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The *Vibrio cholerae* Fatty Acid Regulatory Protein, FadR, Represses Transcription of *plsB*, the Gene Encoding the First Enzyme of Membrane Phospholipid Biosynthesis

Youjun Feng[1] and **John E. Cronan**[1,2,*]

[1]Department of Microbiology, University of Illinois, Urbana, Illinois 61801, USA

[2]Department of Biochemistry, University of Illinois, Urbana, Illinois 61801, USA

SUMMARY

Glycerol-3-phosphate (*sn*-glycerol-3-P, G3P) acyltransferase catalyzes the first committed step in the biosynthesis of membrane phospholipids, the acylation of G3P to form 1-acyl G3P (lysophosphatidic acid). The paradigm G3P acyltransferase is the *Escherichia coli plsB* gene product which acylates position-1 of G3P using fatty acids in thioester linkage to either acyl carrier protein (ACP) or CoA as acyl-donors. Although the *Escherichia coli plsB* gene was discovered about 30 years ago, no evidence for transcriptional control of its expression has been reported. However Kazakov and coworkers (Kazakov, A. E. *et al.* (2009) *J Bacteriol*, 191, 52–64) reported the presence of a putative FadR-binding site upstream of the candidate *plsB* genes of *V. cholerae* and three other *Vibrio* species suggesting that *plsB* might be regulated by FadR, a GntR-family transcription factor thus far known only to regulate fatty acid synthesis and degradation. We report that the *V. cholerae plsB* homologue restored growth of *E. coli* strain BB26-36 which is a G3P auxotroph due to an altered G3P acyltransferase activity. The *plsB* promoter was also mapped and the predicted FadR-binding palindrome was found to span positions -19 to -35, upstream of the transcription start site. Gel shift assays confirmed that both *V. cholerae* FadR and *E. coli* FadR bound the *V. cholerae plsB* promoter region and binding was reversed upon addition of long chain fatty acyl-CoA thioesters. The expression level of the *V. cholerae plsB* gene was elevated 2–3 fold in an *E. coli fadR* null mutant strain indicating that FadR acts as a repressor of *V. cholerae plsB* expression. In both *E. coli* and *V. cholerae* the β-galactosidase activity of transcriptional fusions of the *V. cholerae plsB* promoter to *lacZ* increased 2–3 fold upon supplementation of growth media with oleic acid. Therefore, *V. cholerae* coordinates fatty acid metabolism with 1-acyl G3P synthesis.

INTRODUCTION

Our current knowledge of bacterial phospholipid biosynthesis is largely derived from studies of model organisms including *E. coli* (Cronan & Bell, 1974, Larson *et al.*, 1980, Lightner *et al.*, 1983, Lightner *et al.*, 1980), *Streptococcus pneumoniae* (Lu *et al.*, 2006) and *Bacillus subtilis* (Paoletti *et al.*, 2007). The universal precursor of membrane phospholipid formation (Lu *et al.*, 2006, Zhang & Rock, 2008), phosphatidic acid (PtdOH), is synthesized by acylation of *sn*-glycerol-3-phosphate (G3P) to 1-acyl-G3P followed by a second acylation to give phosphatidic acid (Fig. 1A) (Paoletti *et al.*, 2007). Two different enzyme systems are known to catalyze the first G3P acylation reaction. PlsB (G3P acyltransferase) which acylates the 1-position of G3P using acyl thioesters of either CoA or acyl carrier protein

*To whom correspondence should be addressed: Dept. of Microbiology, B103, Chemical and Life Sciences Laboratory, 601 S. Goodwin Ave., Urbana, IL 61801. Tel.: 217-333-7919; Fax: 217-244-6697; j-cronan@life.uiuc.edu.

