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The LysR-type regulator LeuO regulates the acid tolerance response in *Vibrio cholerae*

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Vibrio cholerae is a neutrophilic enteric pathogen that is extremely sensitive to acid. As *V. cholerae* passages through the host gastrointestinal tract it is exposed to a variety of environmental stresses including low pH and volatile fatty acids. Exposure to acidic environments induces expression of the *V. cholerae* acid tolerance response. A key component of the acid tolerance response is the *cad* system, which is encoded by *cadC* and the *cadBA* operon. CadB is a lysine/cadaverine antiporter and CadA is a lysine decarboxylase and these function together to counter low intracellular and extracellular pH. CadC is a membrane-associated transcription factor that activates *cadBA* expression in response to acidic conditions. Herein we investigated the role of the LysR-type transcriptional regulator LeuO in the *V. cholerae* acid tolerance response. Transcriptional reporter assays revealed that *leuO* expression repressed *cadC* transcription, indicating that LeuO was a *cadC* repressor. Consistent with this, *leuO* expression was inversely linked to lysine decarboxylase production and *leuO* overexpression resulted in increased sensitivity to organic acids. Overexpression of *leuO* in a *cadA* mutant potentiated killing by organic acids, suggesting that the function of *leuO* in the acid tolerance response extended beyond its regulation of the *cad* system. Collectively, these studies have identified a new physiological role for LeuO in *V. cholerae* acid tolerance.

INTRODUCTION

Vibrio cholerae is a neutrophilic bacterium that is extremely sensitive to even mild acidic conditions (Wachsmuth *et al.*, 1994). *V. cholerae* naturally persists in aquatic reservoirs with a neutral pH, variable nutrient availability and ambient temperatures. It is also an enteric pathogen that encounters a variety of environmental stresses while passing through the human gastrointestinal tract. Following ingestion *V. cholerae* encounters a dramatic change in pH from near neutral to ≤ 2 in the human stomach. Passage of *V. cholerae* from the stomach into the small intestine further exposes the bacterium to an environment that contains a combination of inorganic acids and organic acids (Audia *et al.*, 2001). Exposure of *V. cholerae* to acidic conditions results in the induction of an acid tolerance response. The acid tolerance response can be divided into two distinct branches: an inorganic acid tolerance response and an organic acid tolerance response (Merrell & Camilli, 1999).

The *V. cholerae* acid tolerance response encompasses diverse genes that function together to mitigate the effects of acid stress. This includes alterations in the outer membrane, the expression of genes that function in the regulation of K^+ and Na^+ homeostasis, and biofilm production (Merrell *et al.*, 2001, 2002; Zhu & Mekalanos, 2003). The acid tolerance response is probably an important factor for *V. cholerae* pathogenesis. For example, biofilm production has been shown to enhance acid tolerance, which contributes to pathogenesis by providing protection from acid stress during passage through the gastric acid barrier of the stomach (Tamayo *et al.*, 2010; Zhu & Mekalanos, 2003). In addition, pre-activation of the *V. cholerae* acid tolerance response has been shown to impart a competitive advantage for colonization of the infant mouse intestine relative to unadapted cells (Merrell & Camilli, 1999). Taken together these results suggest that the acid tolerance response may play a crucial role in both the initial infection with *V. cholerae* and the subsequent development of hyper-infectivity that has been observed in human and animal-shed *V. cholerae* (Alam *et al.*, 2005; Angelichio *et al.*, 2004; Merrell *et al.*, 2002).

An important subset of genes that are induced in both the inorganic and the organic acid tolerance response of *V. cholerae* is the *cad* system. The contribution of the *cad* system to acid resistance is conserved among a number of enteric bacteria (Bearson *et al.*, 1997). The *cad* system includes three genes that are involved in maintaining the intracellular pH while also neutralizing the external pH. CadC is a ToxR-family transcriptional regulator that positively regulates expression of the *cadBA* operon (Merrell & Camilli, 2000). CadA is a lysine decarboxylase that converts

Abbreviations: Cb, carbenicillin; Cml, chloramphenicol; EMSA, electrophoretic mobility shift assay; Km, kanamycin; MBP, maltose binding protein; Sm, streptomycin.

lysine to cadaverine while consuming a proton and producing carbon dioxide. CadB is a lysine–cadaverine antiporter that is localized to the cytoplasmic membrane. Tight regulation of the *cad* system are necessary as alterations in the intracellular pH are detrimental to the cell (Booth, 1985).

In *V. cholerae* it has been shown that AphB, a cytoplasmic DNA-binding protein, positively regulates the *cad* system in response to low pH or low oxygen by directly binding to the *cadC* promoter (Kovacikova *et al.*, 2010). Upregulation of the *cad* system contributes to the maintenance of the intracellular pH. Expression of the *cad* system returns to a low constitutive level upon neutralization of the external environmental. The molecular mechanisms by which *V. cholerae* downregulates the *cad* system are not known. In *Escherichia coli*, the *cad* system is repressed in two ways: the first is through feedback inhibition by cadaverine, and the second is through the transcriptional regulator LeuO which functions by repressing *cadC* expression (Shi & Bennett, 1995). In *V. cholerae* cadaverine does not repress the *cad* system (Merrell & Camilli, 1999), but it is unknown if LeuO influences *cadC* expression.

LeuO is a LysR-type transcriptional regulator that shares 50 % identity and 75 % similarity with *E. coli* LeuO. Our laboratory has shown that *V. cholerae leuO* is positively regulated by the virulence regulator ToxR, often in response to environmental signals (Ante *et al.*, 2015; Bina *et al.*, 2013). Expression of *leuO* is induced by the endogenously produced cyclic dipeptide cyclo(Phe–Pro). In response to cyclo(Phe–Pro) LeuO has was shown to repress the production of essential virulence factors by downregulating the ToxR regulon. Expression of *leuO* is also induced by bile salts and contributes to *V. cholerae* bile resistance (Ante *et al.*, 2015). Preliminary transcriptomic profiling experiments performed in our laboratory indicated that the *cad* system was differentially regulated in a *V. cholerae leuO* mutant, suggesting that LeuO may regulate the *cad* system. In the present study, we expanded upon this observation and tested the hypothesis that LeuO functioned as a regulator of the *V. cholerae cad* system. The results showed that LeuO was a repressor of *cadC* expression and directly bound to the *cadC* promoter. LeuO was also shown to regulate the production of CadA (lysine decarboxylase) and to contribute to *V. cholerae* survival after exposure to organic acid. LeuO overproduction in a *cadA* mutant also resulted in increased acid sensitivity, suggesting that the contribution of LeuO to acid tolerance extends beyond the *cad* system. Taken together, our studies have identified a new physiological role for LeuO and indicate that LeuO is a component of the *V. cholerae* acid tolerance response.

