AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Transcriptional Repression of the VC2105 Protein by *Vibrio* FadR Suggests that It Is a New Auxiliary Member of the *fad* Regulon

Rongsui Gao, Jingxia Lin, Han Zhang, Youjun Feng

Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China

ABSTRACT

Recently, our group along with others reported that the *Vibrio* FadR regulatory protein is unusual in that, unlike the prototypical *fadR* product of *Escherichia coli*, which has only one ligand-binding site, *Vibrio* FadR has two ligand-binding sites and represents a new mechanism for fatty acid sensing. The promoter region of the *vc2105* gene, encoding a putative thioesterase, was mapped, and a putative FadR-binding site (AA CTG GTA AGA GCA CTT) was proposed. Different versions of the FadR regulatory proteins were prepared and purified to homogeneity. Both electrophoretic mobility shift assay (EMSA) and surface plasmon resonance (SPR) determined the direct interaction of the *vc2105* gene with FadR proteins of various origins. Further, EMSAs illustrated that the addition of long-chain acyl-coenzyme A (CoA) species efficiently dissociates the *vc2105* promoter from the FadR regulator. The expression level of the *Vibrio cholerae vc2105* gene was elevated 2- to 3-fold in a *fadR* null mutant strain, validating that FadR is a repressor for the *vc2105* gene. The β-galactosidase activity of a *vc2105-lacZ* transcriptional fusion was increased over 2-fold upon supplementation of growth medium with oleic acid. Unlike the *fadD* gene, a member of the *Vibrio fad* regulon, the VC2105 protein played no role in bacterial growth and virulence-associated gene expression of *ctxAB* (cholera toxin A/B) and *tcpA* (toxin coregulated pilus A). Given that the transcriptional regulation of *vc2105* fits the criteria for fatty acid degradation (*fad*) genes, we suggested that it is a new member of the *Vibrio fad* regulon.

IMPORTANCE

The *Vibrio* FadR regulator is unusual in that it has two ligand-binding sites. Different versions of the FadR regulatory proteins were prepared and characterized *in vitro* and *in vivo*. An auxiliary *fad* gene (*vc2105*) from *Vibrio* was proposed that encodes a putative thioesterase and has a predicted FadR-binding site (AAC TGG TA A GAG CAC TT). The function of this putative binding site was proved using both EMSA and SPR. Further *in vitro* and *in vivo* experiments revealed that the *Vibrio* FadR is a repressor for the *vc2105* gene. Unlike *fadD*, a member of the *Vibrio fad* regulon, VC2105 played no role in bacterial growth and expression of the two virulence-associated genes (*ctxAB* and *tcpA*). Therefore, since transcriptional regulation of *vc2105* fits the criteria for *fad* genes, it seems likely that *vc2105* acts as a new auxiliary member of the *Vibrio fad* regulon.

Most of our knowledge about bacterial fatty acid metabolism is derived from extensive studies with *Escherichia coli* (*E. coli*) (1, 2). Apart from participating in the formation of bacterial membrane phospholipids, fatty acids also provide precursors for biotin and lipoic acid, two essential sulfur-containing vitamins (3). In *E. coli*, the fatty acid metabolism comprises both *de novo* synthesis of fatty acids and fatty acid degradation (FAD; also referred to as β-oxidation here). The repetitive cycle for type II fatty acid synthesis (FAS II) comprises four successive reactions: (i) FabB/FabF-mediated condensation, (ii) reduction by FabG, the β-ketoacyl-acyl carrier protein (ACP) reductase, (iii) dehydration by the β-hydroxyacyl-ACP dehydratase (FabZ)/β-hydroxydecanoyl-ACP dehydratase/isomerase (FabA), and (iv) reduction by the enoyl-ACP reductase, FabI (1, 4). The aerobic β-oxidation of long-chain fatty acids (LCFAs) requires the coordinated participation of no fewer than five Fad family members (FadL, FadD, FadE, and FadBA) (5, 6), while the anaerobic β-oxidation of fatty acids depends on the FadJ-FadI-FadK system (7). To maintain a precise balance between catabolism and anabolism of fatty acids, energetically expensive molecules essential for all three domains of life, *E. coli* has developed two opposing regulatory systems: a FadR regulator belonging to the GntR family of transcription factors (8, 9) and a FabR repressor, the TetR-like regulator (10–12). The FadR regulatory protein plays dual roles in fatty acid metabolism. It acts as a repressor for no fewer than six members of the *fad* system (*fadBA* [6, 7], *fadL* [6], *fadD* [6], *fad* [13, 14], *fadH* [15], and *fadM* [5]), and it also functions as an activator of *fabA* and *fabB*, the two essential genes for unsaturated fatty acid biosynthesis (9, 16). In contrast, the *E. coli* FabR negatively regulates the expression of *fabA* and *fabB* to control membrane lipid homeostasis (10–12). Long-chain fatty acyl-coenzyme A (CoA) thioesters are the physiological ligands/effectors of small molecules for the fatty acid-responsive FadR regulator in that their binding to FadR causes its release from the cognate DNA targets (8, 17). The accumulated structures of FadR alone and in complex with cognate DNA (and/or ligand) have initially established the biochem-

Received 28 January 2016 **Accepted** 25 February 2016

Accepted manuscript posted online 4 March 2016

Citation Gao R, Lin J, Zhang H, Feng Y. 2016. Transcriptional repression of the VC2105 protein by *Vibrio* FadR suggests that it is a new auxiliary member of the *fad* regulon. Appl Environ Microbiol 82:2819–2832. doi:10.1128/AEM.00293-16.

Editor: M. Kivisaar, Tartu University

Address correspondence to Youjun Feng, fengyj@zju.edu.cn.

R.G. and J.L. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00293-16.

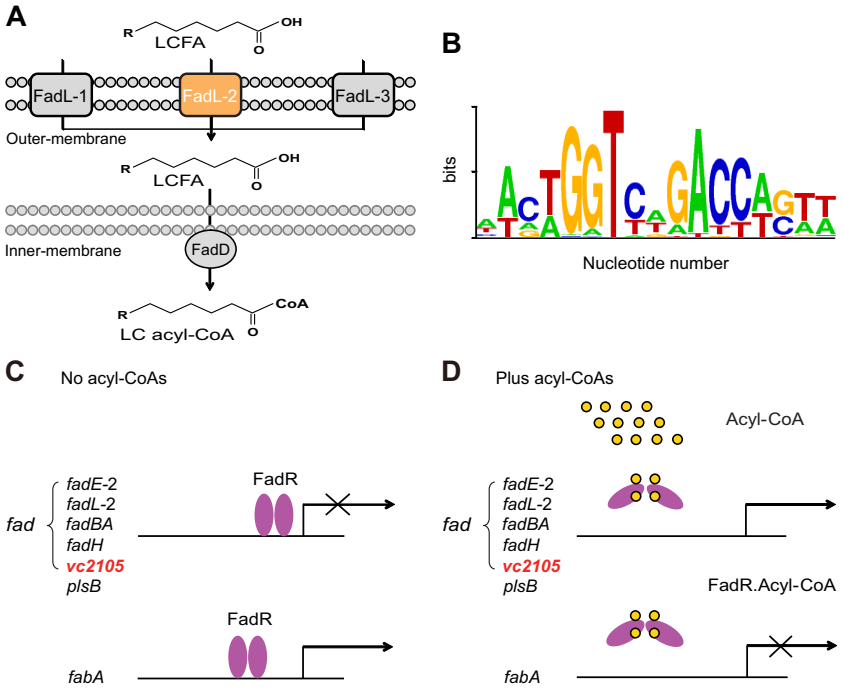

FIG 1 A working model for regulation of fatty acid metabolism by FadR in *Vibrio*. (A) Uptake of long-chain fatty acids (LCFAs) by the FadL-FadD system and generation of the activated forms (LC fatty acyl-CoAs) in *Vibrio*. The exogenous LCFA species are transported by the FadL membrane protein into the periplasm and activated by the FadD inner membrane-associated protein into LC acyl-CoA pools. Note that among three FadL homologues in the *V. cholerae* N16961 genome, only FadL2 (in orange) has a FadR-recognizable palindrome. (B) Sequence logo for DNA palindrome sequences recognized by *Vibrio* FadR. The sequences of the putative *Vibrio* FadR sites were collected from nine *Vibrio* species (http://regprecise.lbl.gov/RegPrecise/index.jsp). (C) When acyl-CoA is poor, *Vibrio* FadR negatively regulates the transcription of both the contracted *fad* members and *plsB*, whereas it positively controls *fabA* expression; *vc2105* is proposed to be a new *fad* member in that it is predicted to have a FadR-specific palindrome (Fig. 2). (D) LCFA acyl-CoAs can neutralize the DNA-binding activity of *Vibrio* FadR, which consequently derepresses the expression of limited *fad* members but quenches *fabA* transcription. The small yellow circles denote the acyl-CoA pool, and the purple ovals indicate the *Vibrio* FadR regulator. Unlike the scenarios seen with *E. coli* FadR, which has only one ligand-binding site in each monomer, the studies by our group and by others found that *Vibrio* FadR has evolved two ligand-binding sites in each monomer.

ical basis for FadR-mediated regulation of lipid metabolism (18–20). Further genetic analyses have suggested that unexpected functional diversity is present among bacterial FadR homologues (21, 22). Therefore, it seemed likely that the paradigm of the *E. coli* FadR-centric regulatory network does not fit its counterparts in other organisms well.

The genera of the *Vibrionaceae* family are Gram-negative bacteria from a diversified ocean environment and/or ecosystem that consist of more than 140 heterogeneous species (23, 24). A notorious food-borne pathogen, *Vibrio cholerae* (*V. cholerae*) has the ability to infect humans through consumption of uncooked seafood, resulting in gastroenteritis and septicemia. The mechanism by which *V. cholerae* establishes a successful infection is closely associated with multiple virulence factors, including cholera toxin (CT) and toxin-coregulated pilus (TCP) (25, 26). It seemed very likely that bacterial fatty acid metabolism is relevant to *Vibrio* pathogenicity in that no fewer than three lines of independent evidence have already been accumulated: (i) the FadR of *Vibrio vulnificus* is required for successful infection (27), (ii) inactivation of the *fadD* gene that encodes the long-chain acyl-CoA ligase attenuates full virulence of *V. cholerae* (28, 29), and (iii) unsaturated fatty acids, including linoleic acid and its conjugated form, quench expression/production of virulence genes (e.g., CT and TCP) in *V. cholerae* (25, 26, 30). More intriguingly, bioinformatics-based gene discovery suggests that *Vibrio* species might be far different from their closely related cousins, *E. coli* bacteria, in the context of lipid metabolism (Fig. 1). The following observations are relevant: (i) the number of FadR-controlled *fad* or *fab* genes varies greatly (7 for *Vibrio* and 12 for *E. coli*), suggesting *fad* contraction in *Vibrio*, which is consistent with scenarios seen in another marine bacterium, *Shewanella* (31); (ii) unlike the scenarios seen in *E. coli*, the *plsB* gene is repressed by FadR in *V. cholerae* (32); (iii) *V. cholerae* has three FadL orthologues, only one of which is regulated by FadR, whereas *E. coli* has only one FadL transporter; (iv) in contrast to the two known FadR-activated genes (*fabA* and *fabB*) of *E. coli*, *fabA* is the only *V. cholerae* counterpart for which a putative FadR-binding has been site detected (31); (v) a new gene, *vc2105*, encoding a putative thioesterase is predicted to have a possible FadR palindrome lacking functional/experimental evidence. Very recently, structural and functional studies by Shi et al. and our group demonstrated that *Vibrio* FadR defines a new mechanism for fatty acid sensing in that an extra 40-amino-acid (aa) insert in *Vibrio* FadR constitutes a second ligand-binding site (see Fig. S2 in the supplemental material) (33). We also proved that this new ligand-binding site is critical for the role played by FadR in *Vibrio* infections (Y. Feng, D. Li, R. Gao, Y. Lin, H. Zhang, X. Xia, J. Lin, H. Zhang, H. Wang, L. Bi, J. Zhu, and S. Wang, submitted for publication).

