Central metabolism controls transcription of a virulence gene regulator in *Vibrio cholerae*

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ToxT is the central regulatory protein involved in activation of the main virulence genes in *Vibrio cholerae*. We have identified transposon insertions in central metabolism genes, whose disruption increases toxT transcription. These disrupted genes encode the primary respiration-linked sodium pump (NADH:ubiquinone oxidoreductase or NQR) and certain tricarboxylic acid (TCA) cycle enzymes. Observations made following stimulation of respiration in the nqr mutant or chemical inhibition of NQR activity in the TCA cycle mutants led to the hypothesis that NQR affects toxT transcription via the TCA cycle. That toxT transcription increased when the growth medium was supplemented with citrate, but decreased with oxaloacetate, focused our attention on the TCA cycle substrate acetyl-CoA and its non-TCA cycle metabolism. Indeed, both the nqr and the TCA cycle mutants increased acetate excretion. A similar correlation between acetate excretion and toxT transcription was observed in a tolC mutant and upon amino acid (NRES) supplementation. As acetate and its tendency to decrease pH exerted no strong effect on toxT transcription, and because disruption of the major acetate excretion pathway increased toxT transcription, we propose that toxT transcription is regulated by either acetyl-CoA or some close derivative.

INTRODUCTION

Cholera is a severe waterborne diarrhoeal disease that remains a public health concern in many developing countries. The causative agent of cholera is *Vibrio cholerae*, a Gram-negative bacterium. The two main essential virulence factors of *V. cholerae* are toxin-coregulated pilus (TCP) and cholera toxin (CT). TCP is a type IV pilus that is required for colonization of the small intestine. CT is a potent enterotoxin responsible for inducing most of the cholera symptoms. The genes that encode TCP, CT and many other virulence determinants comprise a network of genes called the ToxT regulon, whose expression is modulated by a hierarchy of transcriptional regulators (Matson et al., 2007). These regulators include the transcription factors AphA and AphB, which positively regulate transcription of tcpPH. The membrane-bound protein complex TcpPH works with the membrane-bound protein complex ToxRS to activate transcription of toxT, which encodes an AraC-type transcriptional factor (Matson et al., 2007). While it is ToxT that directly activates the genes that encode TCP, CT and the rest of the ToxT regulon, additional positive regulators have been reported. In contrast, our knowledge concerning factors that negatively regulate the ToxT regulon (reviewed by Matson et al., 2007) is restricted to the nucleoid-associated protein H-NS, which represses transcription of toxT, tcpA and ctxAB (Nye et al., 2000; Stonehouse et al., 2011), and the cAMP-receptor protein (CRP), which is reported to negatively regulate tcpPH (Kovacikova & Skorupska, 2001).

In a previous attempt to identify negative regulators of toxT transcription, we found that elevated toxT transcription results from loss of the primary respiration-linked sodium pump (NADH:ubiquinone oxidoreductase or NQR) (Häse & Mekalanos, 1998, 1999). As NQR is expected to generate a sodium motive force (smf), lack of a functional NQR can be expected to reduce smf-dependent flagella rotation. Because a correlation between motility and virulence gene expression exists (Gardel & Mekalanos, 1996), we previously proposed that NQR might affect toxT expression via flagella rotation (Häse & Mekalanos, 1999). Further characterization of this link, however, revealed that NQR-associated toxT induction is flagellum-independent.

**Abbreviations**: acetyl-P, acetyl phosphate; CT, cholera toxin; FRT, FLP recognition target; NQR, NADH:ubiquinone oxidoreductase; OAA, oxaloacetate; PTA-ACK, phosphotransacetylase/acetate kinase; qRT-PCR, quantitative RT-PCR; smf, sodium motive force; TCA, tricarboxylic acid; TCP, toxin-coregulated pilus.

Six supplementary figures are available with the online version of this paper.
(Häse, 2001). An earlier study showed that this induction occurs independently of ToxR (Häse & Mekalanos, 1999). Thus, the detailed mechanism by which NQR affects toxT transcription remains unknown.

Our previous screen also identified transposon insertions into the genes that encode the central metabolism enzymes fumarase (fumA) and L-glutamine-D-fructose 6-phosphate aminotransferase (glmS) (Häse & Mekalanos, 1999). The former is integral to tricarboxylic acid (TCA) cycle function, while the latter is associated with the TCA cycle through its glutamine substrate. V. cholerae experiences diverse nutritional environments as it passes through its human host and it must specifically adapt to the nutrients available at its preferred infection site, the small intestine.

