Malonate Inhibits Virulence Gene Expression in *Vibrio cholerae*

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**Abstract**

We previously found that inhibition of the TCA cycle, either through mutations or chemical inhibition, increased toxT transcription in *Vibrio cholerae*. In this study, we found that the addition of malonate, an inhibitor of succinate dehydrogenase (SDH), decreased toxT transcription in *V. cholerae*, an observation inconsistent with the previous pattern observed. Unlike another SDH inhibitor, 2-thienoyl trifluoroacetone (TTFA), which increased toxT transcription and slightly inhibited *V. cholerae* growth, malonate inhibited toxT transcription in both the wild-type strain and TCA cycle mutants, suggesting malonate-mediated inhibition of virulence gene expression is independent to TCA cycle activity. Addition of malonate also inhibited ctxB and tcpA expressions but did not affect aphA, aphB, tcpP and toxR expressions. Malonate inhibited cholera toxin (CT) production in both *V. cholerae* classical biotype strains O139S5N1 and CA401, and El Tor biotype strain, N16961. Consistent with previous reports, we confirmed that these strains of *V. cholerae* did not utilize malonate as a primary carbon source. However, we found that the addition of malonate to the growth medium stimulated *V. cholerae* growth. All together, these results suggest that metabolizing malonate as a nutrient source negatively affects virulence gene expression in *V. cholerae*.

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**Introduction**

*Vibrio cholerae* is the causative agent of cholera, a severe waterborne diarrheal disease. Toxin-coregulated pilus (TCP) and cholera toxin (CT) are essential for its virulence. TCP is a type IV pilus [1] that is required for colonization in the small intestine [2,3], whereas CT is a potent enterotoxin responsible for inducing cholera symptoms [4]. The expression of TCP and CT are positively regulated by an AraC-type transcriptional factor, ToxT [5,6]. Transcription of toxT is positively regulated by the membrane proteins ToxRS [5] and TcpPH [7]. The expression of tcpPH is positively regulated by the AphA [8] and AphB [9] transcriptional regulators.

Malonate is a dicarboxylic acid and a well-known competitive inhibitor of succinate dehydrogenase (SDH). However, it is also known that some bacteria can utilize malonate as a carbon source both aerobically and anaerobically. Malonate decarboxylase is the key enzyme for the degradation of malonate in aerobic malonate-degrading bacteria [10]. Genes encoding malonate decarboxylase (mdcACDE) are transcribed in an operon with a gene encoding a malonate transporter (mdcF). This operon also includes the mdcB gene, which encodes the biosynthesis of a prosthetic group precursor and mdcG and mdcH, which encode enzymes mediating the transfer of the prosthetic group to the apo-acyl carrier protein (ACP) and transfer of a malonyl residue from malonyl-CoA to the SH moiety of the prosthetic group, respectively.

In a previous attempt to identify negative factors of toxT transcription, we found that loss of the primary respiration-linked sodium pump, a NADH:ubiquinone oxidoreductase (NQR), and the TCA cycle related enzymes, Icd, SucA and AspA, resulted in elevated toxT expression in *V. cholerae* [7,11,12]. These findings suggested a link between central metabolism and virulence gene expression in *V. cholerae*.

In this study, we found that addition of malonate, a known inhibitor of the TCA cycle enzyme SDH, inhibited toxT expression and CT production in *V. cholerae*. This observation was unexpected because our previous study had shown the opposite effects of loss of TCA cycle enzymes on toxT transcription [12]. Further analysis of this phenomenon revealed that malonate inhibited toxT transcription independent to the TCA cycle activities. Although *V. cholerae* did not utilize malonate as a sole carbon source, malonate induced *V. cholerae* growth, prompting us to hypothesize that some malonate metabolizing pathway exists in *V. cholerae* and that some aspect of this pathway negatively affects toxT transcription in this organism.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were kept at −80°C in 20% glycerol stocks. For β-galactosidase assays, bacterial strains were grown
overnight in Luria-Bertani (LB) medium (Difco) at 37°C, washed, diluted to OD_600 = 0.05 in LB (initial pH 6.5) and then grown for 6 hr at 30°C. Yeast extract peptone water (YEP) was used for the AKI growth condition as described previously [13]. Medium pH was adjusted with HCl. Antibiotics were supplemented as appropriate as follows: streptomycin, 100 μg/ml; and kanamycin, 50 μg/ml. Malonate and NaCl were added to LB (pH 6.5) as indicated. 2-thienyltrifluoracetone (TTFA) was added to LB (pH 6.5) at 5 μM.

