Cholera Toxin Production in *Vibrio cholerae* O1 El Tor Biotype Strains in Single-Phase Culture

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Vibrio cholerae O1 serogroup strains have been classified into classical and El Tor biotypes. Cholera, a life-threatening diarrheal disease, can be caused by either biotype through the cholera toxin (CT) that they produce. To increase our knowledge of the pathogenicity of bacteria, we must understand the toxigenicity of bacteria. CT production by classical biotype strains in simple single-phase cell cultures has been established; however, special culture media and growth conditions that are not appropriate for mass production of CT are required to facilitate CT production in El Tor biotype strains. In this report, we produced CT in El Tor biotype strains using simple media and single-phase culture conditions. A single point mutation in ToxT, a transcriptional activator of toxin co-regulated pilus (TCP) and CT, enabled the El Tor biotype strains to produce CT in similar quantities as classical biotype strains in single-phase laboratory culture conditions. CT production capacity varied between El Tor biotype strains. Wave 2 and 3 atypical El Tor strains tended to produce more CT than prototype Wave 1 strains. Wave 2 and 3 strains lack neutral fermentation; however, the capacity for neutral fermentation was not associated with significant differences in CT production by El Tor biotype strains. The Wave 3 strain that caused the 2010 cholera outbreak in Haiti produced CT only when neutral fermentation was abolished. The disparity in CT production between the seventh cholera pandemic strains highlight the differences in virulence between strains and the cause of population changes in *V. cholerae*.

**INTRODUCTION**

Serogroup O1 and O139 *Vibrio cholerae* strains cause a fatal diarrheal disease, cholera (Kaper et al., 1995). The symptoms of cholera are induced primarily by the cholera toxin (CT) that is produced by *V. cholerae* bacteria. The CT gene ctxAB is carried by a filamentous phage, CTXΦ, which can be integrated into both chromosomes in *V. cholerae* (Waldor and Mekalanos, 1996; Kim et al., 2017). Two biotypes of *V. cholerae* O1 serogroup strains—classical and El Tor—have been recognized as the causative agents of the first to sixth and the seventh cholera pandemics, respectively (Safa et al., 2010; Kim et al., 2015).
Classical biotype strains produce classical biotype-specific CT, whereas El Tor biotype strains synthesize El Tor biotype-specific CT (Raychoudhuri et al., 2009). The 2 toxins differ by 2 amino acids at positions 39 and 68 in the binding subunit (CTB) of the toxin, but the amino acid sequences are identical in the active subunit (CTA). Recently, atypical El Tor strains that produce classical biotype CT have replaced prototype El Tor strains globally (Kim et al., 2015).

Cholera is caused by the CT that is produced by and secreted from the bacteria in the intestinal environment of human hosts; however, inducing CT production in vitro is challenging. Laboratory conditions for stimulating CT production in classical biotype strains have been established, whereas efforts to establish CT production in El Tor biotype strains in vitro have demonstrated limited success (Ghosh-Banerjee et al., 2010). Culture in media that contain bicarbonate stimulates CT production in El Tor biotype strains (Iwanaga and Yamamoto, 1985; Abuaita and Withey, 2009). An unusual bi-phasic growth condition—described as AKI conditions—has been created for El Tor biotype strains, in which the bacterial culture is kept under static conditions without shaking for 4 h, followed by vigorous shaking for 16 h in AKI media (Iwanaga et al., 1986). However, monitoring CT production and especially, mass production of CT by El Tor biotype strains remains difficult when using these methods.

Recently, we constructed El Tor biotype strains that can be transduced by CTXΦ by inducing the expression of CTX phage receptor, toxin co-regulated pilus (TCP) (Kim et al., 2017). These El Tor strains were constructed by introducing a point mutation in the ToxT transcription factor, which regulates the expression of TCP and CT (Childers et al., 2011). In V. cholerae strains, ToxT typically contains a tyrosine at position 139 (139Y) and does not induce CT and TCP under conventional laboratory conditions; however, CT and TCP expression in El Tor biotype strains has been affected under laboratory conditions when the Tyr-139 is replaced by phenylalanine (139F) (Kim et al., 2017). CT production in El Tor strains that contain the toxT-139F allele was briefly demonstrated under agglutinating conditions (culture at 30°C in media that was adjusted to pH 6.5 with 50 mM Tris buffer), which had been established for classical biotype strains (Davis et al., 1999). Although CT production by classical biotype strains has been optimized under agglutinating conditions, we expected El Tor biotype strains to have different optimal conditions for CT production.