In this work, we report integrative evidence (ranging from bioinformatics, biochemistry, and biophysics to bacterial genet-

TABLE 1 Bacteria and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference(s) and/or source
Strains		
DH5α	*E. coli* cloning host	Lab stock
BL21(DE3)	*E. coli* protein expression strain	Lab stock
DH5α λ-*pir*	Δ*lac* host for pAH125 and its derivatives	4
MFH8	UB1005 *fadR*::Tn*10* (Tc)	8
MC4100	*E. coli araD139* Δ(*argF-lac*)*169*	5
FYJ187	MC4100 carrying pINT-ts	Lab stock
FYJ360	MC4100 integrated with P*vc2105-lacZ*	This work
FYJ361	MC4100 integrated with P*vc2105*-lacZ, *fadR*::Tn*10*	This work
FYJ372	DH5α λ-pir/pAH-P*vc2105*	This work
FYJ378	DH5α λ-pir carrying pAH-P*fur*_vc	This work
FYJ379	MC4100 integrated with P*fur*_vc-*lacZ*	This work
FYJ380	BL21(DE3) carrying pET28-*vc2105*	This work
V. cholerae C6706	*V. cholerae* El Tor with streptomycin resistance	34
FYJ639	C6706 Δ*fadR* (Δ*vc1900*) *lacZ* mutant	This work
FYJ623	SM10 λ-*pir*	Lab stock; 41
FYJ654	SM10 λ-*pir* carrying pTL61T-P*vc2105*	This work
FYJ655	DH5α carrying pTL61T-P*fabA*$_{vc}$	This work
FYJ656	*V. cholerae* C6706 carrying pTL61T-P*vc2105*	This work
FYJ657	*V. cholerae* C6706 carrying pTL61T-P*fabA*$_{vc}$	This work
FYJ658	FYJ639 Δ*fadR*, carrying pTL61T-P*vc2105*	This work
FYJ659	FYJ639 Δ*fadR*, carrying pTL61T-P*fabA*$_{vc}$	This work
FYJ816	*V. cholerae* C6706 Δ*vc2105* mutant, *lacZ* mutant	This work
FYJ817	*V. cholerae* C6706 carrying pTL61T-P*ctxAB*, Sm^r Amp^r	This work
FYJ818	*V. cholerae* C6706 carrying pTL61T-P*tcpA*, Sm^r Amp^r	This work
FYJ819	DH5α λ-*pir* carrying pWM91-*vc2105*, Amp^r	This work
FYJ820	DH5α λ-*pir* carrying pTL61T-P*tcpA*, Amp^r	This work
FYJ821	DH5α λ-*pir* carrying pTL61T-P*ctxAB*, Amp^r	This work
FYJ824	FYJ816 carrying pTL61T-P*ctxAB*, Sm^r Amp^r	This work
FYJ825	FYJ816 carrying pTL61T-P*tcpA*, Sm^r Amp^r	This work
Plasmids		
pWM91	R6K vector with a *sacB* gene, Amp^r	35
pET28a(+)	T7 promoter-driven expression vector for production of recombinant protein in *E. coli*	Novagen
pBAD24	Arabinose inducible promoter-driven expression vector	42
pAH125	A promoterless *lacZ* reporter plasmid used in *E. coli*, Kan^r	43
pINT-ts	Temperature-sensitive plasmid expressing phage λ integrase	43
pTL61T	A promoterless *lacZ* fusion plasmid used for *V. cholerae*	44–46
pET16-*fadR*$_{vc}$	pET16 carrying the *V. cholerae fadR* gene, Amp^r	32
pET28-*vc2105*	pET28 carrying the *V. cholerae vc2105* gene, Kan^r	This work
pWM91-*fadR*$_{vc}$	The *V. cholerae fadR* gene knockout plasmid, pWM91 that carries the flanking regions of the *fadR*$_{vc}$	This work
pWM91-*VC2105*	The knockout plasmid of the *V. cholerae vc2105* gene, pWM91 that carries the flanking regions of the *VC2105* gene	This work
pAH125-P*vc2105*	pAH125 carrying the promoter region of the *V. cholerae vc2105* gene, Kan^r	This work
pAH125-P*fur*	pAH125 carrying the promoter region of the *V. cholerae fur* gene, Kan^r	This work
pTL61T-P*vc2105*	pTL61T carrying the promoter region of the *V. cholerae vc2105* gene, Kan^r	This work
pTL61T-P*tcpA*	pTL61T carrying the promoter region of the *V. cholerae tcpA* gene, Kan^r	This work
pTL61T-P*ctxAB*	pTL61T carrying the promoter region of the *V. cholerae ctxAB* gene, Kan^r	This work
pTL61T-P*fabA*$_{vc}$	pTL61T carrying the promoter region of the *V. cholerae fabA* gene, Kan^r	This work

ics) that the *vc2105* gene is repressed by *Vibrio* FadR and activated by the addition of long-chain fatty acids. Although the biochemical function of VC2105 remains unclear, the data we present still suggest that the *vc2105* gene might be an additional member of the *Vibrio fad* regulon. Given that the context of *Vibrio* lipid metabolism is unusual compared to that of its *E. coli* cousin, it is physiologically possible that *Vibrio* evolved an extraordinary gene *vc2105* (and/or its equivalent) involved in the β-oxidation of certain fatty acids.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacteria used here were *E. coli* K-12 derivatives and *V. cholerae* El Tor C6706 and its derivatives (Table 1) (34). The two media that can be used for *E. coli* and/or *V. cholerae* correspond to Luria-Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) and rich broth (RB) medium (10 g of tryptone, 1g of yeast extract, and 5 g of NaCl per liter). Of particular note, modified LB liquid medium that lacked NaCl was used to cultivate *V. cholerae* for preparation of electroporation-competent cells.

TABLE 2 Primers used in this study

Primer (restriction enzyme or position)	Sequence[a]
VC2105-F1 (NcoI)	5′-TATACCATGGTGAATAAAACACTCTCTAAGCTTTATCAACC-3′
VC2105-R1 (XhoI)	5′-GAGCCTCGAGTCGCCTTTCCGCTGTG-3′
VC2105-F2 (XmaI)	5′-TATACCCGGGATGAATAAAACACTCTCTAAGCTTTATCAACC-3′
VC2105-R2 (SphI) for pBAD24	5′-GAGCGCATGCTTATCGCCTTTCCGCTGTG-3′
FadR-vc2105-F	5′-GTTAAATTATTGT**AACTGGTAAGAGCACTT**TGTTCAGTG-3′
FadR-vc2105-R	5′-CACTGAACA**AAGTGCTCTTACCAGTT**ACAATAATTTAAC-3′
FadR-vp_0834-F	5′-CAAAATTGT**AACTGGTCAGATCTGTA**ATATAATCG-3′
FadR-vp_0834-R	5′-CGATTATAT**TACAGATCTGACCAGTT**ACAATTTTG-3′
FadR-VF_0811-F	5′-GTTAACAAAGAAACTATT**ATGAGGTCTGACCAGAA**TTAAAGAGATTAAATATG-3′
FadR-VF_0811-R	5′-CATATTTAATCTCTTTAA**TTCTGGTCAGACCTCAT**AATAGTTTCTTTGTTAAC-3′
FadR-VH_01340-F	5′-GTCGATTATAT**TACAGATCAGACCAGTT**ACATATTGATAAC-3′
FadR-VH_01340-R	5′-GTTATCAATATGT**AACTGGTCTGATCTGTA**ATATAATCGAC-3′
Pvc2105-F (SalI)	5′-CCGGTCGACATCACTCACTTGCCGTCTAC-3′
Pvc2105-R (EcoRI)	5′-AACCGAATTCCATACCCTGAGTGCCCTAGA-3′
vc2105-promoter-F (XhoI)	5′-CCGCTCGAGCCGTCTACCTGCATCTCAAT-3′
vc2105-promoter-R (BamHI)	5′-CGGGATCCCATACCCTGAGTGCCCTAGA-3′
fabAvc-promoter-F (XhoI)	5′-CCGCTCGAGTCACCAAGGCTTAACTGG-3′
fabAvc-promoter-R (BamHI)	5′-CGGGATCCCATTATTGGTTACTCCATT-3′
fadRvc-L-flank-F (BamHI)	5′-TCCGGATCCGCTTTACCAAGGAAATTCTT-3′
fadRvc-L-flank-R	5′-ATTAGCAATCGACCATTAATGACTAATCCA-3′
fadRvc-R-flank-F	5′-ATTAATGGTCGATTGCTAATCTAGCACTGT-3′
fadRvc-R-flank-R (SacI)	5′-TGCGAGCTCCAAGATTGCCAGTTATGAAA-3′
VC2105-GSP[b]	5′-GCCAAGATCCAATCCAACATC-3′
VC2105-nest	5′-GTCCCATACGTAATAACGCTC-3′
plsBvc_FadR-F	5′-TTAAATT**AAAAGGTTTGACCAGTT**TCTGGTATTCTTGGC-3′
plsBvc_FadR-R	5′-GCCAAGAATACCAGA**AACTGGTCAAACCTTTT**AATTTAA-3′
vc2105-UP-F (BamHI)	5′-TCCGGATCCTTTTGACGTTGTTCAATCACAT-3′
vc2105-UP-R	5′-AGGGAAGCATACCCTGAGTGCCCTAGACTT-3′
vc2105-DOWN-F	5′-CACTCAGGGTATGCTTCCCTGAATTTATTG-3′
vc2105-DOWN-R (SacI)	5′-TGC GAGCTC GGC ATG TTT ACG CAC GGT TA-3′
ctxAB-promoter-F (XhoI)	5′-CCGCTCGAGCGTGGTCTATGGGCGACAG-3′
ctxAB-promoter-R (BamHI)	5′-CGGGATCCCATATAATGCTCCCTTTGTTTAA-3′
tcpA-promoter-F (XhoI)	5′-CCGCTCGAGCACCAGATCACGTAGGTG-3′
tcpA-promoter-R (BamHI)	5′-CGGGATCCCATATTTATATAACTCCACCAT-3′
5 (1041–1061)	5′-CAATCCAACATCTCTTGGGTG-3′
6 (1353–1374)	5′-CACTCTCTAAGCTTTATCAACC-3′
7 (1353–1374)	5′-GGTTGATAAAGCTTAGAGAGTG-3′
8 (1771–1792)	5′-CTTTAAGACCAGCATCCTTTAG-3′
9 (1771–1792)	5′-CTAAAGGATGCTGGTCTTAAAG-3′
10 (2103–2123)	5′-TGGTTAGTCAGTTGCACATTG-3′

[a] Restriction sites are underlined, and the known/predicted FadR sites are in boldface.
[b] GSP, gene-specific primer.

When required, solubilized oleate with Tergitol type NP-40 was supplemented at the level of around 4 mM, as we described previously, with minor changes (5, 32). Antibiotics used here included sodium ampicillin (100 μg/ml), kanamycin sulfate (50 μg/ml), streptomycin (100 μg/ml), and tetracycline-HCl (20 μg/ml for *E. coli* and 2 μg/ml for *V. cholerae*).

Plasmids and DNA manipulations. The following three promoter regions amplified from *V. cholerae* (*vc2105*, *fur*, and *fabA*) were cloned into pAH125, giving the plasmids pAH125-P*vc2105*, pAH125-P*fur*, and pAH125-P*fabA*, respectively (Table 1). Similarly, the promoters of both *vc2105* and *fabA* from *V. cholerae* were inserted into pTL61T, generating the reporter plasmids pTL61T-P*vc2105* and pTL61T-P*fabA*, respectively (Table 1). pAH125/pTL61T plasmids and their derivatives were maintained in strain DH5α λ*pir* (Table 1) since their replication depends on the presence of *pir* protein. To impart antibiotic resistance in *E. coli* MC4100 (a *lacZ* strain lacking *pir*), the pAH125-derived plasmids must specifically integrate into the *att*-λ site of the bacterial chromosome in a reaction catalyzed by the pINT-ts helper plasmid, giving strains FYJ360 (P*vc2105-lacZ* transcriptional fusion) and FYJ379 with the P*fur*_vc-*lacZ* transcriptional fusion (Table 1). To prepare the proteins of FadR and VC2105, the appropriate plasmids (pET16 carrying *V. cholerae fadR* [pET16-$fadR_{vc}$] and pET28-*vc2105*) (Table 1) were introduced into the strain BL21(DE3). The plasmids were validated by PCR detection and direct DNA sequencing.