Indeed, several previous reports have suggested a role for central metabolism in virulence gene regulation (Miller & Mekalanos, 1988; Patra et al., 2012). For instance, the V. cholerae classical strain O395N1 does not produce TCP and CT when grown in M9 medium with glycerol as the sole carbon source (M9-glycerol). However, CT production is stimulated when M9-glycerol is supplemented with a combination of four amino acids [asparagine, arginine, glutamate and serine (NRES)] (Miller & Mekalanos, 1988). Although this phenomenon had been well documented, relatively little was known concerning the underlying mechanism. Recently, however, we reported that the CT production defect in M9-glycerol results from low toxT transcription, that NRES supplementation increases transcription and that the NRES effect involves TolC, a major outer-membrane protein that contributes to bacterial multidrug resistance (Minato et al., 2011). Furthermore, several recent studies have suggested that tolC mutations affect bacterial metabolism (Dhamdhere & Zgurskaya, 2010; Santos et al., 2010).

The most central metabolite is acetyl CoA (acetyl-CoA), which replenishes the TCA cycle and thus facilitates respiration. When respiration and/or TCA cycle activity is low or when the carbon flux into cells exceeds the capacity of the TCA cycle and other central metabolic pathways, acetyl-CoA is metabolized via the phosphotransacetylase and acetate kinase (PTA-ACK) pathway. This pathway catalyses the conversion of acetyl-CoA to acetate via acetyl phosphate (acetyl-P). In the process, it recycles CoA and generates ATP (Wolfe, 2005). Thus, the PTA-ACK pathway is responsible for controlling the levels of two key central metabolites, acetyl-CoA and acetyl-P, which act as acetyl and phosphoryl donors, respectively, to post-translationally modify protein function (Hu et al., 2010; Lima et al., 2011, 2012; Wolfe, 2005, 2010).

To extend our previously identified link between V. cholerae virulence and central metabolism (respiration via NQR and the TCA cycle via fumA and glmS), we again examined transposon-derived mutants that exhibited more toxT-lacZ transcription than their parent. Here, we demonstrate that NQR and TolC influence toxT transcription by affecting the TCA cycle. We further provide evidence that acetyl-CoA is central to the process.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. For β-galactosidase assays, bacterial strains were grown overnight in Luria–Bertani (LB) medium (Difco) at 37 °C, washed, diluted to an OD<sub>600</sub> of 0.05 in LB (initial pH 6.5) or LB buffered with BIS-TRIS propane (BTP) (Sigma) at pH 6.5 (LBB) and then grown for 6 h at 30 °C. Medium pH was adjusted by HCl or NaOH. As required, antibiotics were supplemented as follows: streptomycin, 100 μg ml<sup>−1</sup>; ampicillin, 100 μg ml<sup>−1</sup>; chloramphenicol, 1 μg ml<sup>−1</sup> for V. cholerae and 10 μg ml<sup>−1</sup> for Escherichia coli; and kanamycin, 50 μg ml<sup>−1</sup>. The pta and ackA-1 mutant strains of V. cholerae were constructed essentially as previously described (Donnenberg & Kaper, 1991; Minato et al., 2011; Yamamoto et al., 2009) using the primers listed in Table 2. The pta and ackA-1 mutations in these strains were verified by PCR. As ackA-1 is located upstream of pta and pta is most likely to be transcribed in an operon with ackA-1, the FLP recognition target (FRT)-flanked cat cassette in the ackA-1 mutant was eliminated by using a temperature-sensitive helper plasmid, pCP20 (Cherepanov & Wackernagel, 1995).

**Transposon mutagenesis and screening.** Transposon mutagenesis was performed essentially as previously described (Häse & Mekalanos, 1999).

**Quantitative RT-PCR (qRT-PCR) analysis.** Cells of V. cholerae, grown in LB (initial pH 6.5) at 30 °C until early exponential growth phase (OD<sub>600</sub> of 0.2), were treated with RNA Protect Bacteria Reagent (Qiagen). RNA was extracted using the Qiagen RNaseasy Mini kit (Qiagen). Primers used for qRT-PCR are listed in Table 2. Real-time qRT-PCRs were performed using the SuperScript<sup>®</sup> III Platinum<sup>®</sup> SYBR<sup>®</sup> Green One-Step qRT-PCR kit (Invitrogen) and an ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems) at the Oregon State University State for Genome Research and Biocomputing facility.

