a phase-contrast microscope (model BX2; Olympus, Tokyo, Japan). Cells at day 1 were observed to be arranged in microcolonies (A), with larger aggregations in dark blue, an indication of biofilm, at day 7 (B). Cells appeared as multiple layers, tightly attached to thick biofilm surrounding the aggregations of cells at day 27 (C) and as aggregates of thick biofilm intensifying with time at day 45 (D).

cholerae O1, culturable at day 1, transformed into coccoid cells, gradually becoming smaller and finally appearing as dense clusters of biofilms (Fig. 1D).

In contrast to results obtained for the MW-RT microcosm, DFA counts of the MW-4C microcosm remained unchanged until after incubation for 26 days (SI Table 2). In addition, cell assemblages were not observed early during incubation, as was the case for *V. cholerae* O1 cells in MW-RT. Formation of biofilm was not obvious, even after 40 days, but did begin to occur and increase with time thereafter, up to day 99 (SI Fig. 4), indicating that biofilm formation in *V. cholerae* O1 is temperature dependent and negatively linked to culturability.

The initially culturable curved rods typical of *V. cholerae* O1 (SI Fig. 5A) transformed into small coccoid cells (SI Fig. 5B–D), and eventually become nonculturable. These cells, occurring either as single cells (SI Fig. 5B and D) or in biofilms (separated by filtration), did not grow on agar or in broth, even after prolonged enrichment in APW. Nonculturable *V. cholerae* O1 in biofilms when viewed under a microscope, i.e., cells detectable by DFA but not by culture or M-PCR (SI Table 2), continued to show reduction in size with the time of incubation until barely visible by DFA when examined by phase-contrast microscopy (SI Fig. 5D).

Epifluorescent micrographs of cells from the MW-RT and MW-4C microcosms are shown in Figs. 2 and 3, respectively. The physical appearance of *V. cholerae* O1 cells in successive stages of transformation to nonculturability in the MW-RT and MW-4C microcosms, i.e., altered morphology, reduced size, and accompanying biofilm formation, occurring after initial inoculation up to 450 and 495 days, are shown in Figs. 2 and 3.

To determine whether nonculturable cells in the microcosms could be induced to culturability, a variety of procedures were used (12). Coccoid cells in biofilms were poorly responsive to viability experiment, as reported elsewhere (17). That is, they did not become elongated in response to yeast extract and nalidixic acid. The process of elongation, which occurred in a relatively large number of cells early in incubation (Fig. 1 and SI Fig. 4B–D), took longer for cells that had been nonculturable for a longer time. That

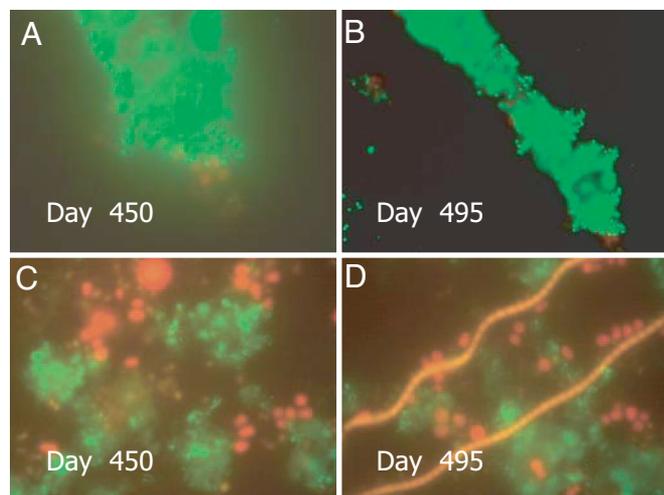


Fig. 2. Epifluorescence micrographs of nonculturable *V. cholerae* O1 in microcosms MW-RT (A and B) and MW-4C (C and D). Samples were stained with DFA reagent specific for *V. cholerae* O1 (New Horizon Diagnostics) and visualized with an epifluorescence microscope (Olympus, model BX51, BX2 Series) (4). Microscopic images were captured digitally (Olympus, DP 20) and processed by using Photoshop Version 5 (Adobe Systems, San Jose, CA). (A and B) Coccoid *V. cholerae* O1 cells attached to a thin biofilm at 450 days and 495 days after inoculation into MW-RT, respectively. (C and D) Coccoid *V. cholerae* O1 cells in biofilm 450 days and 495 days after inoculation into MW-4C, respectively.

is, only a few of these cells within biofilms responded to viability after incubation for 15 months (Fig. 3).