β-galactosidase and Alkaline Phosphatase Assays

β-galactosidase assays were performed as described previously [11]. Alkaline phosphatase assays were performed as described previously [14].

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

qRT-PCR assays were performed essentially as previously described [15]. In brief, cells of _V. cholerae_, grown in LB (initial pH 6.5) at 30°C for 6 hrs, were treated with RNA Protect Bacteria Reagent (Qiagen). RNA was extracted using the QIAGEN RNeasy Mini Kit (Qiagen). Primers used for qRT-PCR are listed in Table 2. Real-time qRT-PCR reactions were performed using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) and an ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems) at the OSU CGRB facility.

CT-ELISA

CT production was determined by a GM₁-based enzyme linked immunosorbsent assays (CT-ELISA), essentially as described previously [16]. CT-ELISA was performed using a cholera toxin-specific monoclonal antibody (Abcam) and Goat-Anti-Mouse (GAM)-HRP Conjugated antibodies (Bio-Rad). An HRP Substrate kit (Bio-Rad) was used to detect the HRP activity and the plates were read at 415 nm on an iMark microplate reader (Bio-Rad). The amount of CT was quantified using known amounts of purified chola tox B subunit (Sigma) as the standard.

Results

Malonate Inhibits toxT, ctxB, and tcpA Transcription

In contrast to our previous finding that inhibition of TCA cycle enzymes increased toxT transcription [12], we found that malonate, a potent inhibitor of SDH, significantly inhibited toxT transcription of the classical biotype strain of _V. cholerae_ O395N1 toxT::lacZ in a concentration dependent manner (Fig. 1A).

Sodium is also known to affect _V. cholerae toxT_ expression [11,16]. Because we used malonate disodium salt for the toxT::lacZ measurement, changes in sodium concentration may be responsible for the decreased toxT transcription. To test this, we investigated the effect of NaCl on toxT transcription. We used 40, 80 and 160 mM NaCl that contained equivalent amounts of sodium ions as the 20, 40 and 80 μM malonate additions. We observed that although NaCl modestly inhibited toxT transcription at these concentrations, the effects were significantly lower than those of the malonate (Fig. 1A and B). In addition, unlike malonate, NaCl did not show concentration-dependent increased inhibition of toxT in the concentration range we tested (Fig. 1B). Malonate also inhibited ctx and tcpA transcription (Fig. 1C and 1D, respectively) further confirming the negative effects of malonate on virulence gene expression in this bacterium.

Malonate Inhibits toxT Independent of the TCA Cycle and ETC Activities

When TCA cycle activity is low, bacteria utilize alternative pathways to generate ATP and to recycle CoASH from acetyl-CoA [17,18]. One such pathway is the PTA-ACK pathway, which consists of phosphotransacetylase (PTA) and acetate kinase (ACK) [17–20]. Metabolic activity through the PTA-ACK pathway results in the excretion of acetate into the external medium [17]. Our previous study revealed that _V. cholerae_ TCA cycle mutants (icd and sucA mutants) and TCA cycle related mutant (aspA mutant) showed increased acetate production that also shifted the medium pH to a slightly acidic pH [12]. In addition, we observed that the TCA cycle mutants showed slower growth compared to the parent strain when grown in LB [12]. Similar effects were also observed when SDH was inhibited by another SDH chemical inhibitor, 2-thienyltrifluoracetone (TTFA) (data not shown). TTFA increased toxT transcription to levels similar to the TCA cycle mutants (data not shown). In contrast, addition of malonate did not affect medium pH and slightly increased the _V. cholerae_ growth (data not shown). Such distinguishable differences prompted us hypothesize

Table 1. Bacterial strains used in this study.