**Phenotype MicroArray (PM) analyses.** Phenotype microarrays were performed using PM1 to PM8 MicroPlate<sup>®</sup> (Biolog) at Biolog's PM Services group.

**Metabolite measurement.** For metabolite measurements, bacteria were grown in LB (initial pH 6.5) for 6 h at 30 °C. To measure total citrate, bacterial culture was boiled at 100 °C for 5 min and centrifuged at 13 000 r.p.m. (4 °C) for 2 min. The citrate concentration in the resultant cell-free lysates was tested using a citric acid enzyme assay kit (R-Biopharm). Total cAMP was measured essentially as described by Inada et al. (1996) using the cAMP Direct Biotrak<sup>®</sup> ELA (non-acetylation protocol) kit (GE Healthcare).

Acetic acid levels in the supernatant were measured using the acetic acid enzyme assay kit (R-Biopharm).

**RESULTS**

**Isolation of V. cholerae transposon mutant strains with increased toxT transcription**

To identify mutants with increased toxT transcription relative to that of the parent strain, we performed a transposon screen of a V. cholerae O395N1 variant that...
carries a toxT-lacZ promoter fusion at the toxT locus (strain TZ, Table 1), essentially as described (Häse & Mekalanos, 1999). We obtained several colonies that, relative to the parent, were darker blue on LB plates supplemented with X-Gal, indicative of increased toxT transcription. We chose seven of these colonies for further characterization. Sequence analysis revealed one insertion in the nqr operon and one insertion in the glmS gene, consistent with our previous screen (Häse & Mekalanos, 1999). We also obtained some novel mutations. Two transposons had inserted into genes that encode TCA cycle enzymes: one in sucA (VC2087) and one in icd (VC1141). Three transposons had inserted into the TCA cycle-related gene aspA (VC2698). The sucA and icd genes encode 2-oxoglutarate dehydrogenase (E1 component) and isocitrate dehydrogenase, respectively. Together, these enzymes convert isocitrate to succinyl-CoA with the production of two molecules of CO₂. The aspA gene encodes

<table>
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<th>Strain or plasmid</th>
<th>Description</th>
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<tr>
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<td>O1 classical biotype strain, lacZ⁺, Sm²</td>
<td>Dr John Mekalanos</td>
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<td>Invitrogen</td>
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<td>Cm³, template for PCR amplification for lambda-red recombinase-mediated recombination</td>
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<td>Cherepanov &amp; Wackernagel (1995)</td>
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Table 1. Bacterial strains and plasmids used in this study

WT, Wild-type.

Table 2. Primers used in this study

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<td>Fup ackA-1</td>
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<td>Rup ackA-1-FRT</td>
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aspartate-ammonia lyase, which converts aspartate to the TCA cycle intermediate fumarate with the production of ammonia (Fig. 1a).

To further characterize these mutants, we grew them in LB at 30 °C and monitored growth and toxT transcription. Similar to comparable mutants in other species (Guccione et al., 2008; Lakshmi & Helling, 1976; Yu et al., 2006), each V. cholerae mutant exhibited a slight growth defect relative to its parent, especially during late exponential growth (Fig. S1 available with the online version of this paper). As expected from the solid media results, each mutant exhibited significantly more β-galactosidase activity than did its parent (Fig. 1b). Subsequent characterization of these mutants suggested that changes in TCA cycle-associated metabolism could affect toxT transcription in V. cholerae. Taken together, our results show that toxT transcription rises in response to the disruption of TCA cycle function.

**NQR affects toxT expression via respiration activity**

We had previously proposed an intimate connection between expression of the main V. cholerae virulence factors and sodium membrane bioenergetics on the basis of the finding that disruption of the sodium pump NQR activated toxT transcription (Häse & Mekalanos, 1999). Further characterization of the link between the smf and toxT expression, however, did not support this theory. First, toxT transcription was unaffected by mutation of other smf generation enzymes (e.g. the sodium/proton antiporters NhaA and NhaD; Fig. S2). Second, the NQR effect on toxT expression was found to be independent of the smf-dependent V. cholerae flagella system (Häse, 2001). Thus, we concluded that smf production per se is probably not directly linked to virulence gene expression.