M-PCR results showed that DNA templates prepared from MW-RT and MW-4C microcosms amplified all of the primers tested, i.e., *wbe*, *ctxA*, and *rstR2*, even after incubation for 40 days (SI Table 2). However, after 54 days, only *wbe* was amplified from the MW-RT microcosm, whereas all primers were amplified for MW-4C (SI Table 2). M-PCR did not amplify all primers for both MW-RT and MW-4C microcosms incubated for 68 days, with the exception being the MW-4C, which amplified only *wbe* (SI Table 2).

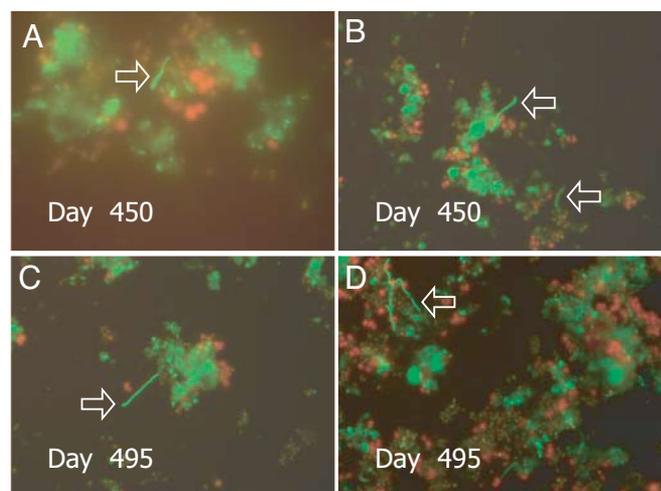


Fig. 3. Epifluorescence micrographs of nonculturable *V. cholerae* O1 in MW-4C microcosms. Samples were pretreated with 0.025% yeast extract and 0.002% nalidixic acid, stained by using cholera DFA (New Horizon Diagnostics), visualized under an epifluorescence microscope (Olympus, model BX51) (1, 4, 17). Images were captured digitally (Olympus DP 20) and processed by using Photoshop Version 5. (A and B) Elongation of *V. cholerae* O1 in a biofilm that had been incubated for 450 days after initial inoculation. (C and D) Elongation of coccoid *V. cholerae* O1 cells in a biofilm 495 days after inoculation into MW-4C.

Further analysis of both MW-RT and MW-4C microcosms by M-PCR at day 85 did not amplify any of the three primer pairs tested (SI Table 2). Note especially that *ctxA* and *rstR2* of bacteriophage CTX Φ were negative for both MW-RT and MW-4C microcosms at day 54 and 68, respectively (SI Table 2). These results suggest, with high probability, that the entire CTX Φ genome is lost during extended incubation in the microcosms. Significantly, in contrast, *wbe* was amplified, even after the CTX Φ gene was no longer detectable in either MW-RT or MW-4C, at days 54 and 68, respectively.

MW Microcosms Inoculated with Cholera Stool. Culturable *V. cholerae* O1 in cholera stool samples inoculated into dialysis bags, which were suspended in 1 liter of autoclaved MW and maintained at room temperature (25–33°C), were examined at selected time intervals by culture, DFA, and M-PCR for genes *wbe*, *ctxA*, and *rstR2*. As shown in SI Table 2, cholera stools in dialysis bags suspended in autoclaved MW at day 1 yielded plate counts of 10⁵ cfu/ml, direct counts of $\approx 10^7$ cells per ml, and amplified primers for *wbe*, *ctxA*, and *rstR2*. The DFA results showed that the majority of the cells had already become coccoid by the first day of incubation (SI Fig. 6A) and that the cells occurred as clusters in biofilm. These cholera stool microcosms amplified primers for *wbe*, *ctxA*, and *rstR2* at day 15, but did not produce colonies when plated. Direct counting by DFA was not possible because of the extensive biofilms that had formed by 15 days (SI Fig. 6). As shown in SI Table 2, results of monitoring up to 68 days revealed the cholera stool microcosm was consistently negative by enrichment culture, with coccoid cells in the biofilm further reduced in size with extended incubation (SI Fig. 5D).