(ACP) as acyl donors, was first identified in *E. coli* (Fig. 1 A&B) (Cronan & Bell, 1974, Larson *et al.*, 1980, Lightner *et al.*, 1980, Lightner *et al.*, 1983, Zhang & Rock, 2008). The second system is the PlsX/Y two-enzyme system first found in *S. pneumonia* and *B. subtilis* (Lu *et al.*, 2006, Paoletti *et al.*, 2007). PlsX activates fatty acids by catalyzing production of fatty acyl-phosphates from fatty acyl-ACP thioesters whereas PlsY transfers the acyl chains of the acyl-phosphates to the 1-position of G3P (Lu *et al.*, 2006, Paoletti *et al.*, 2007). The PlsX-PlsY system is also found in *E. coli*, although PlsB plays the only essential role in 1-acyl-G3P synthesis (Yoshimura *et al.*, 2007) (Baba *et al.*, 2006). Acylation of position-2 of 1-acyl-G3P is catalyzed by PlsC, an acyltransferase found in most bacteria of known genome sequence (Lu *et al.*, 2006). Like PlsB, *E. coli* PlsC utilizes either acyl-ACP or acyl-CoA as acyl donor to form phosphatidic acid although the acyl-donor preference of this enzyme varies among different bacteria (Lu *et al.*, 2006, Paoletti *et al.*, 2007, Zhang & Rock, 2008).

PlsB is a well-characterized membrane-bound enzyme (Fig. 1) (Cronan & Bell, 1974, Bell, 1975, Larson *et al.*, 1980, Lightner *et al.*, 1980, Lightner *et al.*, 1983, Zhang & Rock, 2008). PlsB was discovered by isolation of *E. coli* strain BB26, a G3P auxotroph having a G3P acyltransferase with an elevated Km for G3P (Cronan & Bell, 1974, Bell, 1975). Further studies described the biochemical and enzymatic properties of PlsB (Bell, 1975, Larson *et al.*, 1980, Lightner *et al.*, 1980) and the *plsB* gene (Lightner *et al.*, 1980, Lightner *et al.*, 1983). However, comparative genomics indicates that *plsB* is confined to a subset of gram-negative bacteria, the *Enterobacteriales*, *Vibrionales*, and *Burkholderiales* (Lu *et al.*, 2006). To date there have been no data demonstrating transcriptional regulation of expression of any bacterial G3P or 1-acyl-G3P acyltransferase, although due to the essential nature of phospholipid synthesis, expression was assumed to be constitutive. However, Kazakov *et al.* (Kazakov *et al.*, 2009) recently conducted an extensive bioinformatics study of γ-protebacteria and reported a putative FadR biding site upstream of *plsB* homologues of four *Vibrio* species, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. fischeri*. FadR is a transcription factor of the GntR family having an N-terminal helix-turn-helix DNA-binding domain and a C-terminal ligand-binding domain (van Aalten *et al.*, 2000, van Aalten *et al.*, 2001, Xu *et al.*, 2001, Iram & Cronan, 2005). Long chain fatty acyl-CoA species have been demonstrated to be regulatory ligands for FadR DNA-binding activity. Upon binding of long chain fatty acyl-CoA species the dimeric FadR undergoes dramatic conformational changes that results in rearrangement of the DNA recognition helices and dissociation of FadR protein from its operator sites (van Aalten *et al.*, 2001, Henry & Cronan, 1992, Cronan, 1997). FadR, is known to play plays central roles in modulating lipid metabolism in *E. coli* (Henry & Cronan, 1991, Cronan & Subrahmanyam, 1998). Not only does it serve as a repressor for fatty acid degradation (*fad*) regulon (Iram & Cronan, 2005, Feng & Cronan, 2009b, Cronan & Subrahmanyam, 1998, Campbell & Cronan, 2002), but also functions as an activator of *fabA* and *fabB*, two key genes required for unsaturated fatty acids biosynthesis (Henry & Cronan, 1992, Campbell & Cronan, 2001). In addition, FadR also positively regulates the *iclR* gene which encodes a repressor for glyoxylate bypass operon (Gui *et al.*, 1996). We report that in *V. cholerae* FadR acts as a repressor of *plsB* expression (Fig. 1C). To our knowledge this is the first example of transcriptional control of a membrane phospholipid acyltransferase.