Because V. cholerae does not encode a Complex I homologue, NQR is also expected to function as the main respiratory enzyme. Therefore, loss of NQR should also dramatically decrease aerobic respiration activity. V. cholerae is predicted to encode an inducible l-lactate-ubiquinone oxidoreductase enzyme (VCA0984); thus, the addition of l-lactate would be predicted to stimulate respiration even in the absence of NQR. Indeed, incubation of the nqr mutant in LB supplemented with l-lactate restored growth (Fig. S3a) and returned transcription to parental levels (Fig. 2), suggesting that the loss of
respiration (rather than sodium pumping) is responsible for the effects of the \textit{nqr} mutation on \textit{toxT} transcription.

**The TCA cycle does not affect \textit{toxT} expression via NQR**

As respiration and TCA cycle activity are linked, the TCA cycle mutants may increase \textit{toxT} transcription by negatively affecting respiration activity. To test this idea, we first monitored the effect of the NQR inhibitor 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO) on \textit{toxT} transcription in each TCA cycle mutant. Consistent with our previous report (Häse & Mekalanos, 1999), \textit{toxT} transcription in the parent strain increased in the presence of HQNO, whereas the \textit{nqr} mutant was insensitive (Fig. 2). Like the \textit{nqr} mutant, the TCA cycle mutants were generally insensitive to the addition of HQNO (Fig. 2), suggesting that the TCA cycle and NQR affect \textit{toxT} transcription by a common mechanism. We next investigated whether reduced respiration activity is responsible for the increased \textit{toxT} expression exhibited by the TCA cycle mutants. If a TCA cycle mutation affects \textit{toxT} transcription simply by negatively affecting respiration activity, then restoration of respiration in the TCA cycle mutants by adding L-lactate should restore \textit{toxT} transcription to parental levels. We therefore measured the effects of L-lactate on the TCA cycle mutants. While L-lactate diminished \textit{toxT} transcription in the \textit{aspA} and \textit{sucA} mutants, L-lactate did not restore \textit{toxT} transcription levels of these mutants to the parental level (Fig. 2). This phenotype was clearly distinct from the \textit{nqr} mutant, which showed fully restored \textit{toxT} transcription to the parental level with the addition of L-lactate (Fig. 2). In addition, L-lactate had no effect on the \textit{icd} mutant (Fig. 2). These observations provide support that the common mechanism for increased \textit{toxT} in the TCA cycle mutants is not via respiration.

**The NQR mutant shows decreased TCA cycle intermediate utilization**

As NQR is expected to function as the main respiratory enzyme, we aimed to investigate how lack of NQR affects \textit{V. cholerae} metabolism. For this, we used Phenotype Microarray\textsuperscript{®} (Biolog) analysis to compare metabolism in the \textit{nqr} mutant with that of its parent using PM1–PM8 microplates. The \textit{nqr} mutant had defects in the utilization of 15 of the 190 carbon sources tested with the PM1 and PM2 microplates (Table 3). These 15 carbon sources included several TCA cycle intermediates (fumaric acid, succinic acid and L-malic acid) and many carbon sources that are metabolized into TCA cycle intermediates (L-aspartic acid, L-histidine, L-glutamine and L-glutamic acid), suggesting that TCA cycle activity is low in the \textit{nqr} mutant compared with the parent strain. Because succinic acid was used as the carbon source in the PM3–PM8 (nitrogen source, phosphorus source, sulfur sources and nutrient supplements plates), systematic metabolic defects were observed (data not shown). We propose that the \textit{nqr} mutant exhibits decreased TCA cycle activity and that this reduction is the primary cause of its increased \textit{toxT} transcription.