In-frame deletion of *V. cholerae vc2105*. In-frame deletion of the *vc2105* gene was accomplished by cloning the two neighboring regions into the suicide vector pWM91 containing a *sacB* counterselectable maker (35). The upstream (and/or downstream) DNA fragments (700 bp long) flanking the *vc2105* gene were amplified by PCR using two pairs of specific primers (vc2105-UP-F [BamHI]/vc2105-UP-R and vc2105-DOWN-F/vc2105-DOWN-R [SacI]). Then, the acquired two DNA fragments were overlapped and cloned into pWM91 via two cuts (with BamHI and SacI), giving the *vc2105* knockout (KO) plasmid pWM91-$vc2105_{KO}$. Subsequently, the knockout plasmid was introduced by conjugation into the *Vibrio cholerae* strain. A single-crossover event was selected by resistance to streptomycin and ampicillin, and double-crossover events were ensured by growth selection in the absence of ampicillin and streptomycin. Finally, the *vc2105* deletion strain was produced by screening the strains

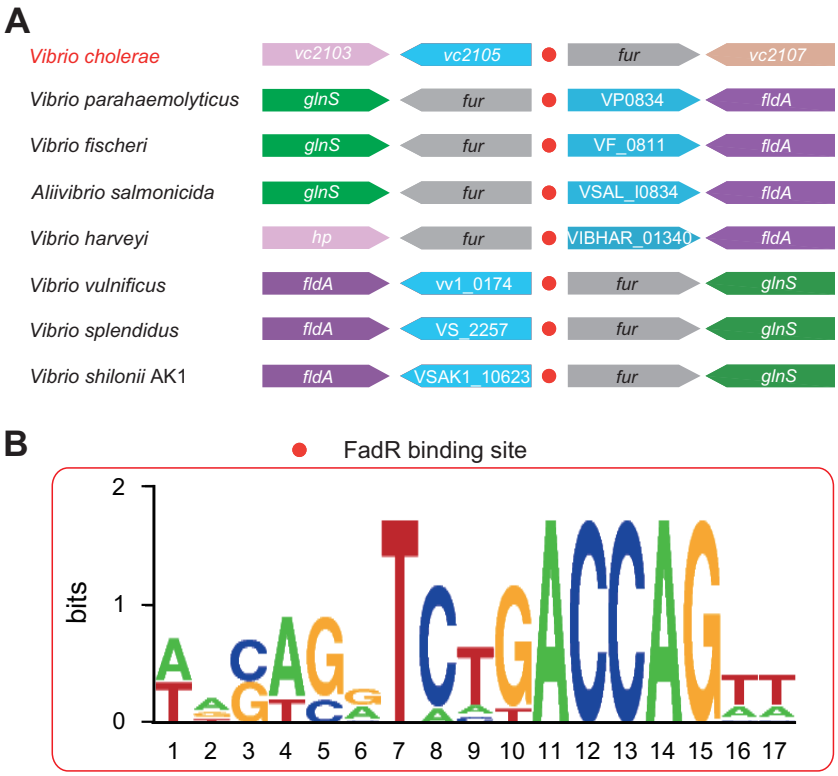

FIG 2 Genetic organization of *vc2105* locus and its FadR-recognizable signal. (A) Genetic loci of *vc2105* (and/or equivalent) and its neighboring genes. The predicted FadR-binding sites (red dots) are located between *vc2105* (and/or equivalent), a putative thioesterase-encoding gene, and *fur*, which encodes the ferric uptake regulator. Blue, *vc2105* (and/or equivalent) gene; gray, *fur*; purple, flavodoxin-1 (*fldA*); green, glutamyl-tRNA synthetase (*glnS*), which is adjacent to *fur* on the opposite strand. (B) Sequence logo for the predictive FadR-binding sites of the *vc2105* gene and other homologues.

with sucrose resistance and validated by multiplex PCR and direct DNA sequencing.

Transformation of *V. cholerae*. As we described previously (32), we adopted the method of electroporation to introduce the *lacZ* reporter plasmids pTL61T-P*vc2105*, pTL61T-P*fabA*, pTL61T-P*ctx*, and pTL61T-P*tcpA* (Table 1) into *V. cholerae*. Electrocompetent cells of *V. cholerae* were routinely prepared as follows. First, 1 ml of overnight culture was inoculated into 100 ml of the modified LB medium lacking NaCl in a 500-ml flask with vigorous shaking (200 rpm) at 37°C. Second, when the bacterial optical density measured at 600 nm (OD_{600}) reached about 0.5, cultures were chilled on ice for 30 min and then sampled by spinning at 3,000 × *g* for 20 min at 4°C. Third, the acquired pellets were resuspended in 30 ml of iced-chilled double-distilled H_2O (ddH_2O) and subsequently centrifuged at 3,000 × *g* for 20 min at 4°C. Last, after two more rounds of washing, the cells were suspended in 1 ml of ice-cold 10% (wt/vol) glycerol, divided into small aliquots (50 μl per tube), and kept at −80°C until use.

Prior to electroporation, the competent cells (50 μl) were mixed with the plasmid pTL61T and/or its derivatives (~1 μg), placed on ice for ~30 min, and then transferred into a chilled 1-cm electrode gap cuvette. Electroporation was performed with a time constant of 25 ms (25 mF capacitance; 1,000 W) at 2.25 kV (22.5 kV/cm). The pulsed cells were subjected to 1 h of premaintenance with warmed LB medium (~1 ml) at 37°C and plated on LB agar plates with ampicillin (100 μg/ml) as selective pressure. The plates were kept at 37°C for ~36 h. The positive colonies that carried the expected plasmids were verified by PCR, β-galactosidase (β-Gal) assay, and direct DNA sequencing.

Size exclusion chromatography. Bacterial FadR proteins used here originated from four sources: *E. coli* FadR (EcFadR), *V. cholerae* FadR (VcFadR), *V. alginolyticus* FadR (ValFadR), and *V. parahaemolyticus* FadR (VpFadR). The sizes of these regulatory proteins in solution were evaluated using the method of size exclusion chromatography. Gel filtrations were carried out using a Superdex 200 column (Pharmacia) run on an Äkta fast protein liquid chromatography (FPLC) system (GE Healthcare) (10, 15) at a flow rate of 0.5 ml/min in running buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.9). The peaks of the proteins of interest were sampled and determined by 15% SDS-PAGE.

Electrophoretic mobility shift assays (EMSAs). The function of the predicted FadR-binding site in front of *V. cholerae vc2105* (and/or its equivalent) was assessed using gel shift assays as we recently performed (21, 31), with minor modifications. In total, four sets of DNA probes (Table 2) were tested for *vc2105* and the equivalent genes *VH_01340*, *vp0834*, and *VF_0811* from *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio fischeri*, respectively. All the probes were prepared *in vitro* by annealing two complementary oligonucleotides (e.g., FadR-vc2105-F and FadR-vc2105-R) (Table 2) by incubation at 95°C in TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) for 15 min, followed by slow cooling to 25°C. Each digoxigenin (DIG)-labeled DNA probe (~0.2 pmol) was mixed with EcFadR and/or VcFadR (in appropriate concentrations) in the binding buffer (Roche) and kept for 15 min at room temperature. The DNA-protein mixtures were separated by native 7% PAGE and transferred onto nylon membrane by contact blotting-aided gel transfer (5). Finally, the chemical luminescence signals of the probes were detected by exposure of the nylon membrane to enhanced chemiluminescence (ECL) films (Amersham).

SPR. As we recently described (31), affinities for the *vc2105* probe binding to different versions of FadR (EcFadR, VcFadR, VpFadR, and ValFadR) were compared via surface plasmon resonance (SPR)-based measurements. The SPR experiments were carried out using a Biacore3000 instrument (GE Healthcare). In the SPR trials, the biotinylated

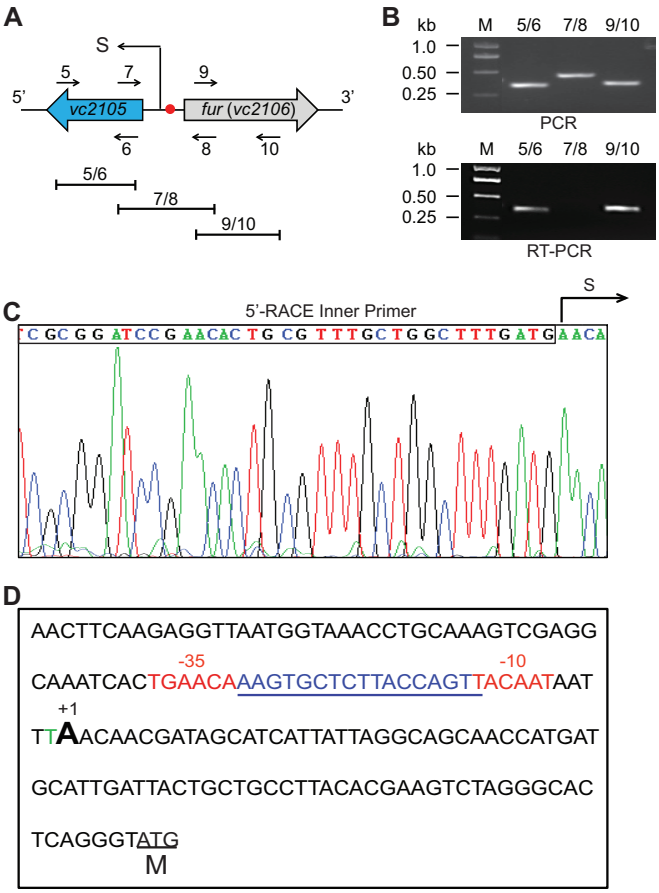

FIG 3 Transcriptional analyses of the *vc2105* gene. (A and B) Genetic organization (A) and transcriptional analyses (B) of the *vc2105* gene from *V. cholerae* are shown. Filled arrows represent *vc2105* and *fur* (*vc2106*). The numbered arrows (5, 6, 7, 8, 9, and 10) denote specific primers, whereas the short lines (labeled 5/6, 7/8, and 9/10) indicate the specific PCR amplicons. The transcription start site (S) is indicated in panel A. In the experiment shown in panel B, the PCR and RT-PCR products were separated by electrophoresis of a 1.5% agarose gel. Lane M, Trans2K Plus IIDNA Ladder (Transgen Biotech, Beijing, China); kb, kilobase pair. (C) Direct DNA sequencing of the 5′-RACE products to determine the transcription start site (S) for *vc2105*. (D) Fine mapping for the *vc2105* promoter. The regions of −35 and −10 are highlighted in red. The transcription start site is indicated (+1). The putative FadR-recognizable site is underlined in blue. M, translational initiation site.

vc2105 DNA probe was immobilized by streptavidin on the chip surface, and a dilution series of the tested FadR protein was passed over the chip surface for 2 min. In addition, the running buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.005% Tween 20) was eluted at a flow rate of 30 μl/min (31). A global data analysis program (BIAevaluation software) was used to determine kinetic parameters, and Origin software was utilized to generate final graphs.

β-Galactosidase assays. Bacterial cultures of *V. cholerae* (and/or *E. coli*) and its derivatives grown in either LB or RB medium were collected by spinning, washed with RB medium, and suspended in Z-buffer for measurement of β-Gal activity (4, 5, 36). The data were recorded in triplicate in more than three independent assays.

