Activation of Cholera Toxin Production by Anaerobic Respiration of Trimethylamine N-oxide in Vibrio cholerae

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Background: The human intestine, in which Vibrio cholerae exerts its virulence, is an anaerobic environment. Results: When grown anaerobically with trimethylamine N-oxide (TMAO), V. cholerae exhibited enhanced growth and cholera toxin (CT) production was remarkably induced. Conclusion: Anaerobic TMAO respiration may serve as a signal to increase V. cholerae virulence. Significance: A novel growth condition that induces CT production is uncovered.

Vibrio cholerae is a Gram-negative bacterium that causes cholera. Although the pathogenesis caused by this deadly pathogen takes place in the intestine, commonly thought to be anaerobic, anaerobiosis-induced virulence regulations are not fully elucidated. Anaerobic growth of the V. cholerae strain, N16961, was promoted when trimethylamine N-oxide (TMAO) was used as an alternative electron acceptor. Strikingly, cholera toxin (CT) production was markedly induced during anaerobic TMAO respiration. N16961 mutants unable to metabolize TMAO were incapable of producing CT, suggesting a mechanistic link between anaerobic TMAO respiration and CT production. TMAO reductase is transported to the periplasm via the twin arginine transport (TAT) system. A similar defect in both anaerobic TMAO respiration and CT production was also observed in a N16961 TAT mutant. In contrast, the abilities to grow on TMAO and to produce CT were not affected in a mutant of the general secretion pathway. This suggests that V. cholerae may utilize the TAT system to secrete CT during TMAO respiration. During anaerobic growth with TMAO, N16961 cells exhibit green fluorescence when stained with 2',7'-dichlorofluorescein diacetate, a specific dye for reactive oxygen species (ROS). Furthermore, CT production was decreased in the presence of an ROS scavenger suggesting a positive role of ROS in regulating CT production. When TMAO was co-administered to infant mice infected with N16961, the mice exhibited more severe pathogenic symptoms. Together, our results reveal a novel anaerobic growth condition that stimulates V. cholerae to produce its major virulence factor.

Cholera is an acute noninflammatory diarrheal disease that affects humans infected with the causative pathogen Vibrio cholerae (1). Cholera has been involved in seven historic pandemics and has posed a huge threat to human health in regional epidemics until very recently (2). Among more than 200 O-antigen serotypes, O1 and O139 serotypes are toxigenic and can cause cholera. O1 serotype strains are further classified into two biotypes, El Tor and Classical, the latter of which is presumed extinct (3). Invading V. cholerae cells that survive the acidic gastric environment enter the intestine, where they produce an array of virulence factors, including cholera toxin (CT)3 and toxin co-regulated pilus (TCP) (4). CT is an ADP ribosylating toxin that creates imbalanced ion transport across the intestinal epithelia leading to loss of electrolytes and water from the epithelial cells (5). TCP is known to play an essential role in the bacterial colonization to the intestinal surface (6). Human intestine is occupied with commensal bacteria, most of which are strict anaerobes (7). This suggests that (i) the microenvironment in the human intestine is anaerobic and (ii) anaerobiosis may serve as a host factor that modulates V. cholerae virulence (8). Consistent with this notion, recent reports showed that under anaerobic conditions, expression of tcpP, a regulator of virulence gene expression (9) was elevated and this increase was mediated by a novel oxygen sensing mechanism of AphB, a LysR-type transcriptional activator (10, 11). These findings were achieved from V. cholerae cells grown anaerobically in AKI media.

As a facultative anaerobe, V. cholerae can support its growth by fermentation of diverse carbohydrates including glucose, sucrose, maltose, mannitol, lactose, dextrin, and starch (12, 13). Sucrose fermentation has been used as a basis for the identification of V. cholerae species among fecal isolates (14). However, whether V. cholerae can also support anaerobic growth by respiration of alternative electron acceptors (AEAs) has not been

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** The abbreviations used are: CT, cholera toxin; TMAO, trimethylamine N-oxide; TMA, trimethylamine; DCF-DA, 2',7'-dichlorofluorescein diacetate; AEA, alternative electron acceptors; DMSO, dimethyl sulfoxide; DIC, differential interference contrast; Tn, transposon; TAT, twin arginine transport; NAC, N-acetylcysteine; TCP, toxin co-regulated pilus.
extensively studied. In addition, studies have not been conducted on (i) which AEA can most efficiently stimulate anaerobic growth of V. cholerae, (ii) how anaerobic respiration contributes to bacterial proliferation in the human intestine, and (iii) how V. cholerae virulence is regulated under such anaerobic respiratory growth.

The genome of the 7th pandemic strain N16961 contains several genes that are likely involved in anaerobic respiration. It appears that N16961 is capable of utilizing fumarate, nitrate, trimethylamine N-oxide (TMAO), or dimethyl sulfoxide (DMSO) as AEAs (15). In this study, we investigated the anaerobic growth and virulence regulation of N16961 under diverse anaerobic respiration conditions. N16961 grew better and showed increased CT production when grown in anaerobic TMAO respiration. This report reveals novel features associated with V. cholerae virulence during a growth mode that may occur inside the human intestine.