**Certain TCA cycle intermediates affect \textit{toxT} transcription**

The loss of a TCA cycle enzyme can be expected to cause accumulation of ‘upstream’ TCA cycle intermediates. Furthermore, it has been shown that certain transcriptional regulators (e.g. CitAB, DctBD and GlnB) can sense changes in the intracellular concentrations of particular TCA cycle intermediates and, consequently, can cause changes in gene regulation (Leigh & Dodsworth, 2007; Mascher \textit{et al.}, 2006; Reid & Poole, 1998; Scheu \textit{et al.}, 2012; Zientz \textit{et al.}, 1998). Therefore, we analysed whether the accumulation of certain TCA cycle intermediates can affect \textit{toxT} transcription. To test this possibility, we grew the parent strain in LB supplemented with TCA cycle intermediates (at 10 or 25 mM) and monitored both growth and \textit{toxT} transcription. When tested at the lower concentration (10 mM), none of the TCA cycle intermediates affected bacterial growth. Even at the higher concentration (25 mM), only citrate inhibited growth (Fig. S3b). Intriguingly, growth in the presence of citrate significantly increased \textit{toxT} transcription, while growth in the presence of oxaloacetate (OAA) and malate inhibited \textit{toxT} transcription (Fig. 3). Whereas the effects of citrate and OAA appeared dose-dependent, the effect of malate was not (Fig. 3). Since exogenous citrate increased \textit{toxT} transcription, we hypothesized that citrate might accumulate in the TCA cycle mutants. We therefore measured total citrate levels and found that all three TCA cycle mutants contained significantly more total citrate than the parent strain, with the \textit{icd} mutant accumulating the largest amount (Fig. S4). Because the citrate levels in these mutants did not directly correlate with the degree of \textit{toxT} induction, these data do not support the notion that citrate accumulation is directly responsible. Since the intracellular acetyl-CoA concentration would be expected to decrease with excess OAA but increase with excess citrate, these data instead suggest the involvement of acetyl-CoA.

**Correlation between \textit{toxT} transcription and acetate excretion**

When TCA cycle activity is low (e.g. in TCA cycle or respiration mutants), bacteria utilize alternative pathways to generate ATP and to recycle CoASH from acetyl-CoA (Wolfe, 2005, 2010). One such pathway is the PTA-ACK pathway, which consists of phosphotransacetylase (PTA) and acetate kinase (ACK) (Brown \textit{et al.}, 1977; Rose \textit{et al.}, 1954; Wolfe, 2005, 2010). Metabolic activity through the PTA-ACK pathway results in the excretion of acetate into the external medium (Wolfe, 2005). Since the TCA cycle and \textit{nqr} mutants are predicted to have reduced TCA cycle function, we monitored acetate excretion during growth in LB and found that the \textit{nqr} mutant and all three TCA cycle
mutants (i.e. aspA, icd and sucA) accumulated higher concentrations of acetate in the external environment than did the parent strain (Table 4). In addition, growth of the parental strain in the presence of citrate, but not of 2-ketoglutarate, resulted in elevated acetate excretion (Table 4). Thus, decreased TCA cycle activity resulted in increased acetate excretion, which correlated with toxT transcription.

**Link between amino acid metabolism and acetate production**

The *V. cholerae* classical strain O395N1 does not produce CT when grown in minimal media. Supplementation with four amino acids (NRES), however, induces CT expression (Miller & Mekalanos, 1988) and involves increased toxT expression (Minato et al., 2011). We therefore investigated whether NRES supplementation also caused acetate excretion and found that it did. When grown in M9-glycerol medium (non-CT-inducing), the parent strain accumulated small amounts of extracellular acetate. In contrast, growth in M9-glycerol medium supplemented with NRES (CT-inducing) caused acetate to accumulate in the medium (Table 4).

The loss of the outer-membrane protein TolC allows *V. cholerae* to produce CT in minimal media even in the absence of NRES, again by increasing toxT transcription (Minato et al., 2011). Based on a previous publication that reported metabolic shut-down in an *Escherichia coli* tolC mutant (Dhamdhere & Zgurskaya, 2010), we predicted reduced TCA cycle activity in the *V. cholerae* tolC mutant compared with the parent strain. To test this prediction, we measured acetate excretion. When grown in M9-glycerol, the tolC mutant strain excreted larger amounts of acetate than its parent (Table 4). Addition of NRES to M9-glycerol further stimulated acetate production in the tolC mutant to levels that resembled those of the parent (Table 4). When grown in LB (a CT-inducing condition), the tolC mutant accumulated acetate at levels similar to that of its parent. Thus, again, toxT transcription correlated with acetate excretion. We therefore propose that some aspect of the acetate excretion process activates toxT transcription.