METHODS

Strains, media and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* strains EC100λ*pir* and SM10λ*pir* were used for cloning and plasmid mobilization, respectively. *E. coli* strain BW25113 was used for the two-plasmid β-galactosidase reporter assays. *E. coli* strain ER2566 was used for purification of LeuO-MBP and maltose binding protein (MBP). The *V. cholerae* strains used in this study were seventh pandemic O1 El Tor clinical isolates. *V. cholerae* strain JB58 (N16961Δ*lacZ* Sm^R) or strain XBV144 (C6706Δ*lacZ* Sm^R) were used as the wild-type (WT) control strains. Bacterial strains were grown at 37 °C in Luria–Bertani (LB) broth or on LB agar. AKI growth conditions which are used to induce the ToxR regulon have been described previously (Iwanaga *et al.*, 1986). An organic acid cocktail (1 ×) consisting of 87 mM acetic acid, 25 mM butyric acid and 37 mM propionic acid was used for the organic acid challenge assays. Acid adaptation media contained 0.1 × organic acid cocktail in LB broth at pH 5.7. Bacterial stocks were maintained at (80 °C in LB broth containing 25 % glycerol. Growth media were supplemented with carbenicillin (Cb) and streptomycin (Sm) at 100 μg ml^{-1}, kanamycin (Km) at 50 μg ml^{-1}, or chloramphenicol (Cml) at 1 μg ml^{-1} for *V. cholerae* or at 25 μg ml^{-1} for *E. coli* as required. Arabinose was added to growth media at the indicated concentrations to induce expression from the arabinose-regulated promoter in pBAD18, pBAD18Km and pBAD33.

Plasmid and mutant construction. Plasmids and oligonucleotides used in this study are listed in Table 1. The *cadC-lacZ* reporter plasmid pXB239 was constructed as follows. Briefly, the P_{cadC}-F/P_{cadC}-R PCR primer pair was used to amplify the *cadC* promoter region from the *V. cholerae* N16961 genome. The resulting PCR amplicon was then digested with *Xho*I and *Xba*I restriction endonucleases before being ligated into similarly digested pTL61T to generate pXB239. The *aphB* deletion plasmid, pWM91: :Δ*aphB*, was constructed by PCR stitching as previously described (Bina & Mekalanos, 2001; Imai *et al.*, 1991). Briefly, the *aphB*-F1/*aphB*-R2/ and *aphB*-F2/*aphB*-R1 PCR primer pairs were used to amplify ∼1 kb regions flanking *aphB*. The resulting PCR amplicons were used as the template for a second round of PCR using the *aphB*-F1 and *aphB*-R1 PCR primers. The resulting ∼2 kb amplicon was digested with *Bam*HI and *Sac*I restriction endonucleases before being ligated into similarly digested pWM91 to generate pWM91: :Δ*aphB*. The *leuO* expression plasmid pVA126 (pBAD33: :*leuO*) was constructed by removing the *leuO* fragment from pXB298 using *Xba*I and *Ssp*I restriction enzymes. The resulting ∼1 kb *leuO* fragment was collected and ligated into pBAD33 digested with *Xba*I and *Sma*I. The LeuO-MBP purification plasmid pVA175 (pMAL-c2: :*leuO*) was constructed by amplifying the *leuO* gene from N16961 using the LeuO-F/LeuO-R PCR primers. The resulting PCR amplicon was then digested with *Xba*I and *Sma*I restriction endonucleases and ligated to pMAL-c2 which had been restricted with *Xba*I and XmnI endonucleases to generate pVA175. This ligation resulted in a translational fusion of *leuO* to the C terminus of *malE* (MBP). The DNA sequence of the protein purification construct was subsequently verified by sequencing.

Deletion of *V. cholerae aphB* (VC1049) was performed by allelic exchange as previously described (Bina *et al.*, 2006). Briefly, *E. coli* SM10λ*pir* was used to conjugate plasmid pWM91: :Δ*aphB* into *V. cholerae* JB58 and co-integrants were selected for Sm/Cb resistance. Several Sm/Cb resistant colonies were cultured on LB agar (without NaCl) containing 5 % sucrose to select for resolution of the integrated plasmid. Sucrose-resistant and Cb-sensitive colonies were then screened by PCR using the *aphB*-F1/*aphB*-R1 PCR primers to confirm *aphB* deletion. Deletion of *lacZ* (VC2338) in JB804 was accomplished in an identical manner using pDLT to generate strain XBV144. Deletion of *leuO* (VC2485) in XBV144 was accomplished as described by Moorthy & Watnick (2005) to generate strain VA412. The C6706 transposon insertion mutants were generously supplied by Dr John Mekalanos (Harvard Medical School).

β-Galactosidase assays. *V. cholerae* strains harbouring the *cadC-lacZ* reporter plasmid pXB239 were grown under AKI conditions and culture aliquots were taken in triplicate at various times to quantify β-galactosidase activity as described by Miller (1972). The effect of