In this study, we determined the optimal conditions for CT production by El Tor biotype strains that contain the toxT-139F allele by varying the pH of the culture medium and growth temperature. CT production in El Tor strains varied, depending on the strain: CT production in prototype (Wave 1) El Tor biotype strains was induced by the toxT-139F allele but was less than 50% of that in classical biotype strains; atypical El Tor strains (Wave 2 and 3) synthesized more CT than Wave 1 strains, with several Wave 2 strains producing 200% more toxin than classical biotype strains under agglutinating conditions. Atypical El Tor strains produced CT at 30°C and 37°C, whereas 30°C was more favorable for toxin production in classical and Wave 1 El Tor biotype strains.

We also constructed a derivative of the classical biotype strain that contained the toxT-139F allele to confirm that this allele also enhances toxin production in classical biotype strains. When compared with CT production by classical biotype strains under agglutinating condition, the engineered classical biotype strain generated 400% more CT in regular LB media and under agglutinating conditions.

Although the CT production condition that we have described in this report might not reflect the physiological conditions in the human host directly, our results provide insights into the pathogenicity of V. cholerae.

**MATERIALS AND METHODS**

**Bacterial Strains and Bacterial Culture**

The bacterial strains used in this study are listed in Table 1.

**toxT Allele Exchange**

Two 843 bp DNA fragments, encompassing the 50 nucleotides upstream of the translation start codon to nucleotide 793 of toxT, were amplified by PCR using primers the toxT-XbaIF: CCG GCC TCT AGA TAG GAG CTC TCT CG and toxT-SacIR: CCG GCC GAG CTC CAC TGG TGA CAT CAT TCA from strains MG116025 and N16961, respectively. The fragments were inserted into a suicide plasmid, pCVD442. The SNP at nucleotide 416 (A416 in N16961 and T416 in MG116025) lies in the center of these fragments. The toxT-139F allele of MG116025 was replaced by the toxT-139Y allele of N16961 by allelic exchange, and similarly, the toxT-139Y alleles of other strains were replaced with the toxT-139F allele of MG116025 (Donnenberg and Kaper, 1991).

**VC1589 Allele Exchange**

Using the VC1589-XbaIF (CGG TCT AGA CGG CGG GCG TCA ACT CAA CG) and VC1589-SacIR (CGG GAG CTC TGG GGC GAT AAG TTG TGT GTC) primer set, a 1,261-bp fragment that encompassed the functional VC1589 allele from the N16961 Wave 1 El Tor biotype strain and a 1,260-bp fragment that contained the nonfunctional VC1589 allele from the B33 Wave 2 atypical El Tor strain were amplified by PCR and subcloned into the suicide plasmid pCVD442 to generate pCVD442-VC1589-T7 and pCVD442-VC1589-T6, respectively. pCVD442-VC1589-T7 was conjugally transferred to Wave 2 and 3 atypical El Tor strains to replace their nonfunctional VC1589 with a functional VC1589 allele. Similarly, functional VC1589 in Wave 1 El Tor biotype strains was replaced by allelic exchange using pCVD442-VC1589-T6. The replacement of the VC1589 allele in each strain was confirmed by sequencing.