| Strains   | Description                        | Source or reference       |
|-----------|------------------------------------|---------------------------|
| V. cholera|                                    |                           |
| O395N1    | O1 classical biotype strain, lacZ’, Sm’        | Dr. John Mekalanos       |
| TZ (toxT::lacZ) | O395N1, toxT::lacZ, Sm’                  | [7]                      |
| TZsucA::TnMar | TZ, sucA::TnMar, Sm’, Km’               | [12]                     |
| TZiicd::TnMar | TZ, iicd::TnMar, Sm’, Km’               | [12]                     |
| TZaspA::TnMar | TZ, aspA::TnMar, Sm’, Km’               | [12]                     |
| TZNqr | TZ, A nqr-A, F, Sm’                        | [34]                     |
| tcpA:phoA | O395N1, tcpA:phoA, Sm’                  | [3]                      |
| ctxA:phoA | O395N1, ctxA:phoA, Sm’                  | [13]                     |
| CA401     | O1 classical biotype strain, Sm’           | [35]                     |
| N16961    | O1 El Tor biotype strain, Sm’             | Dr. John Mekalanos       |

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Table 2. DNA primers used in this study.

| Primer     | Sequence (5’ to 3’) |
|------------|---------------------|
| 5Vc16SrRNAgRT | GATCATGGCCATGATTGTAAACG |
| 3Vc16SrRNAgRT | TGACGACCACCAAGGAAAAC |
| 5VcToxAqRT | GCTGCTCTGGGAAGTGTAATG |
| 3VcToxAqRT | TTCACTCTGTTAGGAAGGAAAG |
| 5VcToxPqRT | GGTTCCAGGAGAAAACGG |
| 3VcToxPqRT | TGACGATTAACTCATGAGGCAAG |
| 5VcToxRqRT | CGGATAGAGGACAACACTAAAGA |
| 3VcToxRqRT | TCATTAGGGAGATCGAGTAAA |
| 5VcAphAgRT | TCACTCAAGTTCGAGACACCTAAC |
| 3VcAphAgRT | TTCTCTGTTAGGGAAGGGACAA |
| 5VcAphBqRT | GCACCATCCATGCAGAAAAAC |
| 3VcAphBqRT | GCACGATCGGAATTCACATC |

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that malonate affects toxT transcription independent to the TCA cycle activity.

To further confirm this idea, we tested the effects of malonate on toxT transcription in the TCA cycle mutants. If malonate affects toxT expression by affecting TCA cycle activity, the TCA cycle mutants should be insensitive to the addition of malonate. We

Table 3. CT production of different V. cholerae strains under various growth conditions.

| Growth conditions | CT production (µg/ml/O.D₆₀₀) |
|-------------------|-----------------------------|
|                   | O395N1 | CA401 | N16961 |
| LB                | 2.2±0.4 | 3.1±0.1 | N.D*   |
| LB+40 mM malonate | 1.4±0.5 | 0.8±0.1 | N.D*   |
| YEP              | 1.5±0.4 | N.D*   | 0.6±0.1 |
| YEP+40 mM malonate| 0.5±0.2 | N.D*   | U.D³   |

*not determined. ³undetected.
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therefore investigated the effects of malonate on toxT transcription in the TCA cycle mutants (\( {\text{aspA}}, {\text{icd}} \text{ and } {\text{sucA}} \)). Consistent with our previous findings, the TCA cycle mutants showed higher toxT transcription compared to the parent strain (Fig. 1A). However, malonate still inhibited toxT transcription in the TCA cycle mutants (Fig. 1A), indicating that the negative effect of malonate on toxT transcription is independent to the TCA cycle activity.

Because SDH is also involved in the electron transport chain (ETC), it could be possible that malonate inhibits toxT transcription by inhibiting ETC activity. However, this notion conflicted with our previous finding that inhibition of one of the major ETC components, NQR, increased toxT transcription [11]. In addition, similar to the TCA cycle mutants, toxT transcription in the \( {\Delta nqA-F} \) mutant was sensitive to malonate (data not shown) indicating that the negative effect of malonate on toxT transcription is independent to the ETC activity.

To further examine the negative effects of malonate on \( V. \text{cholerae} \) virulence gene expression we performed qRT-PCR analyses. As expected from our toxT::\( {\text{lacZ}} \) analyses, we observed that toxT expression levels in \( V. \text{cholerae} \) O395N1 were approximately two-fold lower in the presence of malonate than the control (Fig. 2). Similarly, ctxB and tcpA were also reduced in the presence of malonate (Fig. 2), as both genes are regulated by ToxT. To investigate if other known transcriptional activators in this regulatory cascade respond to the presence of malonate, we next tested whether malonate also affects \( \text{aphA}, \text{aphB}, \text{tcpP} \), or \( \text{toxR} \).