TABLE 1

| Bacterial strains and plasmid used in this study | Relevant characteristics | Ref. or source |
|-----------------------------------------------|--------------------------|---------------|
| **V. cholerae strains**                      |                          |               |
| N16961                                        | O1, El Tor               | Lab collection|
| N16961 PCTXAB::lacZ fusion                   | ctxAB promoter lacZ fusion construct | This study |
| ΔVC1720                                       | N16961, VC1720::TnKGL3   | This study    |
| ΔVC0116                                       | N16961, VC0116::TnKGL3   | This study    |
| ΔVC0205                                       | N16961, VC0205::TnKGL3   | This study    |
| ΔVC0104                                       | N16961, VC0104::TnKGL3   | This study    |
| N16961 Δtat mutant                            | N16961, VC0086-VC0088 deleted | This study |
| N16961 Δsec mutant                            | N16961, VC0742-VC0744 deleted | This study |
| N16961 Δtype II mutant                        | N16961, VC2723-VC2734 deleted | This study |
| QS95                                          | O1, Classical            | Lab collection|
| C6706                                         | O1, El Tor               | Lab collection|
| 5698B                                         | O1, Classical            | Lab collection|
| MO10                                         | O139                     | Lab collection|
| AM19226                                       | Non-O1, non-O139         | Lab collection|
| CVD115                                        | hap, rtxA double mutant of CVD110 (24) |
| **E. coli strain**                            |                          |               |
| SM10/a1pir                                    | Km’ thI-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu pir’, for conjugal transfer | Lab collection |
| **Plasmids**                                  |                          |               |
| pCVD442                                       | sacB suicide vector from plasmid pUM24 | Lab collection |
| pVIK112                                       | Suicide vector for lacZ reporter fusion, Km’ | Lab collection |
| pTnKGL3                                       | Suicide vector bearing TnKGL3, Cm’ Km’ | Lab collection |

**EXPERIMENTAL PROCEDURES**

**Ethics Statement**—All animal experiments were conducted following the national guidelines provided by the Korean government (Ministry for Food, Agriculture, Forestry and Fisheries) and in strict accordance with the institutional guidelines for animal care and use of laboratory animals. The methods for animal experimentation using infant mice were approved by the Committee on the Ethics of Animal Experiments of the Yonsei University College of Medicine (permit number 2011-0166).

**Bacterial Strains and Growth**—Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37 °C in Luria-Bertani media (LB, 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl/liter). The anaerobic growth of V. cholerae strains was performed as described elsewhere (16). To support anaerobic growth, trimethylamine N-oxide, dimethyl sulfoxide, or fumarate (Sigma) was added to the medium and termed LBT, LBD, or LBF, respectively.

**CT ELISA and Western Blot Analysis**—CT ELISA was performed as previously described (17). Purified CT (List Biological Laboratories, Inc., Campbell, CA) was used to provide a standard curve. For Western blot analysis, culture supernatants were first concentrated via TCA (trichloroacetic acid, Sigma) precipitation (18). Western blot analysis was carried out as previously described (19). Rabbit polyclonal antibody raised against CT subunit B (Abcam Inc., Cambridge, UK) was used for both assays.

**Transposon (Tn) Mutant Screening**—A library of Tn-insertion mutants was constructed by the conjugal transfer of TnKGL3, a mariner-based Tn (3). Km’ mutants (>20,000) were screened for their capability to grow anaerobically on LB agar plates containing 50 mM TMAO. N16961 mutants found to form smaller-sized colonies after 2 days of anaerobic growth were selected and individually tested in broth cultures. Arbitrary PCR was performed to determine the location of the Tn insertion site. Information regarding primer sequences and PCR protocol are described elsewhere (3).

**Construction of Mutants and ctxAB Promoter-lacZ Fusion Strain**—V. cholerae mutants were created by allele replacement as previously described (20). The 500-base-pair flanking sequences located at both ends to introduce mutation were amplified by PCR with the primers listed in Table 2. Construction of a single-copy PctxAB::lacZ transcriptional fusion and β-galactosidase activity assay were performed as described previously (3).

**Confocal Microscope**—Differential interference contrast (DIC) and green fluorescent images were acquired using a confocal laser scanning microscope (FV-1000; Olympus Optical Co. Ltd., Japan) and its operating software, FV10-ASW (version 02.01). Detailed procedures are described elsewhere (16, 21). For detection, N16961 was grown anaerobi
cally for 8 h with 50 mM TMAO, DMSO, or fumarate. Aliquots of each culture were removed and stained with 10 μM DCF-DA (2’,7’-dichlorofluorescein diacetate, Sigma) for 30 min. To capture the green fluorescence, samples were scanned at 488 nm and
Anaerobic Respiratory Growth and CT Production in V. cholerae