Bioinformatics analyses. In total, 10 bacterial FadR orthologues were used for multiple sequence alignments. The bacterial organisms included *E. coli*, *Salmonella enterica* (*S. enterica*), *Aliivibrio salmonicida*, *V. cholerae*, *V. splendidus*, *V. shilonii*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, and *V. fischeri*. The known (and/or predicted) FadR-binding sites and the promoter sequences of *vc2105* (and/or its equivalents) were all collected from the RegPrecise database (http://regprecise.lbl.gov/RegPrecise/index.jsp?). ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was employed to perform the multiple sequence alignments. Promoter prediction of the *vc2105* gene was carried out through the running of the corresponding DNA sequence on the PePPER web server (http://genome2d.molgenrug.nl/index.php/prokaryote-promoters) (37). The structural analyses were conducted via PyMol software.

RESULTS

The *Vibrio fad* regulon and *vc2105* promoter. Unlike its closely related enteric cousin *E. coli*, which has only one chromosome of 4.64 Mb (50.8% GC content) and encodes about 4,500 genes (38), the marine bacterium *V. cholerae* N16961 has evolved two genomes, one of which is 2.98 Mb (47.7% GC content) and encodes 2,690 genes and the other of which is 1.07 Mb (46.9% GC content) and corresponds to 1,003 genes (39). In the context of fatty acid metabolisms, the number of FadR-regulated *fad* and *fab* genes in *V. cholerae* (7 genes in total) is much lower that seen in *E. coli* (>12 *fad* and *fab* genes) (31, 40) (Fig. 1). It seemed unusual but not without precedent in that a similar scenario was recently revealed in another marine bacterium, *Shewanella* (31, 40). As the auxiliary member of β-oxidation machinery in *E. coli* (5), the *fadM* gene encoding a type III thioesterase is unexpectedly absent in the closely related *V. cholerae* from the same class, the *Gammaproteobacteria*. In contrast, *V. cholerae* seemed to have acquired a unique thioesterase-encoding gene (designated *vc2105* here), whose functional equivalents (with appreciable similarity ranging from 53.2% to 61.3%) are universally distributed in the genus *Vibrio* (Fig. 2A; see also Fig. S3 in the supplemental material). Unfortunately, functional complementation of *vc2105* into an array of *E. coli* mutants lacking one and/or multiple thioesterases gave an undetectable phenotype in that no colony/growth difference can be seen when the strains are maintained on minimal medium with acetate/long-chain acyl fatty acids as the sole carbon sources (data not shown), implying a cryptic function for this type of thioesterase. A similar scenario was seen in *E. coli* in that the removal of multiple thioesterase-encoding genes (up to six genes) from *E. coli* does not produce any apparent phenotypic changes (John E. Cronan, personal communication). However, a predicted FadR-recognizable palindrome (AAGTGCTCTTACCAGTT) is localized in the intergenic region between *fur* and *vc2105* of *V. cholerae* (Fig. 2A and 3; see also Fig. S1 in the supplemental material). Furthermore, sequence alignment analyses for the promoter regions pinpointed that an appreciably conserved FadR signal is consistently present in the most of species of the *Vibrio* genus (Fig. 2B; see also Fig. S1). Transcriptional analyses suggested that *vc2105* can be transcribed in a transcription unit with the opposite orientation of that of *fur* (Fig. 3A and B). The two combined approaches of 5′ rapid amplification of cDNA ends (5′-RACE) and PePPER web server-based prediction (37) were applied in mapping the transcriptional start site of *vc2105*, giving two adjacent nucleotides, A and T, as the transcriptional start site (Fig. 3C). Apparently, the putative FadR signal (AAGTGCTCTTACCAGTT) overlaps the region from −35 to −10 in front of the transcription start site (Fig. 3D), indicating that *vc2105* is negatively controlled by the FadR repressor.

Contrasting *Vibrio* FadR to *E. coli* FadR. The prototypical *E. coli fadR* protein product, FadR, harbors a polypeptide of 239 residues. However, all the *Vibrio* FadR orthologues seem unusual in that most of them are 279 aa long, having an extra insert of 40

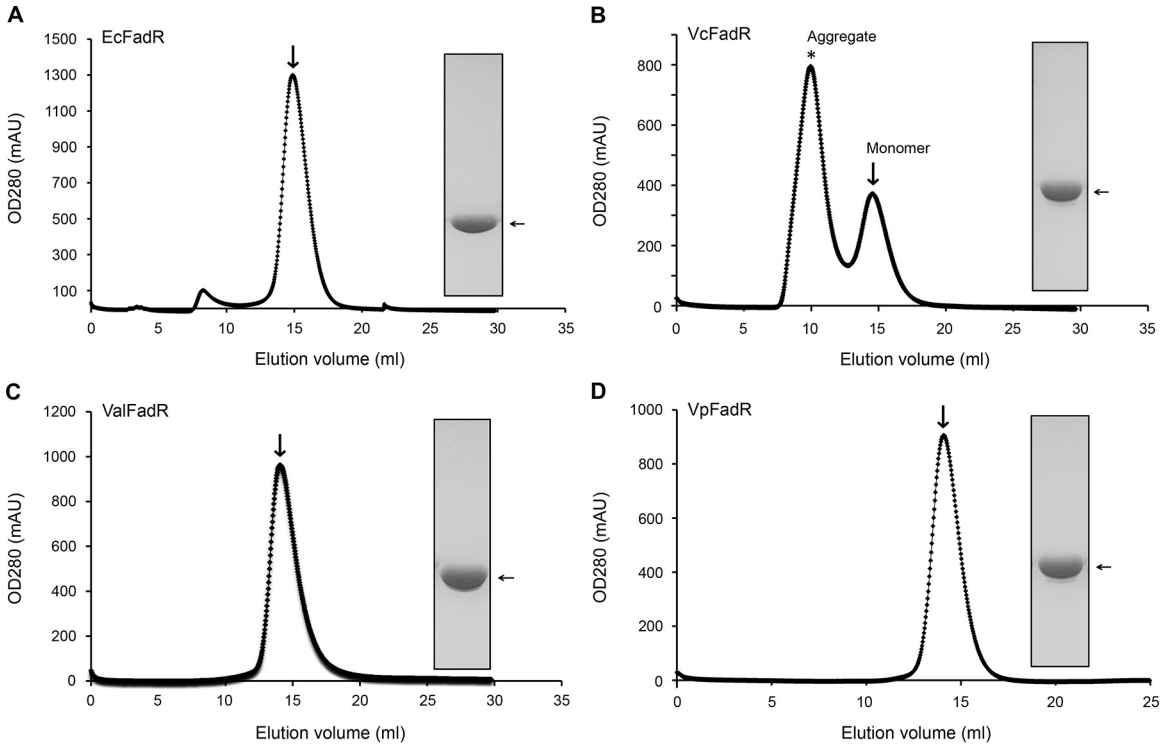

FIG 4 Gel filtration analyses for four bacterial FadR homologues. (A) Gel exclusion chromatographic profile of the *E. coli* FadR. (B) FPLC assay for the *V. cholerae* FadR. *, VcFadR protein aggregate. (C) Gel filtration analysis profile of the *V. alginolyticus* FadR. (D) FPLC profile for the *V. parahaemolyticus* FadR. All four FadR proteins from the three different *Vibrio* species and *E. coli* were run on a Superdex 200HR 10/30 column (GE Healthcare). The expected peak of the bacterial FadR protein was eluted at the position of 14.8 to 15.2 ml (indicated with an arrow). The inset shows the SDS-PAGE gel of the respective purified FadR protein. The apparent masses of the recombinant FadR proteins of *E. coli* and *V. cholerae* are ~27 kDa and ~31 kDa, respectively. Note that *V. cholerae* FadR easily precipitates in solution *in vitro*. *Ec*, *E. coli*; *Vc*, *V. cholerae*; *Val*, *V. alginolyticus*; *Vp*, *V. parahaemolyticus*; OD_{280}, optical density at 280 nm; AU, absorbance units.

residues relative to the sequence of *E. coli* FadR (see Fig. S2A in the supplemental material). The X-ray crystal structures of two different versions of the *Vibrio* FadR protein (that of *V. cholerae* FadR from Shi et al. [33] and of *V. alginolyticus* FadR from our group [Feng et al., submitted]) revealed that the 40-residue insert constitutes an extra functional domain/motif rich in α-helix functioning as a second ligand-binding site (see Fig. S2B to D). In light of the fact that this snapshot of the *Vibrio* FadR-ligand complex structure illustrates a novel FadR architecture with two unexpected ligand-binding sites, it seemed likely that *Vibrio* pathogens have evolved a new mechanism for fatty acid sensing (33) (Feng et al., submitted). For further functional analyses, four sets of the recombinant FadR proteins (EcFadR, VcFadR, ValFadR, and VpFadR) were prepared and purified to homogeneity (Fig. 4). The purity of the acquired protein was judged by SDS-PAGE (Fig. 4), and the protein identity was confirmed with liquid chromatography-mass spectrometry (data not shown).

Gel filtration-based analyses using a Superdex 200HR 10/30 column showed that the elution volumes of all the *Vibrio* FadR proteins (~14.8 ml; ~62 kDa) are consistently slightly smaller than those of the *E. coli* counterparts (~15.2 ml; ~55 kDa), much in agreement with the scenarios seen in our chemical cross-linking assays (data not shown). The FPLC profile suggested that the FadR proteins of both *E. coli* and *Vibrio* predominantly form a dimer in solution.

To probe the interplay between *vc2105* and the FadR proteins of different origins, we performed surface plasmon resonance (SPR)-based measurements. SPR results revealed that the binding affinities (K_D, the equilibrium dissociation constant) of *Vibrio* FadR proteins to *vc2105* (8.66×10^{-9} M for VcFadR, 9.47×10^{-9} M for ValFadR, and 10.5×10^{-9} M for VpFadR (Fig. 5B to D, respectively) are appreciably comparable to the value for EcFadR (8.53×10^{-9} M) (Fig. 5A). Consistent with the results with *Shewanella* FadR, the binding modes for FadR proteins of either *E. coli* or *Vibrio* are nearly 2:1 (a dimeric FadR protein is bound to a target DNA fragment) (data not shown).

Binding of VC2105 to FadR. Very recently, our EMSA-based explorations suggested that the FadR regulatory proteins of *E. coli*, *V. cholerae*, and *Shewanella oneidensis* are functionally exchangeable in the case of FadR-*fabA* interaction (31). To probe if this is true for VC2105 binding of FadR, we conducted gel shift assays similar to those previously described (31). As expected, the *vc2105* probe binds *E. coli* FadR (EcFadR) (Fig. 6A) and *V. cholerae* FadR (VcFadR) quite well (Fig. 6E), which is consistent with observations during SPR-based assays (Fig. 5A and B). In addition to *vc2105*, we also tested the other three equivalents (*VH_01340*, *vp0834*, and *VF_0811*) that are from *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio fischeri*, respectively (Fig. 2A and 3). As a result, EMSAs proved that the EcFadR can interact effectively with *VH_01340* (Fig. 6B), *vp0834* (Fig. 6C), and *VF_0811* (Fig. 6D), with appropriately comparable affinities. Unlike the scenarios seen with EcFadR, the VcFadR binds less tightly to the probes (including *VH_01340*, *vp0834*, and *VF_0811*) (Fig. 6F to H, respectively) in a protein dose-dependent manner; VcFadR also ex-