Table 1. Strains, plasmids and oligonucleotides used in this study

Strain/plasmid/ oligonuncleotide	Characteristic/sequence (5′ to 3′)	Source
E. coli		
EC100λpir	*supE44* Δ*lacU169* (*ϕ*80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (*λpirR6K*)	Epicenter
SM10λpir	*thi-1 thr leu tonA lacY supE recA*: :RP4-2-Tc: :Mu *kmR* (*λpirR6K*)	Lab collection
BW25113	F^- Δ(*araD-araB*)*567 lacZ4787Δ*: :*rrnB-3 LAM*$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Baba *et al.* (2006)
ER2566	fhuA2 lacZ: :T7 gene1 [lon] ompT gal sulA11 R(mcr-73: :miniTn10–TetS)2 [dcm] R(zgb-210: :Tn10–TetS) endA1 Δ(mcrCmrr)114: :IS10	New England BioLabs
V. cholerae		
JB58	*V. cholerae* O1 El Tor strain N16961 Δ*lacZ*, Sm^R	Lab collection
XBV222	JB58Δ*leuO*	Bina *et al.* (2013)
XBV148	JB58Δ*aphB*	This study
JB804	*V. cholerae* O1 El Tor strain C6706, Sm^R	Thelin & Taylor (1996)
XBV144	JB804 Δ*lacZ*	This study
VA412	XBV144 Δ*leuO*	This study
EC20568	C6706 Tn: :VC2485 (*leuO*)	Cameron *et al.* (2008)
EC17926	C6706 Tn: :VC0278 (*cadA*)	Cameron *et al.* (2008)
Plasmid		
pTL61T	*lacZ* transcriptional reporter plasmid, Cb^R	Linn & St Pierre (1990)
pXB239	pTL61T containing the *cadC* promoter region	This study
pXB203	pTL61T containing the *aphB* promoter region	Bina & Bina (2010)
pBAD18	Arabinose-regulated expression plasmid, Cb^R	Guzman *et al.* (1995)
pVA94	pBAD18 expressing *leuO*	Ante *et al.* (2015)
pBAD18Km	Arabinose-regulated expression plasmid, Km^R	Guzman *et al.* (1995)
pXB298	pBAD18Km expressing *leuO*	Bina *et al.* (2013)
pBAD33	Arabinose-regulated expression plasmid, Cml^R	Guzman *et al.* (1995)
pVA126	pBAD33 expressing *leuO*	This study
pWM91	Suicide plasmid vector used for allelic exchange	Metcalf *et al.* (1996)
pDLT	pWM91 containing a fragment of *lacZ* harbouring an internal deletion	Fullner & Mekalanos (1999)
pWM91Δ*leuO*	pWM91 containing a fragment of *leuO* harbouring an internal deletion	Moorthy & Watnick (2005)
pWM91Δ*aphB*	pWM91 containing a fragment of *aphB* harbouring an internal deletion	This study
pMAL-c2	IPTG-inducible expression vector for fusion of proteins to MBP and cytoplasmic expression, Cb^R	New England BioLabs
pVA175	pMAL-c2 expressing *leuO*	This study
Oligonucleotide		
P_{cadC}-F	TTCTCGAGTCGGGCTATCGACTGTACGATG	
P_{cadC}-R	GTTCTAGACACCACACACCGATGAAGAGCGAAATTATAA	
aphB-F1	TTGGATCCGCCCCACGATGGCTCGCG	
aphB-F2	CGACTGGTTGTCACAAAGATCACCAGCCGGAAAAAGTGCGCCTG	
aphB-R1	GCGAGCTCCAGTGGGCGATATGGGCG	
aphB-R2	GGTGATCTTTGTGACAACCAGTCGAAAGAGGTTTAGGTCATCTAG	
LeuO-F	CCCCCGGGTTAGATAAAAAAGACGCAATGAGTGCC	
LeuO-R	CCTCTAGATAGAAACGTAGAATGAACAAAGGATC	
cadC-EMSA-F1	GCGGGAGTCGGCAGCGGATGGTTAAACAACCTAAGTT	
cadC-EMSA-R1	GCGGGAGTCGGCAGCGGAGCGAAATTATAAGTGCAC	
cadC-EMSA-F2	GCGGGAGTCGGCAGCGAATTTCGCTCTTCATCGGTG	
cadC-EMSA-R2	GCGGGAGTCGGCAGCGCATAGAATAGCTCTTTGTATC	
5′BIO	GCGGGAGTCGGCAGCG	

LeuO on *cadC* expression in *V. cholerae* consisted of growing strain JB58 containing the *cadC-lacZ* plasmid pXB239 and pBAD33-*leuO* plasmid pVA126 under AKI conditions in the presence or absence of 0.02 % arabinose. Culture aliquots were collected in triplicate after 5 h to quantify *cadC-lacZ* expression. The effect of LeuO on *aphB* expression in *V. cholerae* was determined by growing strain JB58 containing the *aphB-lacZ* plasmid pXB203 and the pBAD33-*leuO* plasmid pVA126 under AKI conditions in the presence or absence of 0.02 % arabinose. Culture aliquots were collected in triplicate after 5 h to quantify *aphB-lacZ* expression. LeuO repression of *cadC* expression in *E. coli* was accomplished as follows. Overnight cultures of *E. coli* strain BW25113 containing the

cadC-lacZ plasmid pXB239 and the pBAD33-*leuO* plasmid pVA126 were diluted 1 : 100 in LB broth with or without 0.02 % arabinose. The cultures were incubated at 37 °C with shaking and aliquots were collected after 5 h to quantify *cadC-lacZ* expression using β-galactosidase activity. Expression from the reporter plasmids was calculated and displayed as Miller units (MU).

Purification of LeuO-MBP and MBP. Proteins for the gel shift assays were purified as follows. *E. coli* ER2566 carrying plasmid pMAL-c2 or the pMAL-c2: :*leuO* plasmid pVA175 were grown in LB broth overnight at 37 °C with aeration. The cultures were then diluted 100-fold into LB broth containing Cb and incubated at 37 °C with shaking to an OD_{600} of ~0.5 when 0.3 mM IPTG was added and the cultures were incubated for an additional 2 h. The cells were then harvested by centrifugation and the pellet was resuspended in column buffer (20 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA) plus 1 mM PMSF. The cells were then lysed with an M-11P Microfluidizer according to the manufacturer's instructions (Microfluidics). The resulting cell lysates were cleared of particulate matter by centrifugation at 15 000 ***g*** for 20 min at 4 °C. The clarified supernatant (i.e. LeuO-MBP or MBP) was then diluted 1 : 6 with column buffer and loaded onto a 0.8 × 7.0 cm chromatography column containing 1 ml amylose resin (New England Biolabs). The column was equilibrated with 12 ml column buffer before the clarified supernatant was run through. Bound proteins were eluted from the resin using elution buffer (20 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose). The purity of the eluted fusion proteins was analysed by SDS-PAGE with Coomassie brilliant blue R-250 staining. Protein concentrations were determined using the Coomassie Plus (Bradford) Assay kit according to the manufacturer's instructions (Thermo Scientific).

Electrophoretic mobility shift assays (EMSAs). The DNA fragments designated *cadC1* (the nucleotide sequence between (79 and +1 relative to the *cadC* transcriptional start site) and *cadC2* (the nucleotide sequence between (8 and +77 relative to the *cadC* transcriptional start site) were PCR amplified from the N16961 genome using the *cadC*-EMSA-F1/*cadC*-EMSA-R1 and *cadC*-EMSA-F2/*cadC*-EMSA-R2 oligonucleotide primers, respectively. The PCR fragments were then gel purified and 100 ng was used as a template for a second PCR using the biotinylated 5′BIO oligonucleotide primer purchased from Integrated DNA Technologies. The resulting DNA fragments were end-labelled with biotin. The biotin-labelled probes (1.5 nM) were incubated with purified LeuO-MBP or MBP in amounts ranging from 0 to 30 µM in binding buffer containing 10 mM Tris (pH 7.4), 150 mM KCl, 0.1 mM DTT, 0.1 mM EDTA (pH 8.0) and 200 µg sheared salmon sperm ml^{-1}. The binding reactions were incubated at room temperature for 20 min before being subjected to electrophoresis on a non-denaturing 5 % TBE-PAGE in 0.25 × Tris/borate/EDTA (TBE) buffer at 200 V for 45 min. The DNA in the gel was transferred to a nylon membrane in 0.5 × TBE buffer at 380 mA for 1 h. The nylon membrane was then UV cross-linked at 120 mJ using a Stratalinker 1800 crosslinker (Stratagene). Biotin-labelled DNA was detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and visualized using a Fluorchem E Digital Darkroom imager (Protein Simple).