**Measurement of Cholera Toxin Production**

The GM1 enzyme-linked immunosorbent assay was used to determine the CT production in culture supernatant (Lee et al., 2012). Purified whole CT (Cayman Chemical, Ann Arbor, MI, United States) was used to provide a standard curve.
TABLE 1 | V. cholerae strains used in this study.

| Strain           | toxT genotype | VC1589 genotype | ctxB type | Reference and genome sequence information |
|------------------|---------------|-----------------|-----------|-------------------------------------------|
| Classical biotype strain |               |                 |           |                                           |
| O1395            | 139Y          |                 | T7         | Classical (ctxB1) CP000026/CP000027        |
| YJB001           | 139F          |                 | T7         | Classical (ctxB1) (Mutreja et al., 2011)   |
| Wave 1 El Tor biotype strains |               |                 |           |                                           |
| N16961           | 139Y          |                 | T7         | El Tor (ctxB3) AE003862/AE003863           |
| YJB002           | 139Y          |                 | T6         | El Tor (ctxB3) (Heidelberger et al., 2000) |
| YJB003           | 139F          |                 | T7         | El Tor (ctxB3)                             |
| YJB004           | 139F          |                 | T6         | El Tor (ctxB3)                             |
| T19479           | 139Y          |                 | T7         | El Tor (ctxB3) ERS013250                   |
| YJB005           | 139Y          |                 | T6         | El Tor (ctxB3) (Mutreja et al., 2011)     |
| YJB006           | 139F          |                 | T7         | El Tor (ctxB3)                             |
| YJB007           | 139F          |                 | T6         | El Tor (ctxB3)                             |
| Wave 2 El Tor biotype strains |               |                 |           |                                           |
| B33              | 139Y          |                 | T6         | Classical (ctxB1) AEOH00000000             |
| YJB008           | 139Y          |                 | T7         | Classical (ctxB1) (Faruque et al., 2007)  |
| YJB009           | 139F          |                 | T6         | Classical (ctxB1)                         |
| YJB010           | 139F          |                 | T7         | Classical (ctxB1)                         |
| MJ1236           | 139Y          |                 | T6         | Classical (ctxB1) CP001485/CP001486        |
| YJB011           | 139Y          |                 | T7         | Classical (ctxB1) (Grim et al., 2010)     |
| YJB012           | 139F          |                 | T6         | Classical (ctxB1)                         |
| YJB013           | 139F          |                 | T7         | Classical (ctxB1)                         |
| MG116025         | 139F          |                 | T6         | El Tor (ctxB3) ERS013138                   |
| YJB014           | 139F          |                 | T7         | El Tor (ctxB3) (Mutreja et al., 2011)     |
| YJB015           | 139Y          |                 | T6         | El Tor (ctxB3)                             |
| Wave 3 El Tor biotype strains |               |                 |           |                                           |
| IIB4122          | 139Y          |                 | T6         | Classical (ctxB1) ERS013264                |
| YJB016           | 139Y          |                 | T7         | Classical (ctxB1) (Nguyen et al., 2009)   |
| YJB017           | 139F          |                 | T6         | Classical (ctxB1)                         |
| YJB018           | 139F          |                 | T7         | Classical (ctxB1)                         |
| IBS230           | 139Y          |                 | T6         | Haitian (ctxB7) AELH00000000.1             |
| YJB019           | 139Y          |                 | T7         | Haitian (ctxB7) (Chin et al., 2011)       |
| YJB020           | 139F          |                 | T6         | Haitian (ctxB7)                            |
| YJB021           | 139F          |                 | T7         | Haitian (ctxB7)                            |

*139Y: amino acid position 139 in toxT is Tyr, 139F: amino acid position 139 in toxT is Phe. TT6: VC1589 contains 7 thymines from nucleotides 306 to 315 and encodes functional -acetolactate decarboxylase. T6: VC1589 contains 6 thymines at this position and encodes a truncated product.

RESULTS

Cholera Toxin Production in Wave 1 El Tor Biotype Strains

Cholera toxin production by various El Tor biotype strains was compared with that of O1395 under agglutinating conditions (reference CT production). V. cholerae strains usually contain the toxT-139Y allele (1 strain, MG116025, reportedly contains the toxT-139F allele), and no CT is produced in El Tor strains under single-phase laboratory shake culture conditions (Kim et al., 2017). We confirmed that regardless of the culture medium, no measurable CT was produced in El Tor strains that carry the toxT-139Y allele, except for 1 Wave 3 strain, described below. On replacing the toxT-139Y allele with toxT-139F in a Wave 1 El Tor biotype strains—T19479-toxT-139F, cultured under agglutinating conditions—CT production was detected (Figure 1), albeit at levels that were less than 40% versus the classical biotype strain, and no CT was produced at 37°C. The function of the neutral fermentation gene (VC1589) did not influence CT production in this strain (Figure 1).