**TABLE 2**

| Gene name                     | Direction¹ | Primer sequence (5’-3’)² |
|-------------------------------|------------|-------------------------|
| Cloning                      |            |                         |
| ctxA-promoter fusion          | F          | AGCAAGCTGCGTCACGCCACCCA |
| ctxA-promoter fusion          | R          | ACGCACTTGGGTCATCCTCAA   |
| tatABC Left                   | F          | CTCTAGCTCTGCACACACTGTT  |
| tatABC Right                  | F          | CCTCTAGCTCTGCACACACTGTT |
| yajC-seD-seF Left             | R          | GATAGCAGCTGCTCATCTGTGAA |
| yajC-seD-seF Right            | R          | AACTCTGAGCTACTGCGGAGTT  |
| VC2734-VC2723 Left            | R          | AACCTGGAGACACCTCGTGAC   |
| VC2734-VC2723 Right           | R          | GATTAGCAGCTGCTCATCTGTGAA|
| qRT-PCR                       |            |                         |
| rpoD                          | F          | AGCAAGCTGCGTCACGCCACCCA |
| rpoD                          | R          | ACGCACTTGGGTCATCCTCAA   |
| ctxB                          | F          | CCTCTAGCTCTGCACACACTGTT |
| ctxB                          | R          | CTCTAGCTCTGCACACACTGTT  |
| ctxA                          | F          | GATAGCAGCTGCTCATCTGTGAA |
| ctxA                          | R          | AACTCTGAGCTACTGCGGAGTT  |
| toxT                          | F          | CTCTAGCTCTGCACACACTGTT  |
| toxT                          | R          | GATAGCAGCTGCTCATCTGTGAA |
| toxR                          | F          | AACTCTGAGCTACTGCGGAGTT  |
| toxR                          | R          | CTCTAGCTCTGCACACACTGTT  |
| tcpP                          | F          | GAGCAAGCTGATATATATATATATATATA |
| tcpP                          | R          | CTCTAGCTCTGCACACACTGTT  |

¹ F, forward; R, reverse.
² Restriction enzyme recognition sequences are underlined.
³ VC2734-VC2723 are genes encoding components of Type II secretion system.

emission was detected through a 520-nm band filter. The DIC
and green fluorescence images were collected simultaneously.

Two-dimensional Gel Electrophoresis and Protein Identification—N16961 cells grown anaerobically for 16 h in LB, LBT,
LBD, or LBF were harvested by centrifugation at 14,000×g for
5 min. The cell pellet was washed three times with ice-cold PBS
and submitted to Genomics Inc. (Pohang, Korea), where the
entire proteomic analysis was performed.

Infant Mouse Infection—Infant mice (~5 to 6 days old, Central
Lab Animal Inc., Seoul) were orogastrically infected with V.
cholerae strains following procedures previously described (3).
After 24 h infection, intestinal homogenates were prepared and
the number of viable cells was determined by spreading serial
diluents on LB agar containing Sm (for total bacterial cell
count) or LB agar containing both Sm and Km (for ΔtorD
mutant).

qRT-PCR—Transcript levels of virulence-associated genes (ctxA, ctxB, toxT, toxR, and tcpP) were measured by qRT-PCR.
The detailed analysis procedure has been described previously
(21). Transcript levels of the rpoD gene were used to normalize
the transcript levels of the tested genes. The primers used for
qRT-PCR are listed in Table 2.

TMAO Reductase Activity Assay—V. cholerae strains,
N16961, and four Tn-insertion mutants, were grown anaerobi-
cally in LB or LBT for 16 h. To analyze the TMAO reductase
activity in different cellular fractions, the periplasmic and cyto-
plasmic fractions were separated by polymyxin B treatment.
Cell pellets were resuspended with PBS containing 2,000 units
of polymyxin B and incubated for 15 min at 4 °C. After incuba-
tion, reaction mixtures were centrifuged at 12,000 rpm for 10
min and the supernatants were saved for periplasmic fractions.
Cell pellets were then resuspended in PBS and sonicated to
produce cytoplasmic fractions. Equal amounts of proteins pres-
ent in each fraction were resolved by a 9% nondenaturing poly-
acrylamide gel, and a native gel-based enzyme assay was per-
fomed as previously described (22).

Statistical Analysis—Data are expressed as mean ± S.D. An
unpaired Student’s t test was used to analyze the data. A p value
of <0.05 was considered statistically significant. All experi-
ments were repeated for reproducibility.

RESULTS

Anaerobic Growth of the V. cholerae Strain N16961 Was
Enhanced by TMAO Respiration and CT Production Was
Remarkably Induced under Such Conditions—V. cholerae
was reported to support anaerobic growth by using diverse AEAs,
such as TMAO, fumarate, and DMSO (15). To examine the
relative anaerobic growth achieved using each AEA, N16961
was grown in LB supplemented with TMAO, fumarate, or
DMSO at three different concentrations. As shown in Fig. 1A,
bacterial growth was enhanced when grown with TMAO or
fumarate. When TMAO was added at 50 mM concentration,
the final 600 values were not observed when DMSO was used.
Such a dramatic induction was 2-fold higher than that of control
growth in plain LB media (black bar to the left). N16961
was also grown in LB supplemented with TMAO, fumarate,
or DMSO at three different concentrations. As shown in Fig. 1A,
bacterial growth was enhanced when grown with TMAO or
fumarate. When TMAO was added at 50 mM concentration,
the final 600 values were not observed when DMSO was used.

The level of CT secreted to culture supernatants during each
anaerobic culture was then measured. Surprisingly, CT produc-
tion was strikingly induced during TMAO-stimulated anaero-
bic respiratory growth (Fig. 1B). Such a dramatic induction was
not detected in other types of anaerobic growth. Notably, CT
was not produced when N16961 was grown aerobically with

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equal amounts of TMAO (Fig. 1B), demonstrating that TMAO-induced CT production occurred only under anaerobic growth conditions. To confirm the CT ELISA results, Western blot analysis was also performed using an antibody against CT subunit B. As shown in Fig. 1C, the band specific to CT subunit B was only detected in the cell-free culture supernatant of N16961 grown by anaerobic TMAO respiration. These results suggest that among various AEAs, TMAO can most efficiently stimulate anaerobic growth of the V. cholerae strain N16961. In addition, CT production is specifically and substantially induced during TMAO respiration.