Lysine decarboxylase assays. Strains were grown in AKI media static at 37 °C until the cultures reached an OD_{600} of ~0.1 (4 h). Strains containing pBAD18Km-*leuO* were grown in AKI media in the presence or absence of 0.02 % arabinose. After 4 h, culture aliquots were collected and processed for the quantification of lysine decarboxylase activity as described by Lemonnier & Lane (1998) with slight modification. Briefly, the cells were collected by centrifugation and normalized to an OD_{600} of 1. The cell pellet was then washed with 1 ml of cold (4 °C) Buffer A (1 M NaCl, 0.05 M potassium Pi buffer pH 6.5) before being centrifuged and resuspended in 200 µl cold (4 °C) Buffer B (20 mM potassium Pi buffer pH 5.8). $CHCl_3$ (20 µl) was then added to each sample followed by vortexing for 15 s to disrupt the cell membrane. Quantification of lysine decarboxylase activity was then carried out in triplicate by combining 10 µl of the cell lysate with 110 µl prewarmed Buffer C (5 mM lysine, 0.1 mM pyridoxal 5′-phosphate, 16 mM potassium Pi biffer pH 5.8); a parallel mixture without lysine was also prepared to control the level of endogenous polyamines, as these react in the assay as cadaverine. The enzymic reaction was incubated at 37 °C for 15 min before adding 120 µl of Stop Solution (1 M Na_2CO_3) and placing on ice. Lysine and cadaverine were then derivatized by adding 120 µl of 10 mM 2,4,6-trinitrobenzene sulphonate to the mixture and incubating at 40 °C for 4 min. After incubation, samples were chilled on ice. For phase separation, 1 ml toluene was added and thoroughly vortexed for 20 s; *N*,*N*′-bistrinitrophenylcadaverine (TNP-cadaverine) is soluble in toluene and *N*,*N*′-bistrinitrophenyllysine (TNP-lysine) is toluene-insoluble. Samples were then centrifuged at 2000 r.p.m. for 5 min to allow the phases to separate. The concentration of TNP-cadaverine was measured by removing the upper aqueous phase and reading the A_{340} in quartz cuvettes with a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific). Lysine decarboxylase activity was determined as the difference in A_{340} between the sample incubated with lysine and that incubated without. Specific activity was calculated using the equation $(A_{340}/(\text{time} \times OD_{600})) \times 1000$, and is a measure of lysine converted to cadaverine per time (min) per unit cell density.

Organic acid challenge assays. The acid challenge assays were facilitated by obtaining mutant strains from an ordered *V. cholerae* C6706 transposon library (Cameron *et al.*, 2008). C6706 is highly conserved with N16961 (Reimer *et al.*, 2011) and we have not observed differences in the LeuO regulon or acid tolerance between the two strains. Overnight cultures of each test strain were diluted 1 : 10 000 into 10 ml of AKI broth, in the presence or absence of 0.2 % arabinose, in a test tube and incubated statically at 37 °C for 4 h before the cultures were normalized to an OD_{600} of 0.1 before use. The analysis of unadapted cells was performed as follows. Aliquots (100 µl) of the respective normalized cultures were distributed into the wells of a 96-well microtitre plate that contained a linear range of the organic acid cocktail in LB broth. For the acid adaptation analysis, the cells were resuspended in organic acid adaptation media at pH 5.7 and incubated for 1 h at 37 °C. The cells were then collected by centrifugation and resuspended in fresh LB broth from which 100 µl aliquots were distributed into the wells of a 96-well microtitre plate that contained a linear range of the organic acid cocktail in LB broth. The inoculated microtitre plates were then incubated at 37 °C and ~10 µl aliquots from each well were replica-plated at the indicated time points onto LB agar plates using a 96-pin replicator. The agar plates were then incubated at 37 °C for 18 h after which they were imaged using a Fluorchem E Digital Darkroom imager (Protein Simple).

RESULTS

LeuO regulates *cadC* expression

Our preliminary transcriptome studies suggested that LeuO may regulate the *V. cholerae cad* system. The *cad* system is regulated by AphB, which functions as an activator of *cadC*. Once CadC is produced, it directly activates expression of the *cadBA* operon. Therefore, we tested if LeuO affected the expression of either of these two regulatory genes in *V. cholerae*. We first investigated *cadC* transcription by quantifying *cadC* expression levels in WT strain JB58 and an isogenic Δ*leuO* strain XBV222 using the *cadC-lacZ* transcriptional reporter pXB239. The test strains were cultured under AKI virulence-gene-inducing conditions and *cadC-lacZ* expression was quantified using β-galactosidase assays. The

results showed that *cadC* expression peaked at 5 h and declined thereafter (Fig. 1a). Growth of *V. cholerae* under AKI conditions resulted in acidification of the culture media during static growth (i.e. the first 4 h). The reduction in pH appeared to correlate with the generation of organic acid byproducts from fermentation metabolism. After the initial 4 h of static growth, the cultures shifted to aerobic growth, which resulted in alkalinization of the media (data not shown). Thus, *cadC* expression appeared to correlate with the changes in the pH of the growth medium during growth under AKI conditions. Expression of *cadC* in the Δ*leuO* mutant mirrored expression in the WT strain except that the expression level was elevated in the Δ*leuO* mutant compared with WT. The elevated *cadC* expression observed in the absence of *leuO* supported the hypothesis that LeuO was a regulator of the *cad* system and was acting as a repressor of *cadC*.