MW Microcosms. Microcosms prepared with unfiltered MW that had tested positive for *V. cholerae* O1 revealed biofilm aggregates containing both curved rods and coccoid cells of *V. cholerae* O1 when examined by DFA. Also, M-PCR, using template DNA from the same microcosm, amplified primers for the genes *wbe* and *ctxA* (1). See SI Table 2. However, samples at day 15 and up to day 99 were negative for *V. cholerae* O1 when tested by both enrichment culture and M-PCR. In sharp contrast, biofilm aggregates containing coccoid cells were positive by DFA.

Ileal Loop Analysis. The nonculturable *V. cholerae* O1 cells from various microcosms described above were harvested, and equal volumes of cell pellets were used in animal challenge experiments. None of the harvested nonculturable coccoid cells grew on agar even after prolonged enrichment in APW. Adult RILs were inoculated (18) with nonculturable coccoid cells in biofilm aggregates, confirmed as such by DFA (18). After incubation for 18 h, the contents in RIL were removed aseptically, and the volume was measured to determine fluid accumulation. Contents from each of the RILs, separately enriched in APW for 6–8 h, and ≈ 5 –10 μ l from the APW streaked onto TTGA agar and incubated for 18 h at 37°C, yielded significantly large numbers of colonies on TTGA, confirmed by serotyping and M-PCR (1) to be *V. cholerae* O1.

The nonculturable *V. cholerae* O1 cells from originally cholera-positive stools and maintained in MW microcosms at room temperature for 59 days were culturable after passage in RIL assay. Resuscitation by animal passage in the RILs was also achieved for *V. cholerae* O1 in biofilm aggregates collected from pond water and maintained at room temperature for up to 54 days. Nonculturable *V. cholerae* O1 in biofilm from MW-RT and MW-4C microcosms were recoverable by means of RILs up to 495 days. Interestingly, none of these nonculturable cells inoculated into the RILs produced significant fluid accumulation at 18 h, even though they were able to be cultured.

Filtration Experiment. Water samples from microcosms containing nonculturable cells were filtered through 0.22- μ m membrane filters

(1). The *V. cholerae* O1 cells retained on the 0.22- μ m membranes were coccoid in morphology but occurred as clusters of cells within thick biofilms (SI Fig. 5C). The few cells observed in the filtrates were single, coccoid, and only rarely within a biofilm and usually in a biofilm fragment (SI Fig. 5D).

Animal passage of free cells in the filtrates did not yield culturable cells. In sharp contrast to the free cells, the clusters of nonculturable cells in biofilm collected by filtration were able to be cultured after animal passage.

Discussion

V. cholerae and many related Gram-negative bacteria have been shown to become nonculturable under specific experimental conditions, although the time required for these cells to become nonculturable is variable (8, 9, 19–21). In this study, *V. cholerae* O1 cells in all microcosms became nonculturable on TCBS agar within 10–15 days, as has been reported by other investigators (19–21). *V. cholerae* O1 in biofilms collected from MW and in the biofilm in clinical specimens, when suspended in autoclaved MW that had tested positive for *V. cholerae* O1 by both culture and DFA, became nonculturable within 15 days. Conversely, MW-RT and MW-4C microcosms inoculated with freshly grown *V. cholerae* O1 showed culturability on TTGA and LA for 40 and 68 days, respectively. Miller *et al.* (22) suggested that toxigenic *V. cholerae* O1 could remain culturable for longer periods at a salinity of 0.25–3.0%, a pH of 8.0, and 25°C. The temperature, pH, and salinity of MW used in the studies reported here were not very different from those conditions used previously.

Initial plate counts on TCBS for both MW-RT and MW-4C microcosms were only 1% of the counts obtained on TTGA and LA. The counts on TCBS agar declined sharply within 2 weeks with no growth obtained for either microcosm on TCBS thereafter. However, TCBS, a medium widely used for selective growth of vibrios, has long been known to be inhibitory to a significant portion of a *V. cholerae* population (23) and is not an appropriate medium for direct counting or isolation of *V. cholerae*.