**Media acidification**

Acetate is excreted in its acidic form and thus causes acidification of the environment (Wolfe, 2005). External pH is known to modulate *V. cholerae* virulence factor expression (Gardel & Mekalanos, 1994; Skorupski & Taylor, 1997). Specifically, low pH enhances tcpPH expression (Kovacikova et al., 2010). Thus, a reduction in external pH caused by acetate excretion would be predicted to increase toxT transcription. Indeed, toxT transcription increased when the parent was grown in unbuffered LB with an initial pH of 6.0 compared with the parent grown in unbuffered LB with an initial pH of 6.5 (Fig. S5a). Since increased acetate excretion is expected to decrease the external pH, we measured the external pH after 6 h growth in unbuffered LB that had an initial pH of 6.5 and found that the parent strain moderately alkalinized its environment, while the TCA cycle mutants slightly acidified theirs (Fig. S5b). To determine if external pH contributes to the increased toxT transcription exhibited by the TCA cycle mutants, we grew cells in unbuffered LB with an initial pH of 6.5 or in LB buffered at pH 6.5 (LBB) and measured toxT transcription. Regardless of buffering status, the parent strain produced similar levels of toxT transcription. In contrast, toxT transcription from the mutants was lower in buffered LB (Fig. S5b). However, this difference

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<td>Ester</td>
<td>Methyl pyruvate</td>
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**Table 3. Carbon metabolism lost in the nqr mutant based on Biolog measurements**

**Fig. 3.** Effects of various TCA cycle intermediates on toxT-lacZ transcription in *V. cholerae* toxT-lacZ (TZ) strain. Overnight growth cultures of bacteria were washed and inoculated into LB (initial pH 6.5) in the presence or absence of TCA cycle intermediates and shaken at 30 °C. The starting OD_600 was adjusted to 0.05. TCA cycle intermediates were added at 10 or 25 mM and the pH was adjusted to pH 6.5 by HCl or NaOH when necessary. The β-galactosidase activities were measured after 6 h growth. The β-galactosidase activities are shown as a percentage relative to strain TZ grown in LB. All experiments were repeated more than three times. Error bars, SD. P values were calculated by one-way ANOVA followed by post hoc Bonferroni test; **P < 0.05.
was small, suggesting that medium acidification caused by acetate excretion exerts only a minor effect on toxT transcription. We conclude that acidification of the external environment increases toxT transcription, but that it cannot explain the elevated toxT transcription of the TCA cycle mutants.

**Acetyl-P does not affect toxT transcription**

Since the addition of sodium acetate, which did not change the external pH, did not alter toxT expression (Fig. 3), we conclude that the accumulation of excreted acetate per se is not responsible for increased toxT transcription. Instead, we propose that some other process associated with acetyl-CoA results in increased toxT transcription. Likely candidates are acetyl-CoA itself and the PTA-ACK pathway intermediate acetyl-P. Recent findings have revealed that these two central metabolites can affect gene regulation by protein acetylation and protein phosphorylation, respectively (Hu et al., 2010; Lima et al. 2012; Wolfe, 2005, 2010).

To test whether acetyl-P is responsible for increased toxT transcription, we constructed V. cholerae pta and ackA-1 mutants, which should be defective for the synthesis and degradation of acetyl-P, respectively. We measured toxT expression levels in these mutants and found that both showed more toxT transcription than did their parent (Fig. 4). Because cells that should not synthesize acetyl-P (pta) and those that should accumulate acetyl-P (ackA-1) both produced similarly elevated levels of toxT transcription, we conclude that acetyl-P does not increase toxT expression. Since both of the pta and the ackA-1 mutants should accumulate acetyl-CoA and its derivatives, we propose that toxT transcription is regulated by either acetyl-CoA or some close derivative.

**DISCUSSION**

V. cholerae, the causative agent of cholera, tightly regulates virulence factor production primarily by ToxT. Although many positive regulators of toxT transcription have been described, relatively little is known about factors that negatively regulate toxT transcription. A previous attempt to identify negative factors led to our discovery that the loss of NQR, a primary respiration-linked sodium pump, results in elevated toxT expression. To identify additional negative regulators of toxT transcription, we again isolated transposon mutants with increased toxT expression. As in our previous screen, we identified an nqr mutant, but this time we also isolated several additional mutants. Some of these mutant strains had transposon insertions in genes encoding TCA cycle enzymes (sucA and icd) or the TCA cycle-related enzyme (aspA). Because our previous screen also identified genes that encode a TCA cycle enzyme (fumA) and a TCA cycle-associated enzyme (glmS), we chose to investigate the link between TCA cycle and toxT transcription.