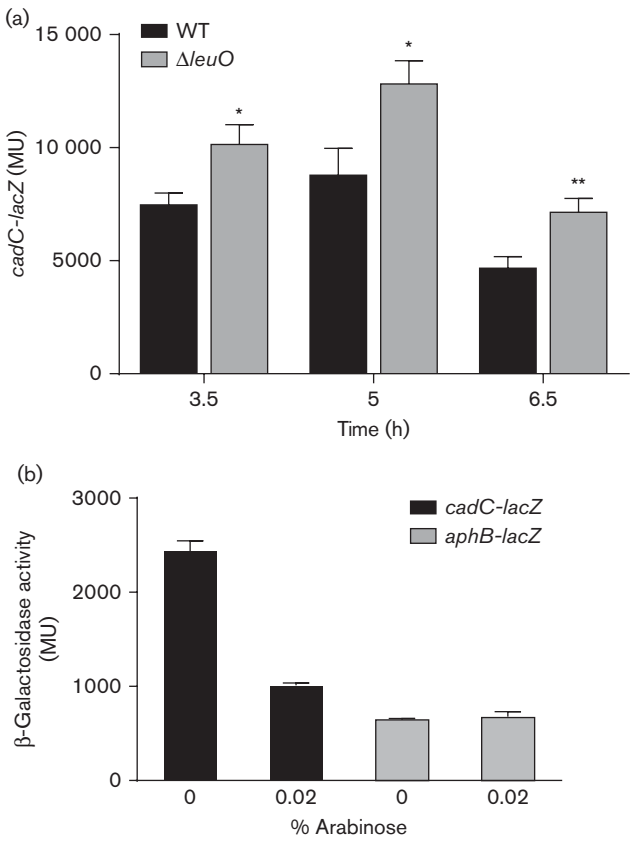

Fig. 1. Effect of LeuO on *cadC* and *aphB* expression. (a) WT *V. cholerae* strain JB58 and Δ*leuO* strain XBV222 carrying the *cadC-lacZ* reporter plasmid pXB239 were grown under AKI conditions. Culture aliquots were taken at the indicated times and assayed for β-galactosidase activity as described in Methods. The presented data are the mean ± SD of three independent biological replicates. (b) WT strain JB58 bearing pBAD33-*leuO* plasmid pVA126 and either the *cadC-lacZ* transcriptional reporter pXB239 (black bars) or the *aphB-lacZ* transcriptional reporter pXB203 (grey bars) were grown under AKI conditions in the presence or absence of 0.02 % arabinose. Expression of the indicated reporter gene was assessed at 5 h by measuring β-galactosidase production. The presented data are the mean ± SD of three technical replicates and are representative of two independent experiments. Statistical significance was determined using a *t*-test comparing the sample mean with the WT control mean; **P*<0.05, ***P*<0.01.

If LeuO was a *cadC* repressor, then we hypothesized that *leuO* overexpression would repress *cadC* transcription in *V. cholerae*. To test this hypothesis, *V. cholerae* WT strain JB58 was transformed with the expression plasmid pVA126 (pBAD33-*leuO*) and the *cadC-lacZ* reporter plasmid pXB239. The resulting strain was cultured under AKI growth conditions in AKI broth alone or AKI broth containing arabinose to induce *leuO* expression. Expression of *cadC* was then quantified at 5 h post-inoculation. The results showed that the induction of *leuO* expression by the addition of 0.02 % arabinose resulted in an ~60 % reduction in *cadC* expression (Fig. 1b). This further supported the conclusion that LeuO was a *cadC* repressor in *V. cholerae*.

There are several potential mechanisms for LeuO to affect *cadC* expression. LeuO could act directly at *cadC* by binding to its promoter and inhibiting transcription. Alternatively, LeuO could repress *cadC* expression indirectly by repressing the expression of its upstream activator *aphB*. To differentiate between these two possibilities, we examined the effect of *leuO* overexpression on *aphB* transcription. We therefore repeated the above experiments using WT strain JB58 carrying pVA126 (pBAD33-*leuO*) and an *aphB-lacZ* transcriptional reporter plasmid (pXB203). The results showed that induction of *leuO* expression by the addition of 0.02 % arabinose did not alter *aphB* expression (Fig. 1b). This indicated that *aphB* is not regulated by LeuO and that the effects of LeuO on *cadC* transcription were probably independent of *aphB*.

LeuO represses *cadC* expression by directly binding to its promoter

The above results suggested that LeuO reduced *cadC* expression independently of *aphB*, but did not discriminate between LeuO affecting *cadC* expression directly or indirectly. To address this we examined whether *leuO* expression affected *cadC-lacZ* expression in a heterologous host. We introduced both the pBAD33-*leuO* expression plasmid pVA126 and the *cadC-lacZ* reporter plasmid pXB239 into *E. coli* and quantified *cadC-lacZ* expression following growth in LB broth for 5 h in the presence and absence of arabinose. The results showed an ~65 % decrease in *cadC-lacZ* expression in LB broth containing 0.02 % arabinose (Fig. 2a). This result indicated that genes unique to *V. cholerae* were not required for LeuO repression of *cadC* and suggested that LeuO may act directly at the *cadC* promoter. Note that these results do not exclude the possibility that LeuO could be acting indirectly through an intermediate gene present in *E. coli*.

To confirm further that LeuO was acting directly at the *cadC* promoter we performed gel shift assays. For these experiments, we purified LeuO as a translational fusion

to MBP and generated two biotin-labelled DNA probes from the *cadC* locus. The first DNA probe, named *cadC1*, contained the *cadC* promoter region from (79 to +1 relative to the *cadC* transcriptional start site as defined by Merrell & Camilli (2000) (Fig. 2b). This region of the *cadC* promoter also included the AphB binding site which was mapped to nucleotides (71 to 55 (Kovacikova *et al.*, 2010). The second DNA probe, called *cadC2*, was used as a negative control and contained nucleotides (8 to +77 relative to the *cadC* transcriptional start site. The results of the gel shift assays showed that LeuO-MBP bound to the *cadC1* DNA probe, but not to the *cadC2* DNA probe (Fig. 2b). Incubation of the *cadC1* DNA probe with MBP alone did not result in a shift, confirming that LeuO was responsible for the shift of the *cadC1* probe by the LeuO-MBP fusion protein. Taken together these results confirmed that LeuO directly binds to a region in the *cadC* promoter that is present in the *cadC1* probe.

Lysine decarboxylase activity is influenced by LeuO. CadC positively regulates the expression of *cadBA*, and thus the production of lysine decarboxylase (CadA), in response to low environmental pH (Merrell & Camilli, 2000). Based on this, we hypothesized that if LeuO repressed *cadC*, then deletion of *leuO* should result in increased *cadC* expression, and a corresponding increase in *cadBA* expression and lysine decarboxylase production. Likewise, *leuO* overexpression should result in decreased *cadC* expression and a corresponding decrease in *cadBA* expression and lysine decarboxylase activity. To test this hypothesis we quantified lysine decarboxylase activity in *V. cholerae* strains lacking *leuO* or *aphB* and in a *V. cholerae leuO*-negative mutant in which we ectopically expressed *leuO*. In contrast to *E. coli* (Tabor & Tabor, 1985), *V. cholerae* encodes only one lysine decarboxylase (i.e. CadA), which facilitates direct measurement of lysine decarboxylase production in *V. cholerae* cell lysates as a reporter for *cadA* expression (Merrell & Camilli, 1999).

We first quantified lysine decarboxylase production in WT strain JB58, Δ*leuO* strain XBV222 and Δ*aphB* strain XBV148. The results revealed a 29 % increase in lysine decarboxylase activity in the *leuO* mutant relative to WT (Fig. 3). Although this increase in lysine decarboxylase activity did not reach statistical significance (P=0.16), lysine decarboxylase activity was consistently elevated in the *leuO* mutant in multiple independent experiments. By contrast, deletion of *aphB* resulted in a 79 % reduction in lysine decarboxylase activity. This was expected, as AphB is a positive regulator of *cadC*. To provide further evidence that LeuO negatively regulated lysine decarboxylase production we quantified the effect of *leuO* overexpression from pBAD18Km-*leuO* (pXB298) on lysine decarboxylase production in a Δ*leuO* mutant (XBV222). The results showed that the addition of 0.02 % arabinose to the growth media resulted in a 53 % reduction in lysine decarboxylase activity (Fig. 3). The observation that *leuO* deletion appeared to increase lysine decarboxylase activity, while *leuO* overexpression decreased it, provided additional evidence to support the conclusion that LeuO was a negative regulator of the *cad* system in *V. cholerae*.