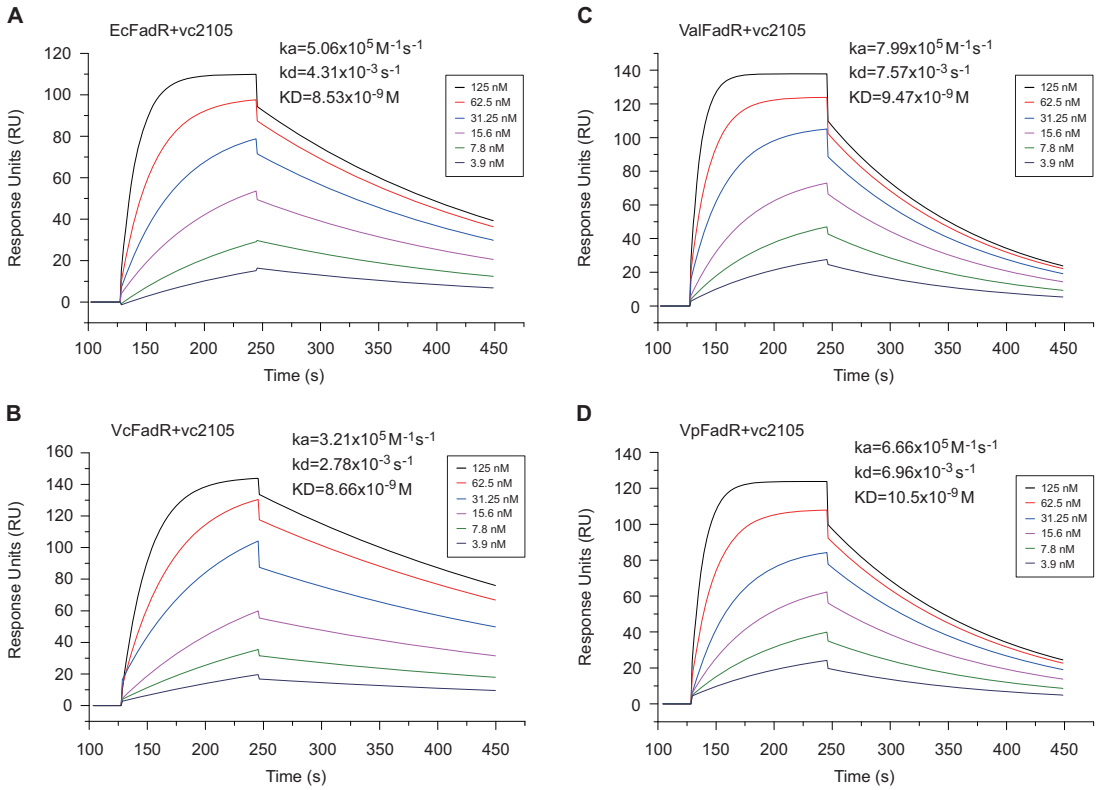

FIG 5 SPR-based dynamic assays for binding of *vc2105* to different FadR homologues. (A) SPR assay for physical interaction of *E. coli* FadR with *vc2105*. (B) SPR-based evaluation for dynamic binding of *V. cholerae* FadR to *vc2105*. (C) SPR-based determination of interplay between *V. alginolyticus* FadR and *vc2105*. (D) SPR analyses for direct binding of *V. parahaemolyticus* FadR to *vc2105*. k_a, association rate constant; k_d, dissociation rate constant.

hibited consistently a supershift band in the *in vitro* trials. Although this is unusual, it is not without precedent, as seen in similar scenarios of cross talk between VcFadR with both *plsB* (see Fig. S4A in the supplemental material) (32) and *fabA* (31). It seemed likely that the *Vibrio* FadR binds its cognate palindrome well and predominantly forms the supershift band in the gel shift assay *in vitro*. Also, the affinity of *vc2105* to *Vibrio* FadR seemed to be comparable to that of *plsB* (see Fig. S4). Given the accumulated data of *Vibrio* FadR protein combined with the newly resolved X-ray structure (33) and its previously reported superior robust regulatory ability (22), we believed that this unique EMSA profile might be relevant to the configuration shift due to the second ligand-binding pocket formed by the unusual 40-residue insert (see Fig. S2). Together, the FadR-recognizable palindromes of *vc2105* and its equivalents are functional for VcFadR as well as EcFadR (Fig. 6).

Reversal of VC2105 binding to FadR by long-chain acyl-CoA. Comparative analyses of the promoter sequences of VC2105 and its counterparts allowed us to conclude that these cognate FadR-specific palindromes predicted by Rodionov and coworkers (40) are quite conservative (Fig. 2 and 3). Moreover, our *in vitro* data validated that these FadR-binding sites are functional in binding to FadR proteins of diverse origins (Fig. 4 to 6). It is well known that long-chain (but not short-chain) fatty acyl-CoA species inactivate the activity of FadR in binding to cognate DNA targets (8, 17). Therefore, it is of much interest to test the behaviors of theses ligands toward *vc2105* and its equivalents. By employing the EMSA-based competition trials (Fig. 7; see also Fig. S4B in the supplemental material), we examined six acyl-CoA thioesters of different acyl chain lengths as follows: nonanoyl-CoA ($C_{9:0}$), decanoyl-CoA ($C_{10:0}$), palmitoyl-CoA ($C_{16:0}$), palmitoleic-CoA ($C_{16:1}$), stearoyl-CoA ($C_{18:0}$), and oleoyl-CoA ($C_{18:1}$). As anticipated, the medium-chain acyl-CoAs ($C_{9:0}$ and $C_{10:0}$) failed to impair the interaction of the *vc2105* gene with either EcFadR (Fig. 7A) or VcFadR (Fig. 7E). Similarly, the binding of three other homologous genes (including *VH_01340* [Fig. 7B and F], *vp0834* [Fig. 7C and G], and *VF_0811* [Fig. 7D and H]) to EcFadR (VcFadR) was not affected by the addition of the medium-chain acyl-CoAs. In contrast, the presence of long-chain acyl-CoA species ($C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$) efficiently dissociated the cognate DNA probes of *vc2105* (or its equivalents) from either EcFadR (Fig. 7A to D) or VcFadR (Fig. 7E to H). Consistent with the scenario of *plsB* (see Fig. S4B in the supplemental material) and our previous observations (5, 15, 32), it is clear that the species of long-chain but not medium-chain acyl-CoAs occupy competitively the ligand-binding sites of both EcFadR and VcFadR and subsequently release the EcFadR (VcFadR) protein from the promoter regions of *vc2105* (and/or its equivalents) (Fig. 7). That is why we believe that long-chain acyl-CoAs negotiate the transcription of *vc2105* (and/or its equivalents) through the competitive interaction with the VcFadR regulatory protein.

Repression of *V. cholerae* VC2105 expression by FadR and its induction by oleate *in vivo*. To assess the physiological role of FadR in regulating *V. cholerae* VC2105 expression, we constructed two types of LacZ transcriptional fusion reporter systems, one of which is the single-copy *vc2105-lacZ* fusion integrated into the

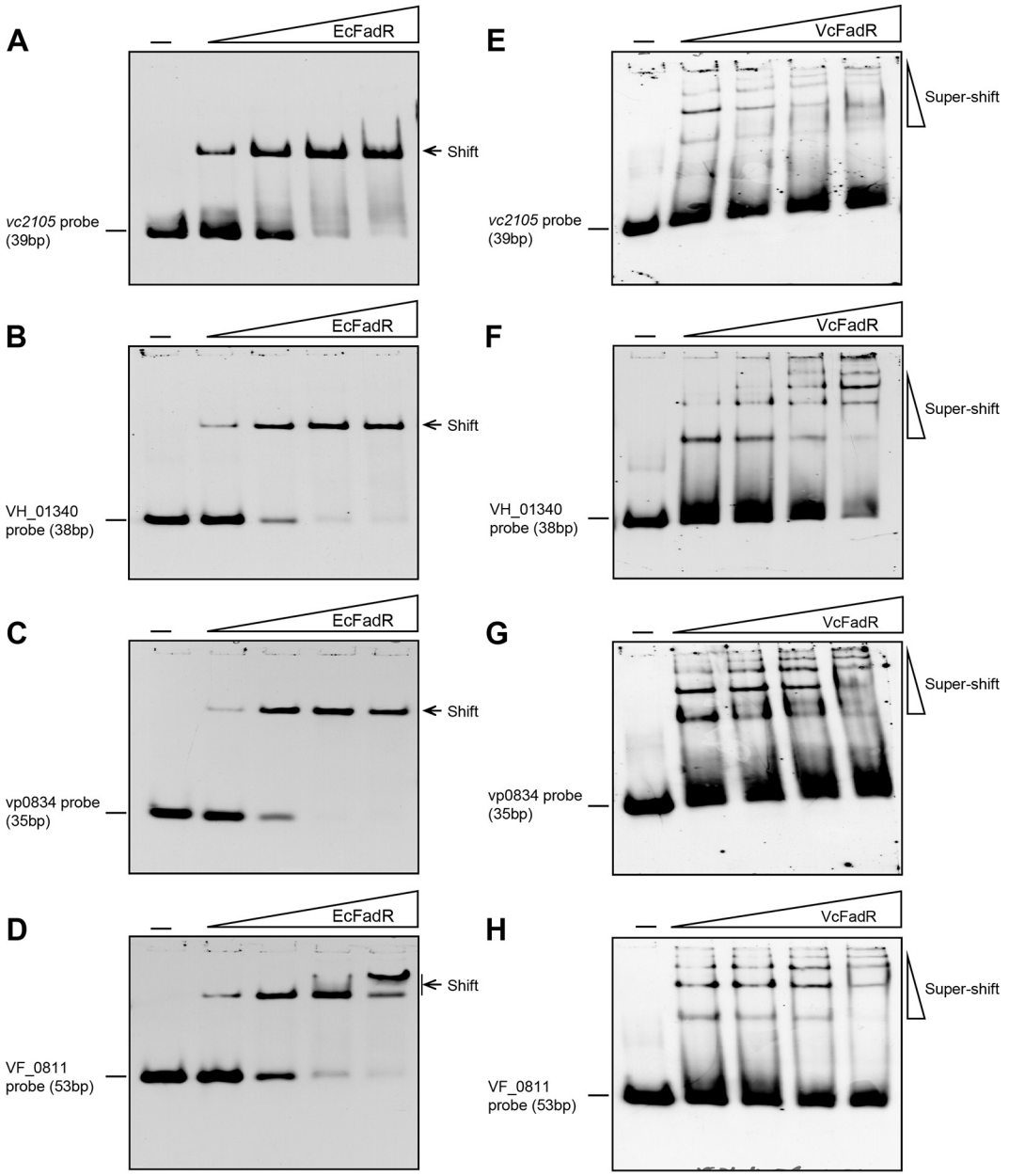

FIG 6 Binding of FadR to the promoter of *vc2105* (and/or its equivalent). (A) EMSAs for *E. coli* FadR binding to the *vc2105* probe. (B) *E. coli* FadR binds to the *VH_01340* probe. (C) Gel shift assay for the interaction of the *vp0834* probe with the *E. coli* FadR. (D) Evidence that the *VF_0811* probe has a functional palindrome recognized by *E. coli* FadR. (E) EMSA-based determination of *vc2105* probe binding to *V. cholerae* FadR. (F) Binding of *V. cholerae* FadR to the *VH_01340* probe. (G) The *vp0834* probe interacts with *V. cholerae* FadR. (H) The *VF_0811* probe is functional in a palindrome recognized by *E. coli* FadR. The gel shift experiments were carried out using 7% native PAGE, and a representative result is given. In each assay, levels of FadR protein (EcFadR and VcFadR) are indicated above the panels (0.1, 0.5, 2, and 5 pmol); all DIG-labeled probes (for *VC2105*, *VH_01340*, *vp0834*, and *VF_0811*) were consistently supplemented to 0.2 pmol. The minus sign above the first lane of each panel denotes the absence of FadR protein.

chromosome of *E. coli*, an alternative model organism [such as FYJ360 (wild type) and FYJ361 (Δ*fadR*)] (Table 1); the other is the plasmid pTL61T-borne *vc2105-lacZ* fusion (low copy number) engineered in *V. cholerae* [e.g, FYJ656 (wild type) and FYJ658 (Δ*fadR*)] (Table 1). Given that VcFadR is functionally replaced by EcFadR, we first evaluated the role of FadR using a system whereby *E. coli* carries a *vc2105-lacZ* transcriptional fusion (single copy). As expected, measurement of the β-Gal level showed that removal of the *fadR* gene from *E. coli* produced a 2- to 2.5-fold increase in *vc2105* expression (Fig. 8A). Subsequently, we attempted to knock out the *fadR* gene from *V. cholerae*, an organism less amenable than *E. coli* to genetic manipulations. Assays for LacZ activity revealed that the level of transcription of plasmid pTL61T-borne *vc2105* was around 2.5-fold higher in the Δ*fadR* strain (FYJ658) than in the wild-type parent strain, FYJ656 (Fig. 8B). Obviously, the results obtained using the alternative organism *E. coli* are in satisfactory agreement with those seen in the native organism *V. cholerae* (Fig. 8). The regulatory strength/extent of *vc2105* by FadR