The study also investigated whether TMAO-stimulated CT production was reflected in the transcriptional activation of CT-coding genes. To address this, the promoter activity of ctxAB genes was monitored by constructing a chromosomal lacZ reporter fusion to this promoter. Consistent with the CT ELISA results, a significant level of β-galactosidase activity was detected only in N16961 grown anaerobically in LB containing 50 mM TMAO (Fig. 1D). The mRNA expression levels of other virulence-associated genes were then measured by qRT-PCR analysis. Transcript levels of five selected genes, ctxA, ctxB, toxT, toxR, and tcpP, invariably increased in N16961 grown in LBT compared with LB (Fig. 1E). Expression of ctxA and tcpP was up-regulated to the highest level at greater than ~50-fold, whereas the mRNA levels of ctxB, toxT, and toxR increased ~13-, ~17-, and ~12-fold, respectively (Fig. 1E).

The 7th pandemic V. cholerae strain N16961 is classified as O1 serogroup and El Tor biotype (23). We therefore asked whether the mechanism of TMAO-stimulated CT production is conserved among other types of V. cholerae strains. Two different Classical biotype strains (O395 and 569B) and another O1 El Tor biotype strain (C6706) produced comparable CT levels under the same anaerobic growth conditions (Fig. 1F). Interestingly, both the O139 serogroup strain MO10 and the non-O1/non-O139 strain AM19226 failed to produce CT. As expected, CT was not produced in the CVD115 strain derived from the N16961 strain grown by anaerobic TMAO respiration in the CVD115 strain derived from CVD110 that has deletions in CT-coding genes (24) or in the N16961 strain grown in plain LB (leftmost lane). Although only a limited number of V. cholerae strains were tested, these results may suggest that the mechanism for producing CT during TMAO respiration is conserved in V. cholerae O1 serogroup strains.

CT Production Was Not Observed in N16961 Tn Insertion Mutants with Defects in Their Ability to Respire TMAO.—To examine whether a mechanistic link exists between enhanced anaerobic growth by TMAO respiration and CT production, an N16961 Tn random insertion mutant library was constructed and screened for mutants that failed to form colonies with a larger size in LB-agar plates supplemented with 50 mM TMAO. In four mutants that were recovered, it was clearly shown that the presence of TMAO did not increase their anaerobic growth (Fig. 2A). In all of these mutants, the final A600 values after 16 h of anaerobic culture in LBT were similar to those obtained from LB growth. Arbitrary PCR amplification followed by DNA sequencing analysis demonstrated that Tn was inserted in the protein coding regions of VC1720, VC0116, VC2053, or VC1024, respectively (Fig. 2B). VC1720, termed torD, encodes a chaperone protein for TorA (VC1692), a major subunit of the TMAO reductase complex (25). VC0116 (HemN) is an oxygen-independent coproporphyrinogen III oxidase involved in heme biosynthesis (26, 27). VC2053 encodes a heme chaperone for...
biosynthesis of the c-type cytochrome that is required for active TMAO reductase (15). TMAO reductase is also featured with the presence of a molybdenum cofactor (28). A mutant of VC1024 (moaA) that is defective in the machinery required to synthesize the molybdenum cofactor was included among the mutants incapable of utilizing TMAO under anaerobic conditions. Consistent with this finding, the synthesis of molybdenum cofactor biosynthesis protein B, the leftmost image represents protein spots detected by gel image analysis.

CT levels induced in all of these mutant strains were negligible during anaerobic growth with TMAO, whereas robust CT production was observed in the wild type strain N16961. Together, our results demonstrate that (i) the ability to produce heme group, cytochrome c, and molybdenum cofactor is necessary to produce functionally intact TMAO reductase and (ii) that CT production during anaerobiciosis occurs in strict association with TMAO respiration.

FIGURE 2. Identification of N16961 mutants defective in anaerobic growth by TMAO respiration. A, anaerobic growth of wild type N16961 and four different mutants recovered from screening Tn mutant library. Strains were grown anaerobically in LB (black bars) or LBT (gray bars) for 16 h, and values of $A_{490}$ (mean ± S.D., n = 3) are displayed. *, p < 0.001 versus A600 values of other cultures. B, information of genes disrupted in each mutant and function of the proteins encoded from each gene. C, up-regulated synthesis of VC1025 protein in N16961 grown in LBT. Bacterial proteins were extracted from N16961 grown anaerobically in LB, LBT, LBD (LB + 50 mM DMSO), or LBF (LB + 50 mM fumarate) and were separated in two-dimensional gels. The same area of each gel containing a spot for VC1025 was compared. The VC1025 protein was identified by Q-TOF. The leftmost image represents protein spots detected by gel image analysis.
It is well established that CT is secreted into the environmental milieu from the periplasmic space via a type II secretion system (T2SS) in *V. cholerae* (33, 34). For facilitated transport across the inner membrane into the periplasm, CT is targeted to either the general secretion pathway (SEC) (35, 36) or the TAT system (22). For this reason, we also examined the effects of SEC or T2SS deficiency on anaerobic TMAO respiration and CT secretion. The ability to support anaerobic growth with TMAO, CT production was antagonized by the presence of N-acetylcysteine, a ROS scavenger—During anaerobic growth, the CT level detected in cell-free culture supernatants of the *torC* mutant strains grown in LB (black bars) or LBT (gray bars). Experimental conditions were identical to those described in the legend to Fig. 1A. Three independent experiments were performed and values of mean ± S.D. are displayed in each bar. *p < 0.05 versus wild type; **p < 0.01 versus CT levels detected in cell-free supernatants harvested from LB grown cells.