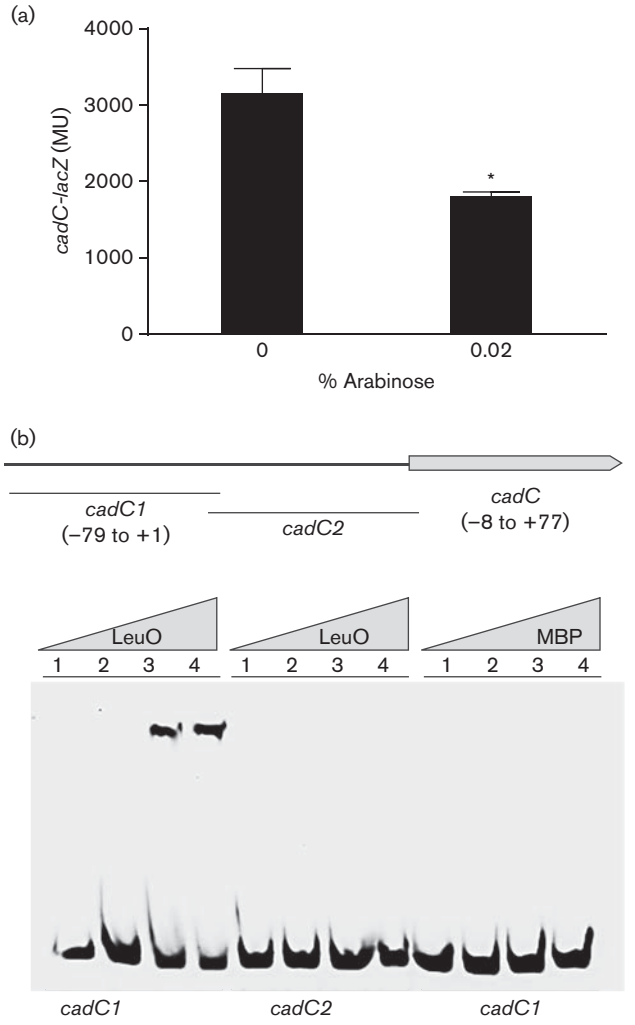

Fig. 2. Influence of LeuO on the *cadC* promoter. (a) *E. coli* containing the *cadC-lacZ* reporter plasmid pXB239 and the pBAD33-*leuO* plasmid pVA126 was grown in LB broth in the presence or absence of 0.02 % arabinose for 5 h when β-galactosidase activity was determined. The presented data are the mean ± SD of three independent experiments. Statistical significance was determined using a *t*-test comparing the mean of the induced strain with the mean of 0 % arabinose control; **P*<0.005. (b) Gel shift assays were performed using purified LeuO-MBP or MBP and the two indicated DNA fragments from the *cadC* promoter. Nucleotide numbering listed for the *cadC1* and *cadC2* DNA fragments are relative to the *cadC* transcriptional start site. Biotin-labelled *cadC1* or *cadC2* DNA fragments (1.5 nM) were incubated with either purified LeuO-MBP or MBP at 0 μM (lane 1), 10 μM (lane 2), 20 μM (lane 3) or 30 μM (lane 4) prior to electrophoresis.

Effect of LeuO on *V. cholerae* survival following organic acidic challenge

Studies have shown that the *cad* system contributes to an inducible acid tolerance phenotype whereby *V. cholerae* cells pre-adapted to mild acid conditions (i.e. pH 5.7) exhibit increased resistance to lethal acid challenge relative to unadapted cells (Merrell & Camilli, 1999). As our genetic and biochemical data suggested that LeuO repressed the *cad* system, we hypothesized that LeuO should also negatively affect *V. cholerae* acid tolerance (Merrell & Camilli, 2000). We tested this by challenging unadapted *V. cholerae* cells with varying concentrations of organic acids as described in Methods. As both *leuO* and the acid tolerance response were expressed *in vivo* (Bina *et al.*, 2013; Merrell & Camilli, 2000; Merrell *et al.*, 2002), we chose to perform these assays using cells cultured under virulence-gene-inducing conditions (i.e. AKI conditions). We cultured WT, *leuO* and *cadA* mutant strains for 4 h under AKI conditions, which is the point at which *cadC* expression was greatest (Fig. 1a), before exposing the cells to varying concentrations of organic acids that were present in the wells of microtitre plates. We then assessed cell viability 15 and 30 min after organic acid challenge with the unadapted cells and 45 and 60 min after challenge with the adapted cells by replica plating culture aliquots from the microtitre plates onto LB agar plates.

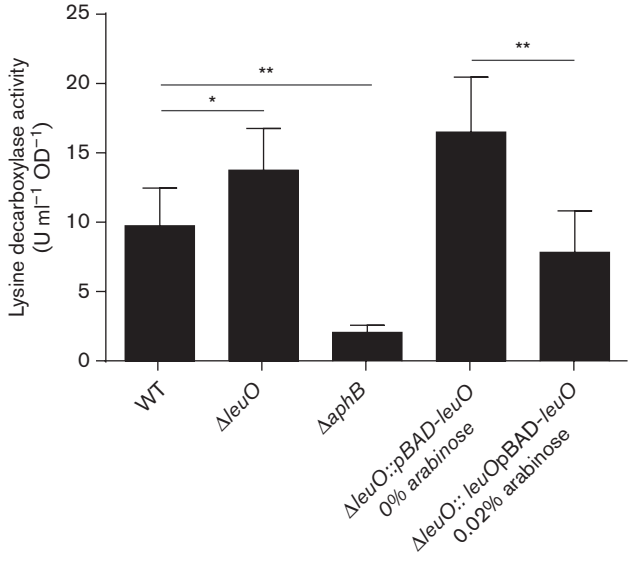

Fig. 3. Impact of *leuO* on lysine decarboxylase production in *V. cholerae*. The WT strain JB58, Δ*leuO* strain XBV222, Δ*aphB* strain XBV148 and Δ*leuO* strain carrying the pBAD18Km-*leuO* plasmid pXB298 were grown in AKI media under AKI conditions at 37 °C for 4 h when lysine decarboxylase activity was quantified as described in Methods. Strains containing the arabinose inducible pBAD18Km-*leuO* were grown in the presence or absence of 0.02 % arabinose. Lysine-decarboxylase-specific activity was defined as the amount of lysine converted to cadaverine per minute divided by the optical density at 600 nm. The presented data are the mean ± SD of three independent experiments. *P=0.16; **P>0.05.