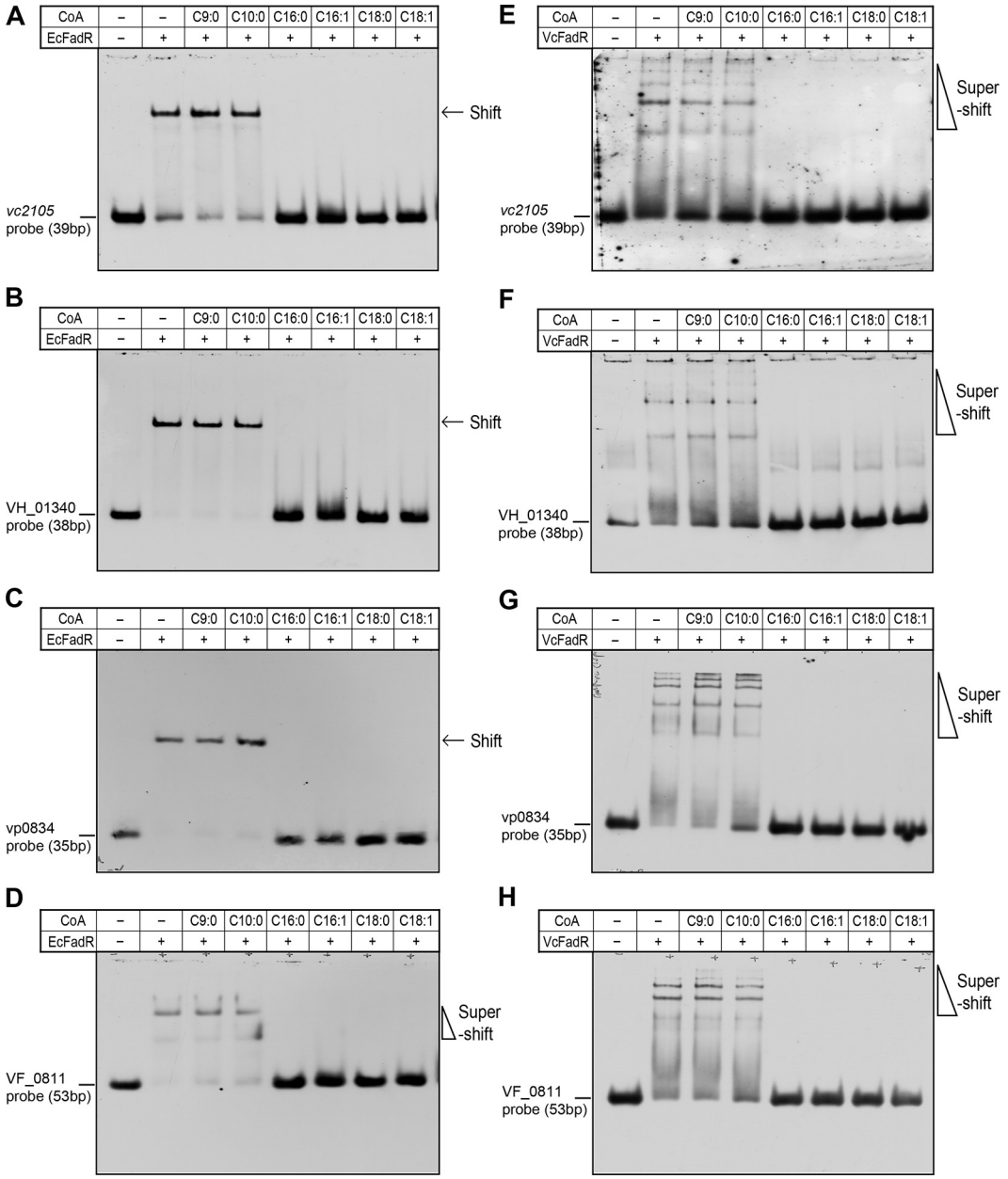

FIG 7 LC acyl-CoA impairs binding of FadR to the promoter of *vc2105* (and/or its equivalent). EMSA-aided visualization for effects of medium- and long-chain acyl-CoA species on binding of the *vc2105* probe to both EcFadR (A) and VcFadR (E). Presence of long-chain acyl-CoA species impairs interaction between the *VH_01340* probe with both EcFadR (B) and VcFadR (F). Long-chain acyl-CoA species release the *vp0834* probe from both EcFadR (C) and VcFadR (G). Addition of long-chain acyl-CoA species results in dysfunction of both EcFadR (D) and VcFadR (H) in binding to the *VF_0811* probe. In the binding reaction mixtures (20 μl in total), the FadR proteins (EcFadR and VcFadR; ~5 pmol) were incubated with 0.2 pmol of DIG-labeled DNA probes (for *vc2105*, *VH_01340*, *vp0834*, and *VF_0811*). When necessary, acyl-CoA species of different acyl lengths (~50 pmol) were added, as we recently described. The gel shift assays were conducted more than three times using 7% native PAGE, and a representative result is given. The shifted DNA probe band is indicated with an arrow, whereas the supershift is highlighted with a triangle. $C_{9:0}$, nonanoyl-CoA; $C_{10:0}$, decanoyl-CoA; $C_{16:0}$, palmitoyl-CoA; $C_{16:1}$, palmitoleic-CoA; $C_{18:0}$, stearoyl-CoA; $C_{18:1}$, oleoyl-CoA. A plus sign denotes addition of either FadR proteins or acyl-CoA species, whereas a minus sign denotes the absence of either FadR protein or acyl-CoA species.

is quite similar to scenarios seen in the *E. coli fadD* gene encoding acyl-CoA synthetase (6) and *V. cholerae plsB* that encodes glycerol-3-phosphate (*sn*-glycerol-3-P, G3P) acyltransferase, which catalyzes the first committed step of the membrane phospholipid pathway (32).

It is known that the regulation (repression and/or activation) by *E. coli* FadR can be greatly reversed by the presence of oleic acid in the medium and that the conversion of oleic acid to oleoyl-CoA, a physiological effector, acts as a robust antagonist of FadR binding of its cognate operator sites (32). Thus, it was not unexpected that the supplementation of 4 mM oleate into RB medium increased the activity of the *vc2105* promoter by about 2.5-fold in *E. coli* (Fig. 9A). We also addressed the effects of fatty acid supplementation on *vc2105* transcription in the biological context of *V.*

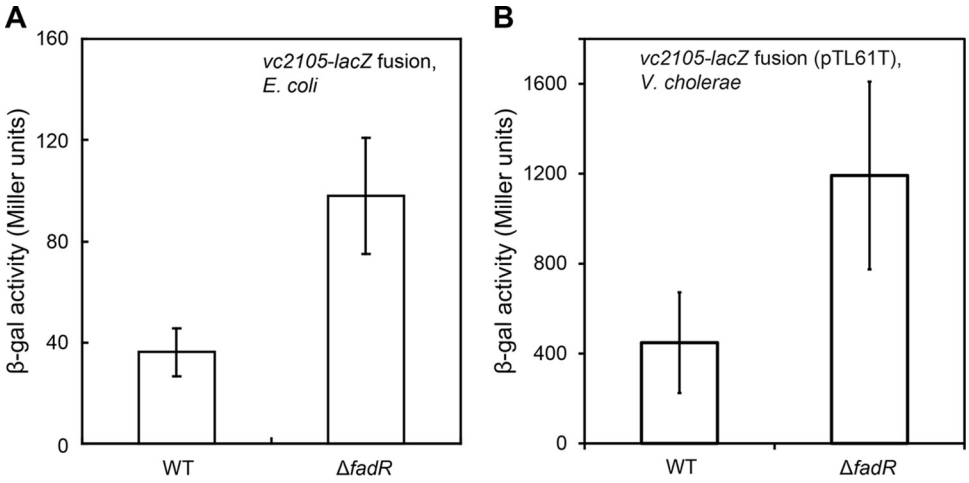

FIG 8 FadR represses the expression of *vc2105*. (A) The β-Gal level of the *vc2105-lacZ* transcriptional fusion is augmented upon the removal of FadR regulator in the alternative model organism *E. coli*. (B) LacZ activity of the plasmid pTL61T-borne *vc2105-lacZ* transcriptional fusion in the Δ*fadR* mutant is increased 2- to 2.5-fold relative to the wild-type level of *V. cholerae*. Bacterial cultures in mid-log phase were collected to assay LacZ (β-Gal) activity. The *E. coli* strains used here, FYJ360 (wild type [WT]) and FYJ361 (Δ*fadR*), carry a single copy of the *vc2105-lacZ* transcriptional fusion integrated on the chromosome, whereas the *V. cholerae* strains, FYJ656 (wild type) and FYJ658 (Δ*fadR*), contain the plasmid pTL61T-borne *vc2105-lacZ* transcriptional fusion. $P < 0.01$ (Student *t* test).

cholerae. The β-Gal activity derived from the plasmid pTL61T-borne *vc2105-lacZ* transcriptional fusion replicating in *V. cholerae* was elevated by around 2-fold upon the addition of oleic acid in RB medium (Fig. 9B). The extent of induced expression of *vc2105* by oleate is comparable to that of derepression by inactivation of the *fadR* gene.

Given that the FadR cognate site localizes in the intergenic region between *fur* and *vc2105* (and/or its equivalent), we raised the possibility that *fur* might also be controlled by FadR in *Vibrio*. To address this question, we engineered the fusion of the *fur* promoter to a *lacZ* reporter gene. β-Gal analyses suggested that the *fur* promoter does not respond to the presence of oleate (see Fig. S5 in the supplemental material), ruling out the possible involvement of FadR with *fur*. Therefore, we believed that *Vibrio* FadR negatively regulates the transcription of *vc2105* (and/or its counterpart) with a regulatory strength similar to that of *V. cholerae plsB* (32).

No role of the *vc2105* gene in bacterial virulence-associated phenotypes. Given that the *fadD* gene, an inner membrane-bound member of the *Vibrio fad* regulon (28, 29), is implicated in bacterial pathogenesis through the expression of the two major virulence genes, *ctxAB* (cholera toxin [CT]-encoding operon) and *tcpA* (toxin-coregulated pilus A [TCP]), which were drastically downregulated in the Δ*fadD* mutant, we were interested in revealing the possible relevance of *vc2105*, an auxiliary *fad* member, to *Vibrio* virulence. Using homologous recombination, we deleted the *vc2105* in frame from *V. cholerae* (Fig. 10A and B). It seemed likely that the removal of *vc2105* from *V. cholerae* would not influence either bacterial growth (Fig. 10C) or the colony phenotype (Fig. 10D). In subsequent assays for swarming, a virulence-associated phenotype, no apparent difference was observed between the Δ*vc2105* mutant and the wild-type strain (Fig. 10E). To test the expression levels of CT and TCP, we engineered two *lacZ* transcriptional fusions (P*ctxAB-lacZ* and P*tcpA-lacZ*). Unlike the scenarios seen with the *fadD* gene, inactivation of *vc2105* failed to exert any significant effects on expression levels of the two leading virulence genes/loci (*ctxAB* and *tcpA*) (Fig. 10F). Together, these results indicate that the *vc2105* gene has no role in bacterial pathogenesis.