Next, we tested the effect of *N*-acetylcysteine (NAC), a ROS scavenger, on CT production and *ctxAB* promoter activity. In the presence of increasing amounts of NAC, the CT level induced during anaerobic growth by TMAO respiration was gradually decreased (Fig. 6A). Likewise, a clear dose-dependent decrease was also observed in the *P_{ctxAB}* activity (Fig. 6B). These results suggest that ROS availability can regulate the degree of *ctxAB* gene expression and CT production. We then investigated whether the CT production level is elevated by exogenous addition of *H₂O₂*. When N16961 was grown anaerobically in LB media that contained increasing concentrations of TMAO, CT production was gradually increased (Fig. 6C, black bars). The level of CT produced during TMAO respiration significantly increased in the presence of 100 μM *H₂O₂* (Fig. 6C, gray bars). During anaerobic growth with 20 or 30 mM TMAO, CT production increased by ~1.8- or ~1.9-fold, respectively, with the addition of 100 μM *H₂O₂*. When grown with 40 or 50 mM TMAO, CT production increased ~1.5-fold in the presence of *H₂O₂* compared with growth in its absence. Notably, CT production was not induced by *H₂O₂* when bacterial cells were grown in plain LB (Fig. 6C, leftmost set of bars). In addition, *H₂O₂*-mediated stimulation of CT production was not observed when fumarate or DMSO were used to support anaerobic growth of N16961 (Fig. 6, D and E). These results suggest that *H₂O₂*, an exogenously added ROS, can promote CT production only when *V. cholerae* cells grow by anaerobic TMAO respiration.

When Infected with TMAO, the Cytotoxicity Exerted by *V. cholerae* Strains Was Elevated in Infant Mouse Intestine—Finally, we sought to examine the effect of TMAO on *in vivo* virulence using an infant mouse model of *V. cholerae* infection. We first measured the fluid accumulation induced by orogastric challenge of *V. cholerae* N16961 cells (*2×10⁵* cells). Higher levels of the intestinal fluid accumulation ratio were observed in mice infected with bacterial cells re-suspended in LB + 100 mM TMAO (fluid accumulation ratio > ~0.12) than in mice infected with bacterial suspensions that contain no TMAO (fluid accumulation ratio > ~0.08) (Fig. 7A). Importantly, mice infected with extraneously added TMAO exhibited higher susceptibility in response to intestinal infection. Although all of the...
FIGURE 5. ROS is spontaneously generated during anaerobic TMAO respiration. Confocal microscope images of N16961 grown anaerobically in LB (A), LBT (B), LBF (C), and LBD (D). LBF and LBD indicate LB + 50 mM fumarate and LB + 50 mM DMSO, respectively. Left images in each panel represent merged DIC and green fluorescent images, whereas right images represent only green fluorescent images. Bacterial cells grown in each media for 8 h were stained with 10 μM DCF-DA for 30 min and processed for confocal microscopic analysis. Images were acquired at ×1,000 magnification.

FIGURE 6. Extraneously added H₂O₂ promotes CT production during anaerobic TMAO respiration. A, effect of the presence of NAC on CT production. N16961 cells were grown anaerobically in LBT for 16 h with the indicated amounts of NAC. Three independent experiments were performed and values of mean ± S.D. are displayed in each bar. *, p < 0.01; **, p < 0.001 versus CT levels detected in cell-free supernatants harvested from cultures with 0 mM NAC. B, the effect of the presence of NAC on ctxAB promoter activity. An N16961 reporter strain harboring a chromosomal copy of ctxAB promoter-lacZ fusion was grown anaerobically in LBT in the presence of increasing concentrations of NAC. Bacterial culture conditions were identical to those described in the legend to Fig. 6A and β-galactosidase activity was measured as described in the legend to Fig. 1D. Three independent experiments were performed and values of mean ± S.D. are displayed in each bar. **, p < 0.001 versus β-galactosidase activity detected in cells grown with 0 mM NAC. C–E, effect of extraneously added H₂O₂ on CT production. LB media with varying concentrations of TMAO (C), fumarate (D), or DMSO (E) were supplemented with no (black bars) or 100 μM H₂O₂ (gray bars). N16961 was grown anaerobically in each media for 16 h and culture supernatants were collected for CT ELISA. Three independent experiments were performed and values of mean ± S.D. are displayed in each bar. *, p < 0.01 versus CT levels produced without added H₂O₂.
mice infected with bacterial suspensions in plain LB survived for 24 h, only one mouse survived at 24 h post-infection when TMAO was added to the inoculum (Fig. 7B). These results suggest that V. cholerae strains exert more severe virulence to infant mice in the presence of added TMAO.