The results for the unadapted cells revealed that there was no significant difference in the susceptibility of WT or the *leuO* mutant to organic acid challenge at either time point (Fig. 4a). This was an expected result given that LeuO appeared to be a *cadC* repressor. In contrast, the *cadA* mutant exhibited an increase in susceptibility to the acid challenge, as shown by decreased survival at 30 min relative to the WT control (Fig. 4a). This confirmed previous reports that *cadA* contributed to the *V. cholerae* acid tolerance response (Merrell & Camilli, 1999). In contrast to the unadapted cells, there was no apparent difference in organic acid susceptibility among any of the acid-adapted mutant strains at either time point (Fig. 4b). This suggests that under virulence-gene-inducing conditions, other components of the acid tolerance response can compensate for the loss of *cadA*.

LeuO is a global regulator in the *Enterobacteriaceae*, a phenotype that appears to be conserved in the *Vibrionaceae*. This suggested the that LeuO might affect the expression of other acid tolerance genes in addition to *cadC*. If this were true, *leuO* overexpression in the *cadA* mutant should result in increased organic acid susceptibility. To test this, we repeated the acid killing assays using *leuO* and *cadA* mutants in which we ectopically expressed *leuO* (Fig. 4c, d). The results showed that *leuO* overexpression in the *leuO* mutant resulted in increased susceptibility of the unadapted cells to organic acid challenge (Fig. 4c). This confirmed that *leuO* expression enhanced *V. cholerae* susceptibility to organic acids and was consistent with the conclusion that LeuO repressed the *cad* system. Interestingly, ectopic expression of *leuO* in the *cadA* mutant also increased *V. cholerae* susceptibility to organic acid challenge (Fig. 4c). This indicated that the function of LeuO in organic acid tolerance extended beyond its regulation of the *cad* system.

We next tested whether LeuO affected the induction of an acid tolerance response phenotype. We therefore repeated the above experiments with AKI cultures that had been pre-adapted at pH 5.7 for 1 h prior to organic acid challenge. The results showed increased organic acid resistance among the adapted cells relative to the unadapted cells with all of the tested strains (Fig. 4). This was evidenced by comparison of cell viability between the 30 min unadapted cultures and the 45 min adapted cultures. Significantly, 60 min post-challenge, there were no observable differences in survival between the WT, *leuO* and *cadA* mutant strains (Fig. 4b), indicating that *V. cholerae* was able to mount an acid tolerance response in the absence of *leuO* and *cadA*. By contrast, when *leuO* was overexpressed in either the *leuO* or the *cadA* mutants, the cells exhibited increased susceptibility to organic acid challenge relative to the empty vector control (Fig. 4d). This indicated that *leuO* overexpression negatively affected the ability of *V. cholerae* to mount an acid tolerance response.

The fact that *leuO* overexpression in the *cadA* mutant resulted in increased acid susceptibility provided additional evidence to suggest that the function of *leuO* to acid tolerance extends beyond the *cad* system.

DISCUSSION

LeuO is a LysR-family regulator that has been shown to function downstream of ToxR in *V. cholerae* (Ante *et al.*, 2015; Bina *et al.*, 2013). Several lines of evidence suggest that LeuO is a global regulator in the *Vibrionaceae* that functions in host adaptation and virulence. In *V. cholerae* LeuO has been shown to affect virulence factor production, biofilm production and bile salt resistance (Ante *et al.*, 2015; Bina *et al.*, 2013; Moorthy & Watnick, 2005). In *Vibrio parahaemolyticus* LeuO has been shown to regulate expression of the type III secretion system, and serine protease production in *Vibrio vulnificus* (Kim *et al.*, 2015; Whitaker *et al.*, 2012). Taken together these results suggest that LeuO probably functions to regulate diverse genes involved in environmental adaptation in the *Vibrionaceae*.

In this study, we identified a new physiological function for LeuO in *V. cholerae* environmental adaptation. We found that LeuO regulated the expression of the *cad* system, suggesting that LeuO contributes to acid tolerance. The *V. cholerae cad* system is constitutively expressed at a low basal level, but is upregulated under conditions of low pH or low oxygen (Kovacikova *et al.*, 2010). Upregulation under these conditions is mediated by AphB binding to the *cadC* promoter. Once CadC is produced, it upregulates