Malonate does not Inhibit Expression of Transcriptional Regulators Operating Upstream of ToxT

Table 4. \( V. \text{cholerae} \) predicted acyl-CoA ligases that show similarity to malonyl-CoA synthetase from \( \text{Rhizobium leguminosarum} \).

| VC numbers | Predicted protein functions                          | Match Values |
|------------|-----------------------------------------------------|--------------|
| VC1985     | long-chain-fatty-acid–CoA ligase (\( {\text{fadD}} \)) | 4.0e\(-53\)   |
| VC0772     | vibriobactin-specific 2,3-dihydroxybenzoate-AMP ligase (\( {\text{vibE}} \)) | 3.8e\(-36\)   |
| VC1340     | propionate–CoA ligase (\( {\text{proP}} \))            | 1.1e\(-25\)   |
| VC0249     | RfbL protein (\( {\text{rfbL}} \))                   | 6.0e\(-21\)   |
| VC2484     | long-chain-fatty-acid–CoA ligase, putative           | 1.2e\(-17\)   |
| VC2341     | long-chain-fatty-acid–CoA ligase, putative           | 2.0e\(-17\)   |
| VC1971     | O-succinylbenzoate–CoA ligase, putative             | 2.3e\(-17\)   |
| VC0298     | acetyl-CoA synthase (\( {\text{acs-1}} \))           | 2.4e\(-17\)   |
| VC1579     | enterobactin synthetase component F-related protein | 3.8e\(-16\)   |
| VCA1110    | long-chain-fatty-acid–CoA ligase, putative           | 9.0e\(-06\)   |
| VCA0829    | acetyl-CoA synthase (\( {\text{acs-2}} \))           | 2.7e\(-3\)     |

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Figure 4. Current working model for the link between malonate and virulence gene expression in \( V. \text{cholerae} \). Malonate is possibly imported by one of the Auxin Efflux Carrier (AEC) Family proteins (VC1229 and VCA0024). We propose that increased levels of malonyl-CoA decrease the available intracellular CoA levels which results in decreased acetyl-CoA (AcCoA) levels. When TCA cycle activity is low, AcCoA levels should be increased and \( V. \text{cholerae} \) produce acetate from AcCoA and excrete it into the external medium most likely via the PTA and ACK system (see text for more details). Such a link between AcCoA levels and toxT transcription, as depicted by the red arrow, has previously been observed [12].

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Figure 3. Effects of malonate, glycerol and glucose on \( V. \text{cholerae} \) growth. Bacteria were inoculated into M9 medium supplemented with different carbon sources (f.c. 0.4%). Bacterial growth was measured after 24 hrs shaking. 40 mM malonate was added to the M9-glycerol and M9-glucose media as indicated. All experiments were repeated more than three times. The error bars indicate standard deviations. \( P \) values were calculated by Student’s t test.
expression. No dramatic effects of malonate were observed on the expression levels of these genes (Fig. 2), however, suggesting that malonate does not appear to affect toxT expression by affecting transcription of known regulators functioning upstream of ToxT.

Malonate Inhibits CT Production in both Classical and El Tor Biotype Strains of V. cholerae

To confirm the negative effects of malonate on V. cholerae virulence, we next investigated the effects of malonate on CT production. Consistent with the gene expression data, addition of malonate inhibits CT production of V. cholerae O395N1 strain when tested in LB (pH 6.5) at 30°C (Table 3). We also observed that the V. cholerae O395N1 strain did not show an autoagglutination phenotype in LB (pH 6.5) at 30°C (Fig. 1C). Because of the known correlation between the autoagglutination phenotype and TCP production [21], these data indicated that malonate inhibits both of the major virulence factors in V. cholerae.