Our results in Fig. 2A demonstrate that wild type N16961, but not the ΔtorD mutant, exhibited enhanced in vitro growth by TMAO respiration. Therefore, we investigated whether in vivo colonization of the ΔtorD mutant is compromised, compared with the wild type strain. To address this issue, we calculated the competitive index between these two strains. Infant mice (n = 7) were co-infected with equal numbers of N16961 and ΔtorD mutant cells re-suspended in LB or LBT (1 × 10⁶ cells each). When TMAO was not added to the inoculum, the level of intestinal colonization by N16961 was slightly less than that of the ΔtorD mutant, yielding a competitive index of ~0.77 (Fig. 7C, closed circles). In contrast, when the mixture of bacterial cells was inoculated with 100 mM TMAO, the number of N16961 cells was higher than that of the mutant cells (Fig. 7C, closed squares). The competitive index in this set of experiments was ~1.78 and the difference between the two competitive indexes was statistically significant (p < 0.005). Together, these results suggest that the ability to metabolize TMAO may be important not only for in vivo virulence but also for intestinal colonization.

DISCUSSION

As a historic enteric pathogen, V. cholerae has been extensively investigated for the regulation of virulence factors. For successful colonization and CT production in host intestinal microenvironments, the organism must alter its phenotypic and metabolic properties from those of its natural aquatic habitat. Because the human intestinal environment is largely anaerobic (7, 41), there is a need for the pathogen to utilize chemicals other than oxygen as AEAs for anaerobic growth. V. cholerae is reported to be capable of metabolizing organic amines including TMAO and fumarate under anaerobic growth conditions (15). However, there is a lack of information regarding the effect of anaerobic respiration on bacterial growth or regulated production of virulence factors for an in vitro culture system that resembles the human intestinal microenvironment.

CT is the major virulence factor that critically influences V. cholerae pathogenesis. Although genetic regulatory systems leading to activation of ctxAB gene expression are relatively well established (4, 42, 43), environmental signals that induce CT expression are not clearly defined. The AKI condition, which has been considered as an efficient in vitro culture method for CT production, involves a biphasic growth of the 4-h static culture followed by vigorous shaking for 16 h (44, 45). In addition, the volume-to-surface ratio of laboratory flask cultures was reported to play a role in regulating CT production (46). Because these culture methods were all developed by trial and error, why they trigger CT production is still unknown. In this study, we identified a previously undescribed culture condition that induces CT production and investigated the molecular basis of such induction and its relevance for in vivo infection.

Our conclusion that CT production was specifically induced when V. cholerae grew by anaerobic TMAO respiration stems from the following evidence: (i) CT production was not induced by other AEAs and (ii) ΔtorD and Δtat mutant strains of N16961 failed to produce CT during TMAO respiration. Because the anaerobic growth of V. cholerae was higher with TMAO than with fumarate or DMSO, we postulated that TMAO respiration can provide both a growth advantage to V. cholerae under anaerobic conditions and a significantly elevated potential for virulence. Consistent with this notion, the infant mouse infection experiments clearly demonstrated effects of extraneously added TMAO on in vivo virulence and competitive intestinal colonization.

In humans, TMAO is produced via an enzyme called flavin-containing monooxygenase, which catalyzes TMAO oxidation. TMAO is derived from ingested food sources, such as phosphatidylcholine and L-carnitine (47). In a recent study by Wang et al., TMAO was not detected in mice when gut microbiota were suppressed by treatment with antibiotics for 3 weeks (54). The TMAO level was restored after the mouse gut was re-colonized by gut microbiota, indicating the key role of gut commensal bacteria for TMAO production (54). As our results showed that CT production could be induced in N16961 with as low as 10 mM TMAO, it seems necessary to determine how much TMAO is present in the human intestine and whether such a level is enough to support both anaerobic growth and CT production of V. cholerae in vivo. Furthermore, it would also be of particular interest to examine the correlation between the altered gut microbiota population profiles and differential susceptibility to V. cholerae infection. Because TMAO is mainly found in marine environments (48, 49), individuals with a marine diet may have elevated levels of TMAO in their intestine and be at a higher risk for pathogenic V. cholerae infection.

Proteins secreted by the Type II secretion system are translocated to the periplasm via the general secretion (SEC) system (50, 51) or TAT system (52). However, our results showed that
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CT secretion was not significantly compromised in the Δsec mutant, demonstrating that CT secretion likely occurs independently of the SEC pathway during anaerobic growth by TMAO respiration. This finding was rather unexpected because the SEC pathway was reported to be critical for CT secretion during aerobic growth (35, 36). In a recent study by Zhang and colleagues (22), CT production in a V. cholerae mutant deficient in the TAT pathway was only mildly affected during aerobic growth under AKI conditions. This result is also in marked contrast to our finding that CT production was completely abrogated in the Δtat mutant during anaerobic TMAO respiration. Together, these results suggest that CT secretion during anaerobic TMAO respiration may occur following different mechanisms than those operational during aerobic growth.