Although the curved rods typical of *V. cholerae* O1 observed at day 1 gradually became coccoid, the DFA counts did not change significantly, evidence for intact nonculturable cells, induced, being present but not culturable. Moreover, highly significant was the observation by microscope that individual planktonic cells in the MW microcosm did form aggregates and produce biofilm that enclosed the aggregates, with those cells thus enclosed becoming coccoid. The clusters of coccoid cells were unequivocally visible within the biofilms, confirming the reports of Kierac and Watnick (24) and Watnick *et al.* (10). Many microorganisms produce polysaccharides, either as capsules or as extracellular matrices, in their immediate environment (25), a function of which is to provide protection against adverse external conditions (26).

The genes *ctxA* and *ctxB*, encoding cholera toxin (CT) subunits A and B, respectively, are constituents of a lysogenic bacteriophage, CTX Φ , whose 7.0-kb genome has the structure of a compound transposable element. The CTX Φ genome consists of a core region encoding CT and other genes that code for virion morphogenesis, as well as an RS2 region encoding proteins for regulation, replication, and integration of the CTX Φ genome (27). In the *V. cholerae* chromosome, the lysogenic CTX Φ genome frequently is flanked by a related genetic element, RS1 (28), the genome of a satellite phage that utilizes CTX Φ machinery to produce RS1 Φ particles (29). RS1 Φ encodes at least four ORFs (*rstABCR*) that jointly determine expression of a site-specific recombination catalyzing integration of plasmids carrying portions of the CTX element into the genome of nontoxigenic *V. cholerae* strains at a 17-bp target sequence, *attRS1* (28). Under appropriate conditions, CTX Φ is self-transmissible and replicates as a plasmid (27). The filamentous CTX Φ propagates by horizontally infecting susceptible strains of *V. cholerae* and *Vibrio mimicus* (30).

Strains of *V. cholerae* O1 lacking CTX Φ prophage have been reported to occur in the aquatic environment (31). However, it is not known whether *ctx⁻* strains exist in the environment as a separate clonal lineage, originally lacking CTX Φ , or whether CTX Φ previously had been excised. Evidence is provided here that the *ctxA* and *rstR2* genes of CTX Φ can be lost simultaneously from toxigenic *V. cholerae* O1, whereas the *wbe* gene encoding the O1 antigen is retained, confirmed by M-PCR. It was not clear, however, from results obtained in this study, whether M-PCR for *ctxA* and *rstR2* was unsuccessful because the entire CTX Φ genome was excised or because truncations of these loci had occurred. Because *ctxA* and *rstR2* are located at two opposite termini of the CTX Φ genome (27), it is concluded that the entire CTX Φ was excised.

Existence of *ctx* progenitors of pandemic strains in the aquatic environment of Mathbaria has been documented (1). Evidence for the loss of CTX Φ prophage, resulting in *ctx⁻* *V. cholerae* O1, as presented here, indicates that toxigenic strains in the environment can become nontoxigenic during interepidemic periods. Thus, our results suggest a mechanism whereby *ctx⁻* *V. cholerae* O1 can occur in the environment and serve as progenitor, because it has long been known that environmental isolates of *V. cholerae* O1 rarely carry the genes for cholera toxin (31).

Because the nonculturable state has been considered to be a survival strategy for many bacterial pathogens (4, 5, 7–9, 19, 32), the mechanism by which such cells return to culturability is important to define. Resuscitation from the dormant state has been reported for an array of bacterial pathogens, mainly by induction by temperature upshift (9), but other explanations include increased nutrient concentration (13, 33, 34), a combination of temperature upshift and increased nutrient concentration (20), or the action of a heat-stable enterobacterial autoinducer (35). However, recovery of culturable bacterial cells by temperature upshift alone has also been suggested to represent regrowth of either one or a few cells remaining culturable (36) or insensitive to H₂O₂ (37).