![Fig. 4. Effect of the pta, ackA-1 mutations on toxT expression. V. cholerae O395N1 and the pta and ackA-1 mutants were grown in LB (initial pH 6.5) at 30 °C. Total RNA was extracted at the early exponential growth phase (OD600 0.2) and analysed by qRT-PCR. Gene expression levels were normalized between the samples by using the 16S rRNA gene. All experiments were repeated three times. Error bars, SD. *P values were calculated by one-way ANOVA followed by post hoc Bonferroni test; **P<0.05.](image-url)
Genome sequencing revealed that *V. cholerae* does not encode an orthologue of Complex I, which often functions as the main respiratory NADH-dehydrogenase in bacteria. This observation led us to consider the possibility that NQR plays a vital role in the respiratory chain of this organism. Indeed, stimulation of respiration by the addition of l-lactate to the *nqr* mutant restored *toxT* transcription to parental levels, demonstrating that the defect in respiration caused by the loss of NQR is directly responsible for the upregulation of *toxT* transcription. How could a change in respiration activity affect *toxT* expression? If respiration were the primary signal for *toxT* transcription, then restoring respiration activity to the TCA cycle mutants should restore *toxT* expression. However, unlike the *nqr* mutant, the TCA cycle mutants did not restore *toxT* expression to parental levels in the presence of l-lactate. Since the *nqr* mutant showed a defect in the TCA cycle activity, we propose that the lack of NQR influences *toxT* transcription by affecting TCA cycle activity.

As expected from other organisms (Lakshmi & Helling, 1976), the *V. cholerae* TCA cycle mutants accumulated citrate; however, intracellular citrate concentrations in these mutants did not directly correlate with *toxT* transcription: the *icd* mutant accumulated the most citrate, but the *sucA* mutant strain had the highest *toxT* transcription. Thus, it is unlikely that citrate is the direct signal to increase *toxT* transcription. We therefore sought an alternative explanation. We were particularly intrigued by the observation that citrate added exogenously to the parent strongly induced transcription from the *toxT* promoter, whereas exogenous OAA strongly inhibited it. Since citrate is formed by the condensation of OAA with acetyl-CoA (Fig. 1a), the intracellular acetyl-CoA concentration would be expected to decrease with excess OAA but increase with excess citrate, regardless of whether the source was exogenous or endogenous. Thus, an increase in the acetyl-CoA concentration also would be anticipated from disruption of the TCA cycle or from a slowing of carbon flux through the TCA cycle by disruption or inhibition of the electron transport chain. We ascertained the likelihood of this scenario by monitoring acetate excretion. To recycle CoA, bacteria often excrete acetate into the external environment (Wolfe, 2005), where it can be easily measured. Like many other organisms, in *V. cholerae* disruption or inhibition of either the TCA cycle or respiration resulted in accumulation of extracellular acetate. Acetate also accumulated during growth in the presence of citrate, a TCA cycle intermediate that induced *toxT* transcription. Acetate did not accumulate, however, in the presence of TCA cycle intermediates that did not induce *toxT* transcription (Table 4). Thus, acetate excretion and *toxT* transcription were highly correlated. Since exogenous acetate did not induce *toxT* transcription, acetate itself is unlikely to be the signal. Instead, the signal that activates *toxT* transcription is more likely to be a metabolite associated with the process of acetate excretion.

In contrast to citrate, OAA and malate inhibited *toxT* transcription. Malate metabolizes to OAA (Fig. 1a) and OAA converts to phosphoenolpyruvate (Debaere et al., 2004). We confirmed that the *V. cholerae* genome has this enzyme, the phosphoenolpyruvate carboxylase (VC2738).