We then asked whether the effects of malonate on CT production are specific to the V. cholerae O395N1 strain. CT production in another V. cholerae classical biotype strain, CA401, was also inhibited by malonate, similar to the O395N1 strain (Table 3). To investigate whether malonate also inhibited CT production in an El Tor biotype strain, we tested the effect of malonate on CT production of V. cholerae N16961. El Tor biotype strains are required to grow under a specific growth conditions, known as the “AKI conditions” [12,22], to produce measurable CT production in vitro. V. cholerae N16961 produced detectable amounts of CT when grown under AKI growth conditions and addition of malonate to the growth media strongly inhibited CT production (Table 3). We also found that addition of malonate into the medium inhibited CT production of the classical biotype V. cholerae strain O395N1 in the AKI condition (Table 3), further demonstrating that the effect of malonate is not growth condition specific. Moreover, the effects of malonate on CT production are not biotype specific.

V. cholerae do not Utilize Malonate as a Sole Carbon Source

Some bacteria are known to utilize malonate as a sole carbon source. Typically, a malonate transporter and malonate decarboxylase are required to utilize malonate. Genetic analysis of the reported V. cholerae genomes, including O395 and N16961 strains, revealed that V. cholerae do not encode neither a malonate transporter gene (mdcF) nor a malonate decarboxylase gene (mdcA,C,D and E), suggesting that V. cholerae do not utilize malonate as the carbon source. Indeed, it was reported that less than 1% of V. cholerae strains can utilize malonate as a carbon source [23].

To confirm this, we performed a growth assay using M9 minimal media supplemented with malonate as the sole carbon source. We observed that all of the V. cholerae strains tested did not grow in M9 minimal media supplemented with just malonate (Fig. 3), indicating that these V. cholerae strains do not utilize malonate as the sole carbon source.

Although the V. cholerae strains we used did not utilize malonate as the sole carbon source, we did notice that the growth of these strains was significantly stimulated when grown in M9 glycerol supplemented with malonate compared to just M9 glycerol (Fig. 3). Similar growth induction by malonate was also observed in M9 glucose (Fig. 3). These observations are consistent with the finding that addition of malonate slightly induced V. cholerae growth in LB (see above). Together, these data indicated that V. cholerae can utilize malonate as a nutrient source but not as a sole carbon source.

Discussion

Recent accumulating evidence suggests that metabolism and bacterial virulence are closely related [24–26]. In V. cholerae, it has been shown that ToxT indirectly inhibits central metabolism pathways, including the TCA cycle and glycolysis [27]. We had previously found that the inhibition of the central metabolic pathway, the TCA cycle and the primary respiration-linked sodium pump, NQR, increased toxT transcription in V. cholerae [11,12]. These findings suggest the existence of a feedback loop between central metabolism and ToxT. In addition, other metabolic pathways, such as the Entner-Doudoroff pathway, the anaerobic trimethylamine N-oxide respiration, and methionine metabolism are involved in V. cholerae virulence [28–30]. Thus, there is a mature link between V. cholerae metabolism and virulence. The current study was initiated by the unexpected finding that malonate, a potent SDH inhibitor, inhibited toxT expression, whereas inhibition of TCA cycle enzymes by mutations increased toxT transcription [12].

When SDH is inhibited, succinate can still be acquired through the glyoxylate shunt. However, we previously showed that the sucA and the icd mutants showed increased toxT transcription [12]. Importantly, these mutants can also acquire succinate through the glyoxylate shunt. Thus, it is unlikely that inhibition of SDH decreases toxT transcription because of the glyoxylate shunt. We have also shown that stimulation of the glyoxylate shunt either by adding glyoxylate or acetate does not affect toxT transcription [12]. Furthermore, since SDH is also a component of the electron transport chain (ETC), inhibition of SDH inhibits ETC activity. We previously reported that inhibition of a major ETC component of V. cholerae, NQR, increased toxT transcription [11] but the increased toxT was primarily caused by decreased TCA cycle activity [12]. Therefore, it is unlikely that inhibition of SDH decreases toxT transcription by affecting ETC activity. Hence, we hypothesized that malonate affects toxT independent to TCA cycle activity. To confirm this hypothesis, we tested another SDH inhibitor, 2-thienoyltrimfluoroacetone (TTFA). We found that addition of TTFA increased toxT transcription, an observation similar to the toxT increases observed in the sucA, the icd and the aspA TCA cycle mutants [12]. TTFA also inhibited growth similar to the TCA cycle mutants [12]. In contrast, malonate inhibited toxT transcription and slightly induced growth. These data strongly suggested that inhibition of SDH increases toxT transcription similar to the TCA cycle mutants and that the effect of malonate on toxT is different from the TCA cycle inhibition effect. In addition, we investigated the effects of malonate on several TCA cycle mutants and an ETC mutant (the napA-F mutant) and found that malonate still inhibited toxT transcription in these mutants. These data clearly showed that malonate inhibits toxT transcription independent to TCA cycle and ETC activities.