What is not disputed is the fact that cholera epidemics occur seasonally in endemic areas, but during interepidemic periods, nonculturable cholera bacteria are detectable in water only when molecular and immunochemical methods are used. That is, culturable *V. cholerae* O1 rarely can be isolated. It is recognized that, in addition to naturally occurring *V. cholerae* O1 that give rise to initial diarrheal epidemics in the spring months of the year, the aquatic environment receives large quantities of these bacteria released into the water during diarrheal epidemics. From an ecological point of view, if return from the nonculturable state to culturability were simply a temperature and/or nutrient response, the characteristic seasonality of cholera would be expected to result in a series of sporadic outbreaks, particularly during the summer months, when temperature and nutrient levels of shallow water bodies in tropical cholera-endemic areas of the world are higher than in spring and fall, the two seasons of annual cholera, which actually occurs in a distinctive bimodal pattern (38) related to blooms of the zooplankton host of *V. cholerae* O1 (2, 3, 7).

Despite nonculturability having long been proposed as a survival strategy for cholera bacteria in the aquatic environment between epidemics, the reservoir and the mechanism by which nonculturable cells regain culturability to initiate seasonal cholera are not clear. In this study, nonculturable *V. cholerae* O1 cells in MW microcosms were observed to occur in aggregates within biofilms. Also, in animal challenge experiments, nonculturable *V. cholerae* cells in biofilms converted to actively growing *V. cholerae* O1, even after having been nonculturable for more than 1 year, in microcosms from which culturable cells could not be recovered. The results presented in this study explicitly demonstrate that those nonculturable cells, after exposure to MW microcosms for 495 days, that proved viable occurred only within biofilms. It is concluded that biofilm-bound, nonculturable cells in the natural aquatic environment play a significant role in the seasonal epidemics of cholera (1, 16).

A recent study carried out in Dhaka, Bangladesh, reported enhanced infectivity of *V. cholerae* O1 in human stools being largely caused by the presence of *V. cholerae* O1 in biofilms formed *in vivo* (39). Furthermore, a Sm-supplemented primary isolation medium was used to obtain improved recovery of cholera vibrios and those that were isolated by using the Sm-supplemented medium were referred to as “conditionally viable environmental cells (CVEC)” (17), the inference being that conditions of conventional culture methods underestimate the numbers of *V. cholerae* O1, because the antibiotic-supplemented medium suppresses competition from other aquatic bacteria. In the study reported here, a component of a 10-year, more extensive program of research on the ecology and pathogenicity of *V. cholerae* O1 in selected coastal environmental sites within cholera endemic areas located adjacent to the Bay of Bengal (1), both Sm-supplemented and nonsupplemented media were used. Poor recovery of *V. cholerae* O1 was achieved when the drug was used, in sharp contrast to results reported for a study carried out at sites in Dhaka City, Bangladesh (17), where the yield of *V. cholerae* O1 from the antibiotic medium was >2-fold compared with the standard culture medium without antibiotic. Unlike the Dhaka City study, where *V. cholerae* O1 was isolated from the environment consistently and for an extended period (August–December) (17), isolation of culturable *V. cholerae* O1 from the Mathbaria and Bakerganj environments was successful only within a narrow time period, namely spring and fall, the very times when seasonal epidemics of cholera occur in Bangladesh. Also, noteworthy was the observation in this study that the frequency of isolation of *V. cholerae* O1 was higher for environmental sites linked to a river. The only sample in the present study that yielded *V. cholerae* O1 when antibiotic media were used was collected at the Mathbaria site no. 2, which is linked to a flowing river, analogous to the site in the Dhaka City study (17). The Mathbaria site yielded *V. cholerae* O1 in two preceding samplings regardless of whether antibiotic-supplemented media were used. In fact, only when sampling was done immediately after the seasonal peak of endemic cholera did the antibiotic medium prove successful in isolating *V. cholerae* O1. This difference is most probably attributable to the ecology of the coastal Bay of Bengal, which is significantly different from Dhaka City, the most populated city in Bangladesh, where epidemics of seasonal cholera are intense and continue for extended periods of time. The bodies of water in and immediately surrounding Dhaka City are heavily contaminated with domestic sewage.