The other Wave 1 El Tor biotype strain, N16961, which produces CT in amounts that are comparable with classical biotype strains under AKI conditions, did not generate CT in any medium under single-phase culture conditions, despite the toxT-139Y allele being replaced by toxT-139F (Supplementary Figure S2). Based on these results, we determined that Wave 1 El Tor biotype strains can be manipulated to produce CT in single-phase culture.

Cholera Toxin Production in Wave 2 Strains

Three Wave 2 El Tor strains that harbored the toxT-139Y allele—B33, MJ1236, and MG116025—were tested with regard to their CT production. None of these strains generated CT under conditions tested in this study (Figure 2).

Derivatives of B33 That Contain the toxT-139F Allele

B33 (toxT-139Y, VC1589-T6) has a tandem repeat of CTX-2 on chromosome 2. YJB009 (toxT-139F, VC1589-T6) and YJB010 (toxT-139F, VC1589-T7) generally produced as much CT as classical biotype strains in the media that were tested. In cultures in AKI media at 37°C, CT production was approximately twice that of the O1395 classical biotype strain. In the same medium, slightly more CT was synthesized at 37°C than 30°C. The function of the V1589 gene (nonfunctional VC1589 in YJB009 and functional VC1589 in YJB010) did not affect CT production significantly (Figure 2A). The CT that was produced from these strains was of the classical type, because the CTX-2 in them contains the classical type ctxB allele (ctxB1).
Derivatives of MJ1236 That Contain the toxT-139F Allele

MJ1236 is closely related to B33, although these strains were isolated in Bangladesh and Mozambique, respectively. They contain the same CTX phage array (a tandem repeat of CTX-2 on chromosome 2). When cultured at 30°C, CT production by YJB012 (toxT-139F, VC1589-T6) and YJB013 (toxT-139F, VC1589-T7), which contained toxT-139F allele, was similar to the reference CT production, regardless of culture medium (Figure 2B). The function of VC1589 (nonfunctional VC1589 in YJB012 and functional VC1589 in YJB013) did not influence CT production significantly in these strains. At 37°C in AKI media, CT production by YJB012 was approximately two-fold that of the reference strain under agglutinating conditions.

MG116025

The original MG116025 strain contains the toxT-139F allele. We constructed a derivative of MG116025, YJB015, that contained toxT-139Y and VC1589-T6 to confirm that native MG116025 produces CT but the strain contained toxT-139Y allele does not (Figure 2C). Another derivative of MG116025 that contained toxT-139Y and functional VC1589 did not produce CT either (data not shown). Although MG116025 is included among Wave 2 atypical El Tor strains, the CT that it produces is the El Tor type (ctxB3). Approximately equal amounts of CT were produced from MG116025 and the reference O395 at 30°C, whereas CT production at 37°C was negligible (Figure 2C).

In summary, CT production varies between Wave 2 El Tor strains, and approximately twice as much CT can be produced from a derivative of B33 that contains the toxT-139F allele versus the reference classical biotype strain. Although MG116025 is a
Wave 2 strain, its pattern of CT production was similar to that of Wave 1 strains, because CT was produced at 30°C. The function of VC1589, and thus the capacity for neutral fermentation, did not affect CT production in Wave 2 strains.

**Cholera Toxin Production in Wave 3 Strains**

Two Wave 3 atypical El Tor strains were examined. IB4122 was isolated from a cholera outbreak in Northern Vietnam in 2009, and IB5230 was isolated from the 2010 cholera outbreak in Haiti (Nguyen et al., 2009; Chin et al., 2011). Both strains originally contained the toxT-139Y allele.