Our results in Fig. 5 clearly demonstrate that N16961 cells grown anaerobically with TMAO exhibited a strong fluorescent signal when stained with DCF-DA, a ROS detector. Because the fluorescent signal was only detected in cells grown by TMAO respiration, the growth mode that resulted in CT production, we postulated that ROS generated during TMAO respiration would trigger a signal leading to CT production. In support of this hypothesis is the finding that both CT production and ctxAB gene transcription decreased in the presence of NAC, a compound that can reduce the availability of cellular ROS. TMAO reductase reduces TMA to TMA. Because TMA is the only product of TMAO reductase, it was also postulated that TMA might be the signal to induce CT production. However, neither growth enhancement nor CT production was observed when N16961 was grown anaerobically with 50 mM TMAO (data not shown). This result further confirms that a signal leading to the CT production (i.e. ROS) is generated during TMAO respiration. The level of CT produced during TMAO respiration was substantially elevated when the culture medium was “spiked” with H2O2. However, the positive effect of H2O2 on CT production was not observed in N16961 grown in plain LB. These results strongly suggest that (i) H2O2 alone may not directly induce CT production in V. cholerae under anaerobic conditions and (ii) extraneously added H2O2 can play a role in expanding the capability of V. cholerae to produce CT during TMAO respiration. One possible interpretation would be that anaerobic TMAO respiration contributes to create conditions for CT production and such conditions are further amplified by the addition of extraneous H2O2. Interestingly, at up to 0.1% concentration (≈28 mM), H2O2 was found to increase the TcpA level in another El Tor strain, A1552, during aerobic growth in LB (53). In contrast, our results showed that only 100 μM H2O2 was enough to promote maximum CT production. It will be necessary to address (i) how much ROS is generated during TMAO respiration, (ii) how potent is it to transduce virulence inducing signals, and (iii) what is the mode of signal transduction. Because CT subunits A and B do not contain the twin arginine motif, a precise mechanism by which CT secretion occurs in association with the activated TAT pathway also needs to be elucidated.

In conclusion, we explored anaerobiosis-induced changes in growth and virulence properties of V. cholerae. Most importantly, we proposed a mechanistic basis for a strict dependence of CT production on the anaerobic TMAO respiration (summarized in Fig. 8). To identify effective strategies to cope with V. cholerae infection, a molecular level understanding of its virulence modulation is necessary. Our results revealed a previously unidentified mechanism for CT production under conditions that likely mimic the environments of the human intestine and therefore, we anticipate that results provided in this study will stimulate further investigations to reduce the severity of intestinal infections caused by this clinically important human pathogen.

REFERENCES
1. Faruque, S. M., Albert, M. J., and Mekalanos, J. J. (1998) Epidemiology, genetics, and ecology of toxigenic Vibrio cholerae. Microbiol. Mol. Biol. Rev. 62, 1301–1314
2. Piéroux, R., Barrais, R., Faucher, B., Haus, R., Piéroux, M., Gaudart, J., Magloire, R., and Raoult, D. (2011) Understanding the cholera epidemic, Haiti. Emerg. Infect. Dis. 17, 1161–1168
3. Yoon, S. S., Cookey, R., Lau, G. W., Lymar, S. V., Gaston, B., Karabulut, A. C., Hennigan, R. F., Hwang, S. H., Buettner, G., Schurr, M. J., Mortensen, J. E., Burns, J. L., Speert, D., Boucher, R. C., and Hassett, D. J. (2006) Anaerobic killing of mucoid Pseudomonas aeruginosa by acidified nitrite derivatives under cystic fibrosis airway conditions. J. Clin. Invest. 116, 436–446
4. Matson, J. S., Withey, I. H., and DiRita, V. J. (2007) Regulatory networks controlling Vibrio cholerae virulence gene expression. Infect. Immun. 75, 5542–5549
5. Spangler, B. D. (1992) Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. Microbiol. Rev. 56, 622–647
6. Iredell, J. R., and Manning, P. A. (1994) The toxin-co-regulated pilus of Vibrio cholerae O1. A model for type 4 pilus biogenesis? Trends Microbiol. 2, 187–192
7. Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I.
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8. Marrero, K., Sánchez, A., Rodríguez-Ulloa, A., González, L. J., Castellanos-Serra, L., Paz-Lago, D., Campos, J., Rodríguez, B. L., Suzarte, E., Ledón, T., Padrón, G., and Fando, R. (2009) Anaerobic growth promotes synthesis of colonization factors encoded at the Vibrio pathogenicity island in Vibrio cholerae El Tor. Res. Microbiol. 160, 48–56

9. Häsé, C. C., and Mekalanos, J. J. (1998) TcpP protein is a positive regulator of virulence gene expression in Vibrio cholerae. Proc. Natl. Acad. Sci. U.S.A. 95, 730–734

10. Liu, Z., Yang, M., Peterfreund, G. L., Tsou, A. M., Selamoglu, N., Daldal, F., Zhong, Z., Kan, B., and Zhu, J. (2011) Vibrio cholerae anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. Proc. Natl. Acad. Sci. U.S.A. 108, 810–815

11. Kovacičková, G., Lin, W., and Skorupski, K. (2010) The LysR-type virulence factor VasX to kill temperature, pH, and osmolarity. Mol. Microbiol. 79, 2941–2949

12. Zhang, L., Zhu, Z., Jing, H., Zhang, J., Xiong, Y., Yan, M., Gao, S., Wu, L. F., Xu, J., and Kan, B. (2009) Pleiotropic effects of the twin arginine translocation pathway of molybdoenzyme TorA. J. Biol. Chem. 284, 1915–1920

13. Johnson, T. L., Abendroth, J., Hol, W. G., and Sandkvist, M. (2006) Type II secretion. From structure to function. FEMS Microbiol. Lett. 255, 175–186