In this study, we were able to confirm that cholera stool, indeed, can contain aggregates of cells in biofilm fragments that remain infective, revive, and become culturable upon animal challenge. However, the aggregates of cells in biofilms in cholera stool that were present immediately after release to the environment contained cells that remained infective only for a short period, thereby having a temporal limitation with respect to contributing to the intensity of cholera epidemics and most probably only for those areas in and around a major city like Dhaka, where conditions of poor sewage treatment expedite the fecal–oral mode of transmission. It cannot, however, account for the annual seasonality of cholera that occurs with significant regularity in the spring and fall months in the remote coastal villages that Mathbaria and Bakerganj, Bangladesh, represent.

In previous studies, we have shown conclusively that biofilms form *in situ* in the aquatic environment and that such biofilms for most of the year will contain cholera bacteria in the nonculturable state (1, 16). Analogous to the proposal of Faruque *et al.* (39), namely that *in vivo*-formed clumps of *V. cholerae* O1 cells shed by cholera patients increase transmissibility of cholera, such *in vivo*-formed clumps of *V. cholerae* O1, when inoculated into MW retained infectivity for at most 2 months. In contrast, *V. cholerae* O1 inoculated into MW microcosms also formed biofilms (1, 16), but those biofilms are more likely to play a significant role in the overall ecology of *V. cholerae* and to account for seasonal epidemics of cholera in cholera-endemic areas of Bangladesh. Interepidemic

periods of seasonal cholera may comprise a few or several months, during which *V. cholerae* O1 is not detectable by culture but is present and detectable when molecular methods are used (1, 16). Seasonal epidemics of cholera in Bangladesh characteristically commence when water harboring cholera bacteria is ingested (2). Therefore, understanding that the cholera vibrios are present throughout the year in the aquatic environment of an endemic area is critical to recognizing the basis of the periodicity of cholera outbreaks that is both predictable and similar year to year.

Preepidemic enrichment of nonculturable *V. cholerae* in the human host as a method of amplification of an epidemic *V. cholerae* strain immediately before onset of an epidemic (17) can contribute to transmission via the fecal–oral route of infectivity of human-amplified clones, but it cannot account for the natural environmental reservoir, nor for the centuries-long consistent and persistent pattern of cholera epidemics that have been historically documented for this disease in Bangladesh (38). However, biofilms formed *in situ* offer significantly greater implication for endemic cholera (1) and provide yet another environmental factor to be considered in understanding and preventing cholera epidemics. In addition, environmental factors driven by climate, including blooms of plankton for which vibrios are commensal and/or symbiotic, play a key role in the endemicity of cholera that cannot be ignored in public health planning and response (3, 7, 13).

Materials and Methods

Collection of Environmental Samples. Samples were collected and analyzed by employing methods described previously (1, 16). This study, part of which includes sample collection from the environment, made use of the availability of samples routinely collected in an ongoing major study of the epidemiology and ecology of *V. cholerae* O1 in Mathbaria and Bakerganj, where cholera is endemic (16). Water and plankton samples collected biweekly from three ponds (one connected to a flowing river) in Mathbaria, and monthly from one river and one pond in Bakerganj (July 2004 to January 2007), were included in this study. All samples were collected while following aseptic techniques described elsewhere (1, 16).

Processing of Environmental Samples. Samples were inoculated into APW (Difco, Detroit, MI) and incubated at 37°C for 6–8 h before plating on TCBS agar (Eiken, Tokyo, Japan) and TTGA (Difco), as described elsewhere (1, 16). To test the selective efficiency of TTGA, with and without antibiotic, samples enriched in APW were streaked on TTGA plates with Sm (70 µg/ml) and also without Sm (17). Inoculated plates were incubated at 37°C for 18–24 h. Presumptive *V. cholerae* colonies were confirmed, as described elsewhere (1, 16).

Collection and Analysis of Clinical Samples. Stool samples from cholera patients who had not taken antibiotics before reporting to Dhaka hospital of the International Center for Diarrhoeal Disease Research, Bangladesh, were collected in sterile 4-oz (118-ml) vials. Aliquots of the samples were used to inoculate TTGA plates. After incubation overnight at 37°C, suspected *V. cholerae* colonies were confirmed, as described elsewhere (1, 16).