Although, V. cholerae did not utilize malonate as the sole carbon source, we observed that addition of malonate, when combined with other carbon sources, induced V. cholerae growth, indicating that V. cholerae can indeed utilize malonate as a nutrient source. Thus, once inside the V. cholerae cells, malonate might be metabolized and changed in structure, thereby no longer being able to inhibit SDH. This concept might explain our conflicting finding that inhibition of TCA cycle decreased V. cholerae growth and increased toxT transcription because if malonate were to retain its structure inside the V. cholerae cells, it would inhibit the TCA cycle activity.
cycle and result in decreased growth and increased toxT expression.

Three types of enzymes, malonate decarboxylase, malonamidase and malonyl-CoA synthetase, are known to use malonate as their substrate [31]. Malonate decarboxylase is essential for bacterial growth on malonate as the sole carbon source. V. cholerae strains do not have an ortholog of the known malonate utilizing enzyme complex, malonate decarboxylase (mdcACDE) or malonate transporter (mdcF). Consistent with this, we observed that V. cholerae O935N1 and N16961 strains did not utilize malonate as the sole carbon source. However, our model predicts transport of malonate into V. cholerae. The amino acid sequence of Klebsiella pneumoniae MdcF transporter is known to show similarity to a Bacillus subtilis protein, YwkB [32]. YwkB belongs to the Auxin Efflux Carrier (AEC) Family (TPDB). Interestingly, V. cholerae has two genes encoding proteins that belong to the AEC family (VC1229 and VCA0024) and these two proteins also show some similarity to K. pneumoniae MdcF. Therefore, it might be possible that V. cholerae transports malonate into the cells via these AEC family proteins. We also searched genes that showed similarity with the malonamidase genes in the V. cholerae genome but did not find any genes with significant homology. Although we also did not find a true ortholog of malonyl-CoA synthetase from Rhizobium leguminosarum (Accession: AAC83455.1) in the V. cholerae genome, we found that V. cholerae has several genes that showed some amino acid similarity to the malonyl-CoA synthetase (Table 4). It could be possible that malonate is converted to malonyl-CoA by one of these enzymes in V. cholerae cells.

If malonate is indeed converted to malonyl-CoA, how would such a metabolic change affect toxT expression? As mentioned above, we recently found that inhibition of TCA cycle and NQR, increased toxT transcription in V. cholerae [11,12]. These mutants showed increased acetate production and we found that disruption of the major acetate excretion pathway, the PTA-ACK pathway, increased toxT transcription. Thus, we concluded that toxT transcription is affected by either acetyl-CoA or some metabolic derivative of acetyl-CoA. We are proposing the possibility that malonyl-CoA synthesis from malonate results in the decreased available CoA pool and decreased acetyl-CoA levels (Fig. 4). This idea is consistent with our data that malonate did not affect other virulence related regulators that operate upstream of toxT. It is also interesting to mention that a V. cholerae fadD mutant showed decreased toxT expression, indicating that FadD is related to the virulence gene regulation in V. cholerae [33]. Although the detailed molecular mechanism is still unclear, lack of FadD impairs the membrane localization of TcpP and results in decreased toxT transcription [33]. Similar to the fadD mutant, addition of malonate did not affect tcpP and toxR expression levels. It might be possible that interaction of malonate with FadD modifies its structure and results in impaired TcpP membrane localization.

Together our data demonstrate the intriguing link between malonate and toxT expression. Future research is necessary to understand the mechanisms of 1) how malonate is utilized in V. cholerae and 2) how such changes in metabolism affect toxT expression.

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Author Contributions

Conceived and designed the experiments: YM CCH. Performed the experiments: YM SRF CCH. Analyzed the data: YM SRF CCH. Wrote the paper: YM SRF CCH.

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