14. McCrindle, S. L., Kappler, U., and McEwan, A. G. (2005) Microbial dimethylsulfoxide and trimethylamine N-oxide respiration. Adv. Microb. Physiol. 50, 147–198

15. Ilbert, M., Méjean, V., Giudici-Orticoni, M. T., Samama, J. P., and Iobbi-Nivol, C. (2000) Involvement of the tor operon. Mol. Microbiol. 37, 1013–1021

16. Taylor, C. T., and Colgan, S. P. (2007) Hypoxia and gastrointestinal disease. J. Med. Microbiol. 56, 1295–1300

17. Jørgensen, E. B., Fedorkow, K., Møller, N. J., and Sandkvist, M. (2006) Expression of cholera toxin under non-AKI conditions in Vibrio cholerae El Tor. Mol. Microbiol. 60, 373–395

18. DiRita, V. J. (1992) Coordinate expression of virulence genes by ToxR in Vibrio cholerae. J. Bacteriol. 174, 4066–4072

19. Caplan, A. I., and Kaper, J. B. (1993) CVD110, an aerobic respiratory growth and CT production in V. cholerae. Plasmid 30, 85–96

20. El Tor. J. Bacteriol. 175, 175–186

21. Sandkvist, M., Michel, L. O., Hough, L. P., Morales, V. M., Bagdasarian, M., Koomey, M., and DiRita, V. J. (1997) General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in Vibrio cholerae. J. Bacteriol. 179, 6994–7003

22. Chandel, N. S., Trzyna, W. C., McClintock, D. S., and Schumacker, P. T. (2000) Role of oxidants in NF-κB activation and TNF-α gene transcription induced by hypoxia and endotoxin. J. Immunol. 165, 1013–1021

23. Taylor, C. T., and Colgan, S. P. (2007) Hypoxia and gastrointestinal disease. J. Med. Microbiol. 56, 1295–1300

24. Hall, R. H., and Schumacker, P. T. (1992) Coordinate expression of virulence genes by ToxR in Vibrio cholerae. Mol. Microbiol. 6, 451–458

25. Klose, K. E. (2001) Regulation of virulence in Vibrio cholerae. Int. Med. Microbiol. 291, 81–88

26. Iwanaga, M., Yamamoto, K., Higa, N., Ichinose, Y., Nakasone, N., and Tanabe, M. (1986) Culture conditions for stimulating cholera toxin production by Vibrio cholerae O1 El Tor. Microbiol. Immunol. 30, 1075–1083

27. Iwanaga, M., and Yamamoto, K. (1985) New medium for the production of molybdenum enzymes. Selective catalysis by FMO3. Biochem. Pharmacol. 34, 345–350

28. Iwanaga, M., Yamamoto, K., Higa, N., Ichinose, Y., Nakasone, N., and Tanabe, M. (1986) Culture conditions for stimulating cholera toxin production by Vibrio cholerae O1 El Tor. Microbiol. Immunol. 30, 1075–1083

29. Iwanaga, M., Yamamoto, K., Higa, N., Ichinose, Y., Nakasone, N., and Tanabe, M. (1986) Culture conditions for stimulating cholera toxin production by Vibrio cholerae O1 El Tor. Microbiol. Immunol. 30, 1075–1083

30. Petrov, R. M., and Rhee, S. G. (1988) Isoform specificity of trimethylamine N-oxidase by human flavin-containing monooxygenase (FMO) and P450 enzymes. Selective catalysis by FMO3. Biochem. Pharmacol. 56, 1005–1012

31. Martinez, I., Bathen, T., Standal, I. B., Halvorsen, J., Aursand, M., Gribbestad, I. S., and Axelson, D. E. (2005) Bioactive compounds in cod (Gadus morhua) products and suitability of 1H NMR metabolite profiling for clas-
sification of the products using multivariate data analyses. J. Agric. Food Chem. 53, 6889–6895
49. Bordi, C., Ansaldi, M., Gon, S., Jourlin-Castelli, C., Iobbi-Nivol, C., and Méjean, V. (2004) Genes regulated by TorR, the trimethylamine oxide response regulator of Shewanella oneidensis. J. Bacteriol. 186, 4502–4509
50. Pugsley, A. P. (1993) The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. 57, 50–108
51. Desvaux, M., Parham, N. J., Scott-Tucker, A., and Henderson, I. R. (2004) The general secretory pathway. A a general misnomer? Trends Microbiol. 12, 306–309
52. Voulhoux, R., Ball, G., Ize, B., Vasil, M. L., Lazdunski, A., Wu, L. F., and Filloux, A. (2001) Involvement of the twin arginine translocation system in protein secretion via the type II pathway. EMBO J. 20, 6735–6741
53. Valeru, S. P., Rompikuntal, P. K., Ishikawa, T., Vaitkevicius, K., Sjöling, A., Dolganov, N., Zhu, J., Schoolnik, G., and Wai, S. N. (2009) Role of melanin pigment in expression of Vibrio cholerae virulence factors. Infect. Immun. 77, 935–942
54. Wang, Z., Klipfell, E., Bennett, B. J., Koeth, R., Levison, B. S., Dugar, B., Feldstein, A. E., Britt, E. B., Fu, X., Chung, Y. M., Wu, Y., Schauer, P., Smith, J. D., Allayee, H., Tang, W. H., DiDonato, J. A., Lusis, A. J., and Hazen, S. L. (2011) Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472, 57–63