DISCUSSION

Cholera is a serious diarrheal disease and a widespread food-borne disease that is endemic in more than 50 countries. The causative agent, *V. cholerae*, poses a great challenge to global public health. Long-term evolution employed by this marine bacterium has allowed it to develop unusual strategies to survive in its fatty acid-limited ocean environment/niche habitation. Unlike the scenario observed in *E. coli*, some species of *Vibrio*, like *V. cholerae*, have evolved a novel mechanism that ensures the exact expression of *plsB*, the first gene of membrane phospholipid synthesis, whose physiological advantage might be to allow better scavenging the trace fatty acids available from the inhabited environment and/or host (32). In the context of such an unusual lipid metabolism, it is not surprising that *Vibrio* harbors a new gene (e.g., *vc2105*) encoding a putative thioesterase with some unknown role implicated in the β-oxidation of certain fatty acids. We have expressed the recombinant VC2105 protein product (a polypeptide 124 aa long) (see Fig. S3A in the supplemental material) and verified its identity with 70% coverage by liquid chromatography-mass spectrometry (data not shown). Structural modeling suggested that VC2105 shares a similar structural architecture and a "hot dog-like" folding motif with a *Pseudomonas* thioesterase (PDB accession number 1SH8) (see Fig. S3B to D in the supplemental material). Phylogenetic analyses indicated that VC2105 and its homologues from other *Vibrio* species constitute a subclade distinct from the known PaaI thioesterase (see Fig. S3E). Unfortunately, we failed to detect the activity of this thioesterase *in vitro* and *in vivo*. Given that the *E. coli* mutants lacking multiple genes annotated as putative thioesterases are indistinguishable from those of the parental strains, we preferred to temporarily speculate that VC2105 might be only an auxiliary member of the *fad* regulon without an apparent phenotype under normal growth conditions.

Our data collected here suggest that VC2105 (and/or its equivalents) is a new member implicated in the *Vibrio fad* regulon. The expression of VC2105 has some characteristics in common with the expression of the other members of the *E. coli* regulon but also differs markedly in other parameters. Since VC2105 is generally

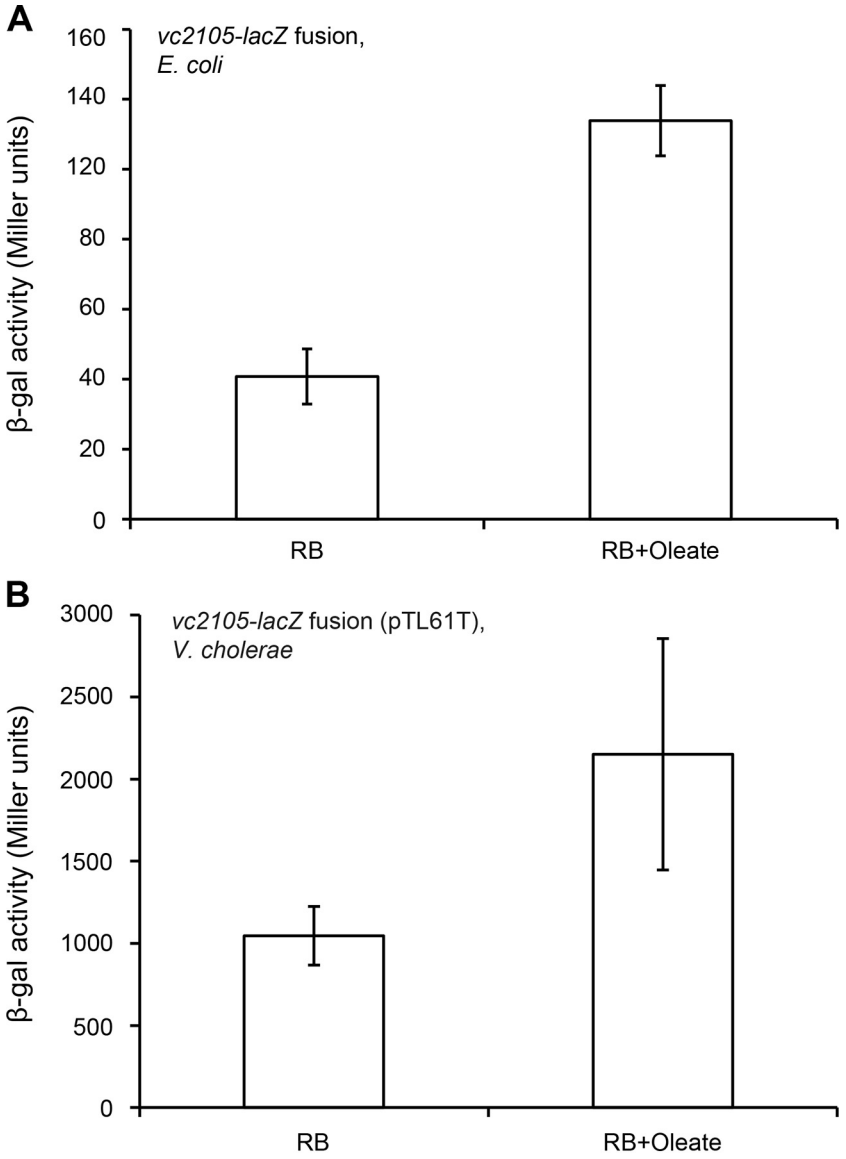

FIG 9 Induction of *Vibrio vc2105* expression by oleate. (A) Transcription of *V. cholerae vc2105* is activated in the alternative model organism *E. coli* upon oleic acid supplementation. (B) Induction of *V. cholerae vc2105* expression in the presence of oleate. The *E. coli* strain FYJ360 used in the experiment shown in panel A carries a single copy of the *vc2105-lacZ* transcriptional fusion which is integrated on chromosomes. Strain FYJ656. (*V. cholerae* carrying pTL61T-P*vc2105*, a plasmid encoding a *vc2105-lacZ* transcriptional fusion) was assayed in the experiment shown in panel B. Overnight bacterial cultures were collected to assay LacZ activity. Values for β-Gal activity represent the means ± standard deviations from no fewer than three independent experiments. When required, oleate was added at the level of 4 mM. $P < 0.01$ (Student *t* test).

similar to FadM (thioesterase III), an auxiliary member of the *E. coli fad* regulon that is only weakly induced by oleate (5), its induction level is also estimated to be about 2-fold (Fig. 9). In contrast, the induction of VC2105 is significantly lower than that of *E. coli fadBA* (10- to 20-fold), a major member of the β-oxidation machinery (6), implying a minor role in the context of fatty acid degradation. It seems true that the number of the FadR-specific sites in the *fad* regulon does not guarantee the extent of regulation/strength in any straightforward manner. Although VC2105 and *E. coli fadBA* both have only one FadR-binding site, VC2105 is weakly regulated by FadR, which is contradictory to the highly regulated *fadBA* promoter (6). In contrast, the *fadD* and *fadL* genes each have two FadR-binding sites (6) and exhibit a weak regulatory profile comparable to that of VC2105 (Fig. 8). The fact that the expression level of *vc2105*, reflected by its promoter-driven LacZ activity in *E. coli*, is only half that of FadM (Fig. 8A) (5) implies a limited/minor cellular role. Unlike the other members of the *E. coli fad* regulon, most of which are activated by the functional cyclic AMP (cAMP) receptor protein (CRP)-cAMP complex, FadM is repressed by this regulatory system, and we failed to observe any evidence that VC2105 expression is connected with cAMP signaling.

In summary, transcriptional regulation of VC2105 by FadR defines a new member of the *Vibrio fad* regulon. It extends our understanding of the diversified regulatory mechanisms by which *Vibrio* catabolizes/utilizes exogenous fatty acids for its survival

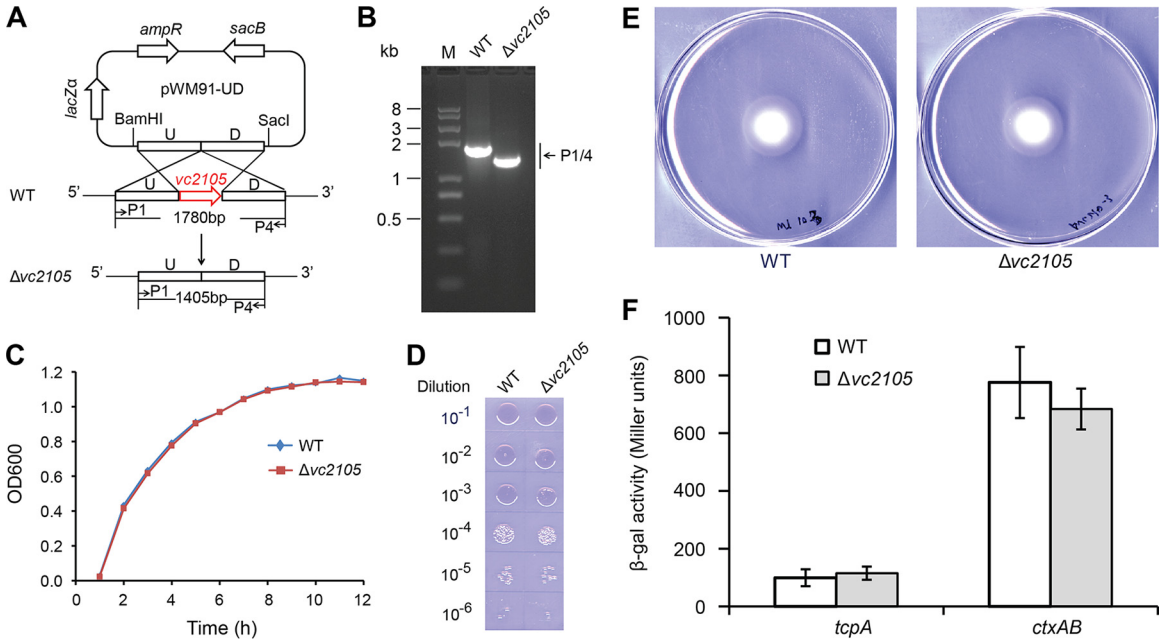

FIG 10 Inactivation of *V. cholerae vc2105* does not affect bacterial growth, the virulence-associated phenotype, or virulence factor expression. (A) Scheme for the strategy for knockout of the *vc2105* gene (in red). The upstream (U) and downstream (D) regions of the *vc2105* gene are indicated. The specific P1/P4 primer pair was used to determine the inactivation mutant of the *vc2105* gene. (B) PCR-based determination of the *vc2105* inactivation mutant of *V. cholerae*. The P1/P4 primers are indicated. The PCR amplicon from the wild-type strain is estimated to be about 1.8 kb, whereas the counterpart amplified from the Δ*vc2105* mutant is expected to be around 1.4 kb (A). (C) Growth curve of the Δ*vc2105* mutant in comparison with that of its parental strain of *V. cholerae*. (D) Colony phenotype of the Δ*vc2105* mutant relative to that of the wild-type strain of *V. cholerae*. The bacterial cultures in log phase were spotted on LB agar plates in a series of dilutions and kept overnight at 37°C. (E) Swarming assays for the role of the *vc2105* gene in the *V. cholerae* virulence-associated phenotype. Ten microliters of the log-phase culture (OD_{600} of 0.7) in appropriate dilution was spotted on semisolid LB agar plates supplemented with only 0.3% agar and maintained overnight at 37°C. (F) Measurement of the LacZ (β-Gal) activity to probe the possible effect exerted by *vc2105* deletion on the expression level of the two well-known virulence-associated genes/loci, *ctxAB* and *tcpA*. Bacterial cultures in mid-log phase were collected to perform the β-Gal analyses. The *V. cholerae* derivatives used here (wild-type and Δ*vc2105* strains) consistently carry the pTL61T-borne transcriptional fusions of either *ctxAB-lacZ* or *tcpA-lacZ* (Table 2). Note that no significant difference was observed.

and even its infection life cycle. Given that *Vibrio* FadR contributes to bacterial infections, our findings might be added into the knowledge contributing to the discovery of promising therapeutics/drug targets against *Vibrio* infections.

ACKNOWLEDGMENTS

We thank Yuan Lin for technical help in SPR analyses and Dmitry Rodionov for constructive discussion on prediction of bacterial FadR regulator and cognate targets. We are grateful to Swaminath Srinivas for critical reading of the manuscript.

Y.F. designed this project; Y.F., R.G., J.L., and H.Z. performed experiments and analyzed the data; Y.F. contributed reagents and tools; Y.F. and R.G. wrote the manuscript.

FUNDING INFORMATION

This work was supported by National Natural Science Foundation of China (NSFC) (31570027) and Zhejiang Provincial Natural Science Foundation of China (LR15H190001). Y.F. is a recipient of the Young 1000 Talents award.