**Derivatives of IB4122 That Contain the toxT-139F Allele**

The CT that was produced by this strain was the classical type toxin (ctxB1). Approximately equal amounts of CT, with the reference strain, were produced by YJB017 (toxT-139F, VC1589-T6) and YJB018 (toxT-139F, VC1589-T7), which contained the toxT-139F allele, at 37°C in AKI media; slightly more CT was generated at 37°C than at 30°C (Figure 3A).

**IB5230 and Derivatives of IB5230 That Contain the toxT-139F Allele**

IB5230 is considered a hypervirulent strain due to its elevated CT production and colonization of the intestine (Satchell et al., 2016; Ghosh et al., 2019). The CT that was produced by this strain is ctxB7, which differs from the classical type ctxB (ctxB1) at amino acid residue 20. However, the secreted form of CT from this strain is the same as classical type CT, because the first 21 amino acids are removed during secretion (Sanchez and Holmgren, 2008).

Notably, although IB5230 harbors the toxT-139Y allele, CT production in PBS-buffered LB or AKI media at 37°C was similar to that by the reference strain (Figure 3B). The function of the VC1589 was not associated with significant differences in CT production in YJB017 or YJB018.

Another unique characteristic of IB5230 is that the function of VC1589, and hence the capacity for neutral fermentation, interfered with CT production when the toxT-139Y allele was replaced with toxT-139F. A derivative of IB5230, YJB20, containing the toxT-139F allele and nonfunctional VC1589, produced as much CT as the reference strain, similar to other strains that contain toxT-139F; however, YJB021, which harbors the toxT-139F allele and functional VC1589, did not produce CT under any culture condition (Figure 3B). Although the function of VC1589 affects the fermentation switch in media that has been supplemented with additional glucose, it appears that the operative metabolic pathway also influences toxin production in *V. cholerae*. More detailed studies of CT production in IB5230 and its derivatives might identify additional important characteristics that underlie the hypervirulent nature of this strain.

**Enhanced Cholera Toxin Production in a V. cholerae Classical Biotype Strain**

A derivative of O395, YJB001, that contained the toxT-139F allele produced 400% more CT than O395 that harbored the toxT-139Y allele when cultured in regular LB media at 30°C and under agglutinating conditions (Figure 4). More CT was produced at 30°C than 37°C. In conclusion, the toxT-139F allele induces classical *V. cholerae* strains to produce more CT under laboratory conditions.
DISCUSSION

The toxigenesis of *V. cholerae* has received much attention with regard to understanding the virulence of bacteria (Clemens et al., 2017). A protocol for generating CT under laboratory conditions has been developed for classical biotype strains—described as “agglutinating conditions” (DiRita et al., 1996). Several special culture methods have been developed to promote CT production in *V. cholerae* strains: the use of a bi-phasic culture method for CT production under the AKI condition (Sanchez et al., 2004); increases in the surface area-to-media-volume ratio to stimulate CT production under laboratory conditions (Iwanaga et al., 1986); and a shallow static culture method for CT production (Cobaxin et al., 2014).

However, these culture methods are not appropriate for monitoring toxigenesis in bacteria or large-scale CT production (Cobaxin et al., 2014). In the present study, we developed laboratory culture conditions under which *El Tor* biotype *V. cholerae* strains produced CT in a simple single-phase culture.

The expression of CT and TCP is stimulated by ToxT, an AraC/XylS-type transcriptional regulator (Yu and DiRita, 2002; Taylor et al., 2004). *toxT* expression is tightly regulated by ToxR/ToxS and TcpP/TcpH, which in turn are controlled by various regulatory proteins and stimuli in the intestinal environment (Sanchez and Holmgren, 2008). We identified a *V. cholerae* Wave 2 atypical *El Tor* strains, MG116025, that contains a point mutation in *toxT* (Phe at amino acid position 139, instead of Tyr in most other strains) (Kim et al., 2014, 2017). Amino acid position 139 of ToxT belongs to the N-terminal domain (amino acids 1–160), which has been implicated in regulation of binding to the cognate DNA sites of ToxT (Thomson and Withey, 2014; Thomson et al., 2015).