A high phosphoenolpyruvate/pyruvate ratio results in phosphorylation of EIAlag, which activates the cAMP-producing enzyme adenylate cyclase (Deutscher, 2008; Görke & Stülke, 2008). Since cAMP-CRP is known to inhibit tcpPH transcription (Kovacikova & Skorupski, 2001), and because TcpPH controls *toxT* transcription, we tested whether OAA inhibited tcpPH transcription and/or increased cAMP levels. Surprisingly, OAA decreased rather than increased cAMP concentrations similar to the glucose and pyruvate cAMP concentrations (Fig. S6). Although further experiments are needed to fully understand the effects of OAA, we conclude that oxalacetate does not inhibit *toxT* transcription via cAMP.

The observed correlation between acetate excretion and *toxT* transcription was supported by the behaviour of the *V. cholerae tolC* mutant. TolC is a major outer-membrane protein frequently involved in bacterial multidrug resistance and survival of many Gram-negative pathogens during infection (Koronaś et al., 2004). TolC forms a channel that interacts with certain inner membrane transporters and directly mediates the efflux of a broad range of substrates from the cytosol or periplasm to the extracellular environment (Postle & Vakharia, 2000). Several recent studies have suggested that disruption of *tolC* affects bacterial metabolism (Dhamdhere & Zgurskaya, 2010; Santos et al., 2010), possibly via the accumulation of metabolites (Chubiz & Rao, 2010; Rosner & Martin, 2009) or by inducing envelope stress responses (Santos et al., 2010). We recently reported that a *V.cholerae tolC* mutant strain showed increased *toxT* transcription when grown in M9-glycerol, but not when that medium was supplemented with four amino acids that induce CT production (M9-glycerol-NRES) or during growth in a rich medium, e.g. LB (Minato et al., 2011). Interestingly, the *tolC* mutant strain only accumulated extracellular acetate when grown in M9-glycerol, further supporting the positive correlation between *toxT* expression and acetate excretion in *V. cholerae*. The mechanism underlying the observed metabolic shutdown in the *E. coli tolC* mutant is not known, but probably involves reduced respiration activity (Dhamdhere & Zgurskaya, 2010; Zgurskaya et al., 2011). Thus, in *V. cholerae*, it is feasible that the loss of TolC negatively affects respiration, perhaps via NQR activity. The loss of respiration would in turn diminish TCA cycle activity. It is intriguing that the effects of TolC on metabolism, in both *E. coli* and *V. cholerae*, are only observed with cells grown in minimal media and not in LB.

*E. coli* uses two independent pathways to excrete acetate, the PTA-ACK pathway and pyruvate oxidase (POXB). It is likely that *V. cholerae* only uses the PTA-ACK pathway, as its genome lacks a *poxB* homologue. In contrast to *E. coli*, which encodes a single *ackA* gene, *V. cholerae* encodes two
(ackA-1 and ackA-2). The purpose for having two ackA genes remains unknown, but their existence suggests the importance of this pathway in *V. cholerae*. In *E. coli* and other organisms, the PTA-ACK pathway converts acetyl-CoA into the excreted byproduct acetate and, as a result, recycles CoA and produces ATP (Brown *et al.*, 1977; Rose *et al.*, 1954; Wolfe, 2005, 2010). The PTA-ACK pathway also regulates the intracellular levels of two central metabolites, acetyl-P and acetyl-CoA. Acetyl-P is known to work as an intracellular signal by directly phosphorylating the response regulators of certain two-component regulatory systems (Wolfe, 2005, 2010). Acetyl-CoA can modify protein function by working as the acetyl donor for regulatory systems (Wolfe, 2005, 2010). Acetyl-CoA can also regulate the intracellular levels of two central metabolites, acetyl-CoA (acCoA) activate gene transcription either directly or indirectly. A recent study also showed the indirect repression of TCA cycle gene expression by ToxT (Davies *et al.*, 2012), suggesting the existence of a feedback loop between central metabolism and ToxT. The structure of TolC was adopted from Koronakis *et al.* (2004). AcP, acetyl phosphate.

Virulence gene expression in *V. cholerae* is tightly regulated and this bacterium expresses toxT only under limited conditions, including at the early stage of infection in the small intestine. At the early stage of infection *V. cholerae* adopts its metabolism based on the available nutrients in the small intestine and expresses virulence genes including toxT. The described feedback loop between central metabolism and ToxT suggests that *V. cholerae* might sense changes of its metabolic condition in the small intestine and use these changes as a signal to induce toxT. Future studies are necessary to better understand the detailed molecular mechanism(s) of this intriguing link.

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