Preparation of Laboratory Microcosms. Pond water microcosms. Microcosms were prepared with MW positive for *V. cholerae* O1 when tested by both culture and DFA (1). The MW microcosms (1 liter in 2.5-liter conical flasks) were autoclaved and *V. cholerae* O1 (EM-226) El Tor cells of exponential phase, grown in Luria-Bertani (LB) broth at 37°C and washed with PBS (pH 8) and finally MW, were inoculated at a final concentration of 10⁷ cfu/ml into duplicate microcosms. One was maintained at room temperature (25–33°C), designated MW-RT, and the other at 4°C (MW-4C). At selected time intervals, samples from microcosms were examined by plating on TTGA, TCBS, and LB agar,

and DFA counts and M-PCR (as described below) were also done.

Clinical sample microcosms. Microcosms, prepared as above, were treated as follows. Aliquots of *V. cholerae* O1 culture positive stool samples were transferred to a series of PBS-pres soaked and washed 25 mm × 16 mm dialysis tubes (Sigma, St. Louis, MO) containing MW, prepared as described above. That is, the tubes were sealed and placed in MW microcosms incubated at room temperature. Each of the dialysis tubes was aseptically sampled periodically for culturable *V. cholerae* O1 and also tested by both DFA and M-PCR.

MW and plankton microcosms. Microcosms were constructed in sterile 2.5-liter conical flasks by using 1-liter MW aliquots (not autoclaved) seeded with plankton fractions collected by filtering 100 liters of MW (1, 16) through a 20- μ m filter, the 1 liter of water being from the pond that tested positive for *V. cholerae* O1 by culture, DFA, and M-PCR. DFA slides of MW, prepared before collection of plankton and examined under the microscope, revealed biofilm clusters containing cells typical of *V. cholerae* O1 (1). The mouth of each flask was sealed aseptically with sterilized aluminum foil and then incubated at room temperature. At selected time intervals, *V. cholerae* O1 total culturable counts, DFA, and M-PCR were determined.

DFA. *V. cholerae* O1 counts by DFA were made with samples preincubated with, or without, 0.025% yeast extract (Difco) and 0.002% nalidixic acid (Sigma-Aldrich, St. Louis, MO), overnight in the dark. Samples were centrifuged and pellets were stained with cholera DFA reagents (New Horizon Diagnostics, Columbia, MD). *V. cholerae* O1 in stool samples were counted in samples diluted 10-fold in PBS before fixing on slides for staining with DFA (40). Fluorescent stained cells were counted by using an epifluorescence microscope (Olympus BX51) and recorded with a digital camera attachment (Olympus DP20).

M-PCR. The genes *wb* encoding O antigens of *V. cholerae* O1 (*wbe*) and O139 (*wbf*), *ctxA* encoding cholera toxin subunit A, and *rstR2*,

encoding a repressor for El Tor CTX Φ , were amplified by using M-PCR (1). Samples preincubated overnight with 0.025% yeast extract and 0.002% nalidixic acid were subjected to M-PCR, using the procedure described in ref. 41. Primer sequences for genes, *wbe*, *ctxA*, and *rstR2* are shown in SI Table 3.

Animal Passage of Nonculturable *V. cholerae* O1. Cells of nonculturable *V. cholerae* O1 in MW microcosms were collected by filtering 10 ml through a 0.22- μ m membrane to a final volume of 2 ml. Bacteria washed from the surface of the membrane filters were aseptically transferred to sterile Eppendorf tubes in duplicate (1.0 ml each) of which 1.0 ml was enriched in APW for 6–8 h at 37°C to determine that no culturable cells were present. An aliquot was also inoculated into RILs, following methods described elsewhere (19). The rabbits were killed after 18–20 h and contents of the RIL were collected aseptically and enriched in APW at 37°C for 6–8 h before plating (4). A loopfull of enriched APW was streaked onto TCBS agar and TTGA and incubated at 37°C for 18–24 h. Presumptive *V. cholerae* colonies were confirmed by biochemical, serological, and, finally, molecular methods (1, 16).

Filtration. Nonculturable *V. cholerae* O1 planktonic cells (free cells in the water phase of the microcosms) and cells bound within biofilm in the microcosms were separated by filtration employing 0.22- μ m membrane filters (1). The filtrate (planktonic-phase cells) and cells retained on the membrane filters (biofilm-bound cells) were separately subjected to enrichment culture and animal passage.

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