The expression of TCP and CT is higher in this strain, and we anticipated the *toxT*-139F allele to be useful for examining and producing CT in *V. cholerae* strains (Kim et al., 2017). As expected, CT production in *El Tor* strains was stimulated by the same point mutation in *toxT*, but the toxin production varies by strain. These results also imply that the expression of TCP and CT is constitutively stimulated by pre-existing ToxT but not by newly synthesized ToxT.

We determined the optimum single-phase culture methods for CT production in *El Tor* strains. A representative Wave 1 *El Tor* biotype strain, N16961, produces CT under specific culture conditions (Lee et al., 2012; Satchell et al., 2016). However, this strain failed to produce CT under regulation of the *toxT*-139F allele. When *toxT*-139F was introduced, another Wave 1 strain, T19479, generated approximately 40% of the CT that was produced by the classical biotype strain O395 under agglutinating conditions. CT production varies between atypical *El Tor* strains. Atypical *El Tor* strains tend to produce more CT than prototype *El Tor* biotype strains under regulation of the *toxT*-139F allele. A notable characteristic of classical and Wave 1 *El Tor* strains is that they produce more CT at 30°C than at 37°C, whereas Wave 2 and 3 atypical *El Tor* strains synthesize similar amounts of CT at both temperatures. This might be an important factor for population changes or the Waves in *El Tor* biotype strains within the seventh cholera pandemic.

Recently, we found that Wave 2 and 3 atypical *El Tor* strains lack neutral fermentation due to the loss of function of the acetolactate decarboxylase that is encoded by VC1589 (Lee et al., 2020). This change in phenotype did not affect CT production in most *El Tor* strains, except for the hypervirulent 2010 Haiti cholera outbreak strain that produced CT only when neutral fermentation was disrupted (Chin et al., 2011). However, acidic fermentation occurred when glucose was sufficiently supplied in the media, and the bacteria did not experience much difference in growth without fortified glucose, regardless of whether the VC1589 was functional. More detailed follow-up studies are required to determine the link between the fermentation pathways and toxigenesis in this strain, especially because it is considered a hypervirulent *V. cholerae* strain.

We also found that CT production increased approximately four-fold under agglutinating conditions in classical biotype strains when the *toxT*-139Y allele was replaced by *toxT*-139F. The optimal culture conditions for CT production by classical biotype strains that contained the *toxT*-139F allele were regular LB media at 30°C and agglutinating conditions.

Cholera toxin has tremendous potential as a mucosal vaccine adjuvant (Terrinoni et al., 2019). Efforts to develop mutated CT to avoid enterotoxicity when used as a vaccine adjuvant are ongoing. The method that we have established to mass-produce CT might support the development of a potent CT-based vaccine adjuvant (Lebens et al., 2016).

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.
AUTHOR CONTRIBUTIONS

EK and DK designed the research. YB, DL, EK, JL, and YY performed the research. GN contributed the bacterial strains. YB, DL, EK, JL, and YY analyzed the data and created figures. EK and DK wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00825/full#supplementary-material

FIGURE S1 | Immunoblot analysis of cholera toxin production in V. cholerae strains. As a representative, Western blot analysis of CT in strains MG116205 and one of its derivatives YJB014 is shown in this figure. Bacterial culture that contains approximately 5 × 10^7 cells were loaded onto each lane. Lanes M: Protein molecular weight marker, Lane 1: O395 reference, Lanes 2, 3: MG116205 cultured in PBS-buffered LB at 30°C, Lanes 4, 5: MG116205 cultured in PBS-buffered LB at 37°C, Lanes 6, 7: YJB014 cultured in LB at 30°C, Lanes 8, 9: YJB014 cultured in LB at 37°C. White and black arrows indicate CTA and CTB, respectively.

FIGURE S2 | CT production in Wave 1 strains N16961 and its derivatives. N16961 has been shown to produce CT under the A9i conditions; however, no detectable CT was produced from N16961 or its derivatives that contained toxT-1.0SF allele.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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