REFERENCES

1. **Marrakchi H, Zhang YM, Rock CO.** 2002. Mechanistic diversity and regulation of Type II fatty acid synthesis. Biochem Soc Trans **30:**1050–1055. http://dx.doi.org/10.1042/bst0301050.
2. **Fujita Y, Matsuoka H, Hirooka K.** 2007. Regulation of fatty acid metabolism in bacteria. Mol Microbiol **66:**829–839. http://dx.doi.org/10.1111/j.1365-2958.2007.05947.x.
3. **Cronan JE.** 4 January 2014. Biotin and lipoic acid: synthesis, attachment, and regulation. EcoSal Plus 2014 http://dx.doi.org/10.1128/ecosalplus.ESP-0001-2012.
4. **Feng Y, Cronan JE.** 2009. *Escherichia coli* unsaturated fatty acid synthesis: complex transcription of the *fabA* gene and *in vivo* identification of the essential reaction catalyzed by FabB. J Biol Chem **284:**29526–29535. http://dx.doi.org/10.1074/jbc.M109.023440.
5. **Feng Y, Cronan JE.** 2009. A new member of the *Escherichia coli fad* regulon: transcriptional regulation of *fadM* (*ybaW*). J Bacteriol **191:**6320–6328. http://dx.doi.org/10.1128/JB.00835-09.
6. **Feng Y, Cronan JE.** 2012. Crosstalk of *Escherichia coli* FadR with global regulators in expression of fatty acid transport genes. PLoS One **7:**e46275. http://dx.doi.org/10.1371/journal.pone.0046275.
7. **Campbell JW, Morgan-Kiss RM, Cronan JE, Jr.** 2003. A new *Escherichia coli* metabolic competency: growth on fatty acids by a novel anaerobic beta-oxidation pathway. Mol Microbiol **47:**793–805. http://dx.doi.org/10.1046/j.1365-2958.2003.03341.x.
8. **Henry MF, Cronan JE, Jr.** 1992. A new mechanism of transcriptional regulation: release of an activator triggered by small molecule binding. Cell **70:**671–679. http://dx.doi.org/10.1016/0092-8674(92)90435-F.
9. **Henry MF, Cronan JE, Jr.** 1991. *Escherichia coli* transcription factor that both activates fatty acid synthesis and represses fatty acid degradation. J Mol Biol **222:**843–849. http://dx.doi.org/10.1016/0022-2836(91)90574-P.
10. **Feng Y, Cronan JE.** 2011. Complex binding of the FabR repressor of bacterial unsaturated fatty acid biosynthesis to its cognate promoters. Mol Microbiol **80:**195–218. http://dx.doi.org/10.1111/j.1365-2958.2011.07564.x.
11. **Zhu K, Zhang YM, Rock CO.** 2009. Transcriptional regulation of membrane lipid homeostasis in *Escherichia coli*. J Biol Chem **284:**34880–34888. http://dx.doi.org/10.1074/jbc.M109.068239.
12. **Zhang YM, Marrakchi H, Rock CO.** 2002. The FabR (YijC) transcription

factor regulates unsaturated fatty acid biosynthesis in *Escherichia coli*. J Biol Chem **277:**15558–15565. http://dx.doi.org/10.1074/jbc.M201399200.
13. **Iram SH, Cronan JE.** 2006. The β-oxidation systems of *Escherichia coli* and *Salmonella enterica* are not functionally equivalent. J Bacteriol **188:**599–608. http://dx.doi.org/10.1128/JB.188.2.599-608.2006.
14. **Clark D.** 1981. Regulation of fatty acid degradation in *Escherichia coli*: analysis by operon fusion. J Bacteriol **148:**521–526.
15. **Feng Y, Cronan JE.** 2010. Overlapping repressor binding sites result in additive regulation of *Escherichia coli* FadH by FadR and ArcA. J Bacteriol **192:**4289–4299. http://dx.doi.org/10.1128/JB.00516-10.
16. **Campbell JW, Cronan JE, Jr.** 2001. *Escherichia coli* FadR positively regulates transcription of the *fabB* fatty acid biosynthetic gene. J Bacteriol **183:**5982–5990. http://dx.doi.org/10.1128/JB.183.20.5982-5990.2001.
17. **Cronan JE, Jr.** 1997. In vivo evidence that acyl coenzyme A regulates DNA binding by the *Escherichia coli* FadR global transcription factor. J Bacteriol **179:**1819–1823.
18. **Xu Y, Heath RJ, Li Z, Rock CO, White SW.** 2001. The FadR·DNA complex: transcriptional control of fatty acid metabolism in *Escherichia coli*. J Biol Chem **276:**17373–17379. http://dx.doi.org/10.1074/jbc.M100195200.
19. **van Aalten DM, DiRusso CC, Knudsen J.** 2001. The structural basis of acyl coenzyme A-dependent regulation of the transcription factor FadR. EMBO J **20:**2041–2050. http://dx.doi.org/10.1093/emboj/20.8.2041.
20. **van Aalten DM, DiRusso CC, Knudsen J, Wierenga RK.** 2000. Crystal structure of FadR, a fatty acid-responsive transcription factor with a novel acyl coenzyme A-binding fold. EMBO J **19:**5167–5177. http://dx.doi.org/10.1093/emboj/19.19.5167.
21. **Zhang Y, Gao R, Ye H, Wang Q, Feng Y.** 2014. A new glimpse of FadR-DNA crosstalk revealed by deep dissection of the *E. coli* FadR regulatory protein. Protein Cell **5:**928–939. http://dx.doi.org/10.1007/s13238-014-0107-3.
22. **Iram SH, Cronan JE.** 2005. Unexpected functional diversity among FadR fatty acid transcriptional regulatory proteins. J Biol Chem **280:**32148–32156. http://dx.doi.org/10.1074/jbc.M504054200.
23. **Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J.** 2005. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Appl Environ Microbiol **71:**5107–5115. http://dx.doi.org/10.1128/AEM.71.9.5107-5115.2005.
24. **Boyd EF, Carpenter MR, Chowdhury N, Cohen AL, Haines-Menges BL, Kalburge SS, Kingston JJ, Lubin JB, Ongagna-Yhombi SY, Whitaker WB.** 2015. Post-genomic analysis of members of the family *Vibrionaceae*. Microbiol Spectr **3**(5):VE-0009-2014. http://dx.doi.org/10.1128/microbiolspec.VE-0009-2014.
25. **Plecha SC, Withey JH.** 2015. Mechanism for inhibition of *Vibrio cholerae* ToxT activity by the unsaturated fatty acid components of bile. J Bacteriol **197:**1716–1725. http://dx.doi.org/10.1128/JB.02409-14.
26. **Withey JH, Nag D, Plecha SC, Sinha R, Koley H.** 2015. Conjugated linoleic acid reduces cholera toxin production *in vitro* and *in vivo* by inhibiting *Vibrio cholerae* ToxT activity. Antimicrob Agents Chemother **59:**7471–7476. http://dx.doi.org/10.1128/AAC.01029-15.
27. **Brown RN, Gulig PA.** 2008. Regulation of fatty acid metabolism by FadR is essential for *Vibrio vulnificus* to cause infection of mice. J Bacteriol **190:**7633–7644. http://dx.doi.org/10.1128/JB.01016-08.
28. **Ray S, Chatterjee E, Chatterjee A, Paul K, Chowdhury R.** 2011. A *fadD* mutant of *Vibrio cholerae* is impaired in the production of virulence factors and membrane localization of the virulence regulatory protein TcpP. Infect Immun **79:**258–266. http://dx.doi.org/10.1128/IAI.00663-10.
29. **Chatterjee E, Chowdhury R.** 2013. Reduced virulence of the *Vibrio cholerae fadD* mutant is due to induction of the extracytoplasmic stress response. Infect Immun **81:**3935–3941. http://dx.doi.org/10.1128/IAI.00722-13.
30. **Chatterjee A, Dutta PK, Chowdhury R.** 2007. Effect of fatty acids and cholesterol present in bile on expression of virulence factors and motility of *Vibrio cholerae*. Infect Immun **75:**1946–1953. http://dx.doi.org/10.1128/IAI.01435-06.
31. **Zhang H, Zheng B, Gao R, Feng Y.** 2015. Binding of *Shewanella* FadR to the *fabA* fatty acid biosynthetic gene: implications for contraction of the *fad* regulon. Protein Cell **6:**667–679. http://dx.doi.org/10.1007/s13238-015-0172-2.
32. **Feng Y, Cronan JE.** 2011. The *Vibrio cholerae* fatty acid regulatory protein, FadR, represses transcription of *plsB*, the gene encoding the first enzyme of membrane phospholipid biosynthesis. Mol Microbiol **81:**1020–1033. http://dx.doi.org/10.1111/j.1365-2958.2011.07748.x.
33. **Shi W, Kovacikova G, Lin W, Taylor RK, Skorupski K, Kull FJ.** 2015. The 40-residue insertion in *Vibrio cholerae* FadR facilitates binding of an additional fatty acyl-CoA ligand. Nat Commun **6:**6032. http://dx.doi.org/10.1038/ncomms7032.
34. **Joelsson A, Liu Z, Zhu J.** 2006. Genetic and phenotypic diversity of quorum-sensing systems in clinical and environmental isolates of *Vibrio cholerae*. Infect Immun **74:**1141–1147. http://dx.doi.org/10.1128/IAI.74.2.1141-1147.2006.
35. **Metcalf WW, Jiang W, Daniels LL, Kim SK, Haldimann A, Wanner BL.** 1996. Conditionally replicative and conjugative plasmids carrying *lacZα* for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35:**1–13. http://dx.doi.org/10.1006/plas.1996.0001.
36. **Miller JH.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **de Jong A, Pietersma H, Cordes M, Kuipers OP, Kok J.** 2012. PePPER: a webserver for prediction of prokaryote promoter elements and regulons. BMC Genomics **13:**299. http://dx.doi.org/10.1186/1471-2164-13-299.
38. **Blattner FR, Plunkett G, III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1462. http://dx.doi.org/10.1126/science.277.5331.1453.
39. **Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Varnathevan J, Bass S, Haiying W, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483. http://dx.doi.org/10.1038/35020000.
40. **Rodionov DA, Novichkov PS, Stavrovskaya ED, Rodionova IA, Li X, Kazanov MD, Ravcheev DA, Gerasimova AV, Kazakov AE, Kovaleva GY, Permina EA, Laikova ON, Overbeek R, Romine MF, Fredrickson JK, Arkin AP, Dubchak I, Osterman AL, Gelfand MS.** 2011. Comparative genomic reconstruction of transcriptional networks controlling central metabolism in the *Shewanella genus*. BMC Genomics **12**(Suppl 1):S3. http://dx.doi.org/10.1186/1471-2164-12-S1-S3.
41. **Connell TD, Martone AJ, Holmes RK.** 1995. A new mobilizable cosmid vector for use in *Vibrio cholerae* and other gram-negative bacteria. Gene **153:**85–87. http://dx.doi.org/10.1016/0378-1119(94)00804-2.
42. **Guzman LM, Belin D, Carson MJ, Beckwith J.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. J Bacteriol **177:**4121–4130.
43. **Haldimann A, Wanner BL.** 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J Bacteriol **183:**6384–6393. http://dx.doi.org/10.1128/JB.183.21.6384-6393.2001.
44. **Bellair M, Withey JH.** 2008. Flexibility of *Vibrio cholerae* ToxT in transcription activation of genes having altered promoter spacing. J Bacteriol **190:**7925–7931. http://dx.doi.org/10.1128/JB.00512-08.
45. **Withey JH, Dirita VJ.** 2005. *Vibrio cholerae* ToxT independently activates the divergently transcribed *aldA* and *tagA genes*. J Bacteriol **187:**7890–7900. http://dx.doi.org/10.1128/JB.187.23.7890-7900.2005.
46. **Linn T, St Pierre R.** 1990. Improved vector system for constructing transcriptional fusions that ensures independent translation of *lacZ*. J Bacteriol **172:**1077–1084.