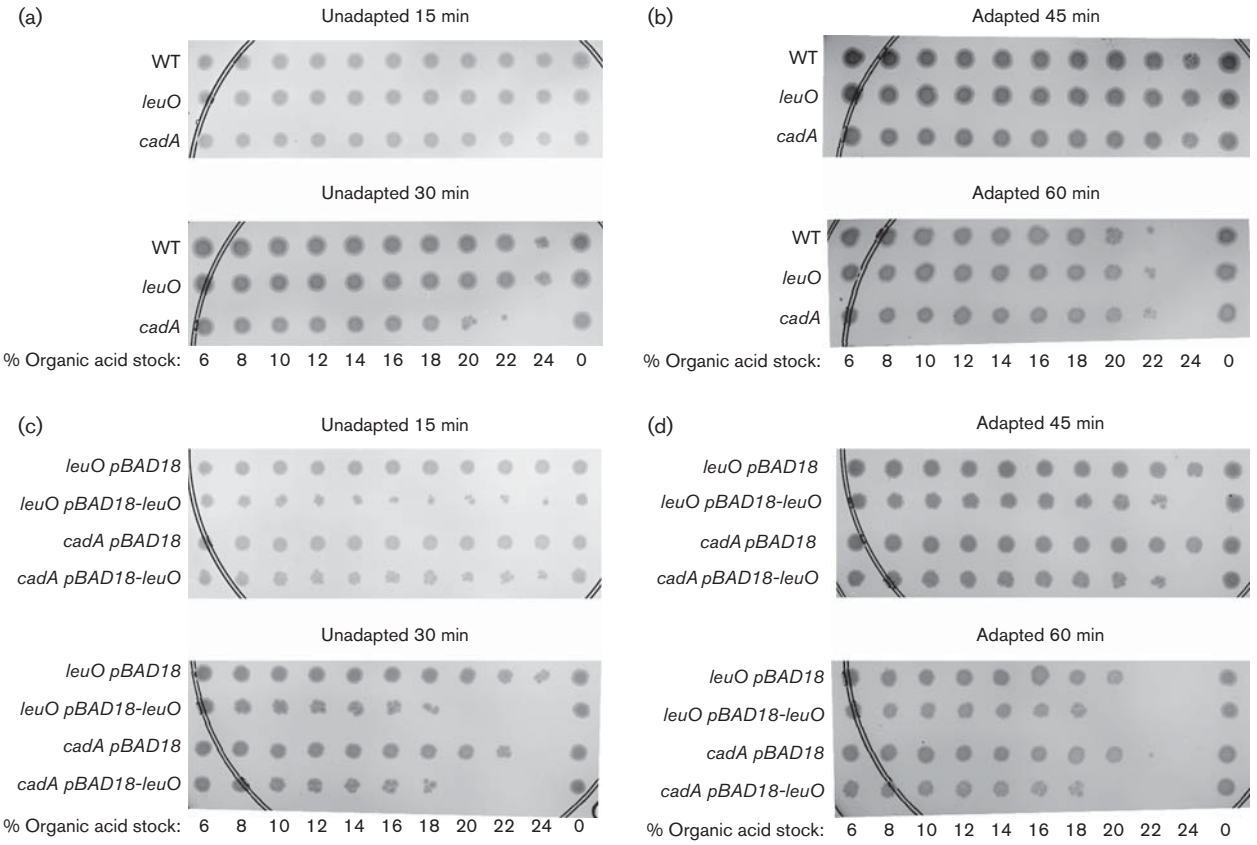

Fig. 4. Effect of *leuO* on *V. cholerae* survival in organic acid. (a) Survival of unadapted WT (XBV144), *leuO* (VA412) and *cadA* (EC17926) mutants following organic acid challenge for 15 and 30 min. (b) Survival of adapted WT (XBV144), *leuO* (VA412) and *cadA* (EC17926) mutants following organic acid challenge for 45 and 60 min. (c) Survival of unadapted *leuO* (EC20568) and *cadA* (EC17926) mutants containing pBAD18 or pBAD-*leuO* following organic acid challenge for 45 and 60 min. (d) Survival of adapted *leuO* (EC20568) and *cadA* (EC17926) mutants containing pBAD18 or pBAD-*leuO* following organic acid challenge for 45 and 60 min. All strains were cultured for 4 h under AKI conditions before the organic acid challenge; 0.02 % arabinose was added to the broth for strains containing pBAD18 or pBAD18-*leuO*. Unadapted cells (a, c) and adapted cells (b, d) were inoculated into microtitre plates containing the indicated final concentrations of the organic acid stock solution. The microtitre plates were then incubated at 37 °C and cell viability was assessed over time by replica plating ~10 µl from each well of the microtitre plates onto the surface of an LB agar plate using a 96-well pin replicator. The agar plates were incubated overnight at 37 °C before being photographed. The presented results are representative of at least three independent experiments.

cadBA expression leading to the production of CadB (a lysine/cadaverine antiporter) and CadA (a lysine decarboxylase). CadA contributes to acid tolerance through its degradation of lysine to the polyamine cadaverine, a reaction that plays a key role in maintaining pH homeostasis within the cell.

While AphB positively regulates expression of the *cad* system, our results showed that LeuO negatively regulates expression of the *cad* system. This was supported by the fact that *leuO* deletion increased *cadC* expression while *leuO* overexpression reduced *cadC* expression (Fig. 1). These results suggested strongly that LeuO was a *cadC* repressor. The negative effects of *leuO* on *cadC* transcription were further shown to affect the production of lysine decarboxylase production, the downstream target of CadC (Fig. 3). Taken together these results indicated that LeuO negatively regulates the expression of the *cad* system by repressing *cadC* transcription.

LeuO appeared to regulate expression of the *cad* system by directly binding to the *cadC* promoter. This suggests that there may be interplay between AphB and LeuO in regulation of the *cad* system. Our results show that expression of the *cad* system increased during static growth under AKI conditions before declining upon shift of the cultures to aerated growth (which is associated with alkalinization of the media). Growth of El Tor strains under static AKI growth conditions results in low oxygen tension and low pH, conditions that have been correlated with AphB activation of *cadC* (Kovacikova *et al.*, 2010). By contrast, *leuO* expression appears to increase with cell density until it reaches its maximum level at the late exponential phase (data not shown). This suggests that LeuO may function to fine-tune expression of the *cad* system by antagonizing AphB binding to the *cadC* promoter. The facts that both LeuO and AphB are LysR-family regulators and that LysR-family regulators bind to T-N_{11}-A motifs (Maddocks & Oyston, 2008) are consistent with this idea. Furthermore, LeuO has been shown to regulate many of its target genes in the *Enterobacteriaceae* by functioning as an antagonist (Shimada *et al.*, 2011). Whether LeuO is functioning as an AphB antagonist in *V. cholerae* will require additional studies.

Overexpression of *leuO* in a *cadA* mutant increased *V. cholerae* susceptibility to organic acid in both adapted and unadapted cells (Fig. 4). This suggested that the contribution of LeuO to organic acid tolerance extended beyond the *cad* system. The mechanism by which this occurred is not known. The acid tolerance response in *V. cholerae* is complicated and involves diverse genes including the virulence regulator ToxR (Merrell *et al.*, 2001, 2002). ToxR was shown to be required for the organic acid tolerance response through its regulation of the OmpU and OmpT porins. The fact that ToxR positively regulates *leuO* expression suggests that the role of ToxR in acid tolerance extends beyond porin regulation. In addition to *cadC*, AphB positively regulates other genes that contribute to acid tolerance (Ding & Waldor, 2003; Kovacikova *et al.*, 2010). While LeuO does not appear to affect production of OmpU or OmpT (Ante *et al.*, 2015), it is possible that LeuO could affect the expression of other AphB-regulated genes that contribute to acid tolerance via a mechanism similar to that with *cadC*. Alternatively, given that LeuO appears to be a global regulator, LeuO could affect acid tolerance through regulation of other unknown genes.

Although our data conclusively show that LeuO represses *cadC* expression, the physiological relevance of LeuO repression of *cadC* and the acid tolerance response is not yet clear. As *leuO* expression is induced by bile and LeuO contributes to bile salt resistance (Ante *et al.*, 2015), one possibility is that downregulation of the acid tolerance response may contribute to bile resistance. In *Salmonella typhimurium* the acid tolerance response increased cell surface hydrophobicity (Leyer & Johnson, 1993), a phenotype that could result in increased susceptibility to detergent-like molecules such as bile salts. If the *V. cholerae* acid tolerance response also resulted in increased cell surface hydrophobicity, *leuO* induction in response to bile salts may function to downregulate the acid tolerance response to decrease cell surface hydrophobicity and positively affect bile resistance. LeuO could also function in a feedback mechanism to modulate cadaverine production via *cadC* repression. Cadaverine is a polyamine that has two positive charges at neutral pH. Excess polyamines are growth inhibitory, which necessitates regulation of their production (He *et al.*, 1993). Cadaverine has also been found to reduce *V. cholerae* auto-agglutination, probably as a result of its positively charged amine groups electrostatically disrupting the pili interactions (Goforth *et al.*, 2013). Thus, excess cadaverine could hinder intestinal colonization.

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