RESULTS

Characterization of *V. cholerae* FadR

The *V. cholerae fadR* encodes a 279 residue polypeptide (Heidelberg *et al.*, 2000) 40 residues longer than that of *E. coli* FadR, the best studied FadR protein (Fig. 2 A&E). Sequence alignment of these two FadR proteins indictaes they share 50.5% sequence identity. As previously noted the *V. cholerae* FadR (FadR_vc) contains a centrally located

40 residue insert (residues 138–177) relative to *E. coli* FadR and other FadR proteins (Fig. 2A) (Iram & Cronan, 2005). Recombinant FadR_vc protein was overexpressed, purified to homogeneity (Fig. 2B) essentially as previously described (Iram & Cronan, 2005) and verified by LC mass-spectrometry with a coverage score of 76% (Fig. 2E). Analysis by size exclusion chromatography (Fig. 2B) indicated that the FadR_vc solution structure was predominantly a dimer with an apparent molecular weight of ~62 kDa, although some larger forms were also present (Fig. 2C). Dimerization was also detected by chemical cross-linking assays (Fig. 2D). Modeling based on the *E. coli* FadR structure indicates that the 40-residue insertion basically extends a loop present in the *E. coli* protein (data not shown).

V. cholerae plsB gene is a Functional Homologue of the *E. coli* protein

The *V. cholerae plsB* gene product (811 residues) aligned well with *E. coli* PlsB protein (807 residues) with 56.3% identity. To test the function of this *plsB* orthologue we assayed the ability to complement growth of an *E. coli plsB* mutant. Due to a point mutation that result in a H306A PlsB *E. coli* strain BB26-36 requires an exogenous supply of G3P to overcome a Km defect in the mutant enzyme (Cronan & Bell, 1974, Bell, 1975). The *V. cholerae plsB* gene carried by vector pCR2.1 allowed growth of strain BB26-36 on minimal medium M9 agar plates lacking G3P, whereas the strain carrying the empty vector failed to grow (Fig. 3A). A similar result was obtained in liquid medium (Fig. 3B). Thus, *V. cholerae plsB* gene encodes a functional G3P acyltransferase.

The *V. cholerae plsB* promoter

The genetic organization of the *plsB* gene on the *V. cholerae* chromosome I differs somewhat from that of the *E. coli plsB* locus. In both cases the *ubiA* gene (4-hydroxybenzoate polyprenyltransferase) is encoded downstream on the other DNA strand. In *E. coli* the the *dgkA* (diacylglycerol kinase) gene is upstream of *plsB* whereas in *V. cholerae*, *dgkA* is replaced by *lexA* which encodes the repressor of the bacterial SOS regulon. In *E. coli* it was recently reported that *plsB* and *dgkA* are inversely regulated by several stress responses at the transcriptional level (Wahl *et al.*, 2011).

To determine the transcriptional start site of *V. cholera plsB*, RLM-RACE, an improved version of 5’-RACE using tobacco acid pyrophosphatase was applied as described (Feng & Cronan, 2011). Although several truncated *plsB* transcripts were encountered we isolated two full-length 5’-RACE PCR products. DNA sequence analyses of several cloned 5’-RACE products showed the same sequence beginning with a C nucleotide adjacent to the RLM-RACE adaptor (Fig. 4B). This located the 5’-end of the transcript 96 nucleotides upstream of the *V. cholerae plsB* initiation codon (Fig. 4B) and defined the promoter (Fig. 4C). Surprisingly, although in most *Vibrio* species *plsB*_vc orthologues are found within the same genetic context, the sequences of *plsB* promoter regions differ very markedly (e.g. *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*). BLAST searches of all the *Vibrionales* of known genome sequences plus whole genome shotgun sequences showed that the only *Vibrio* species having *plsB* promoter sequences highly similar to that of *V. cholerae* were four strains of *V. mimicus* (VM603, VM223, VM573 and MB451and two *Vibrio sp.* isolates (RC586 and RC341, recently named *V. metecus* and *V. parilis*, respectively) (Haley *et al.*, 2010). The conservation of the *V. cholerae plsB* promoter sequences in these bacteria is not surprising since these bacteria were formerly all considered non-toxigenic environmental variants of *V. cholerae* and are regarded as closely related sister species of common ancestry (Haley *et al.*, 2010, Thompson *et al.*, 2008, Thompson *et al.*, 2009). The sequences of these *plsB* promoter regions showed a conserved site upstream of the mapped transcriptional start site, which is the proposed FadR binding site (AAAAGGTTTGACCAGT) of Kazanov and coworkers (Kazakov *et al.*, 2009). The site is centered at 27 bp upstream of the transcriptional start site (Fig. 4B) and thus FadR binding would seem likely to hinder RNA

polymerase binding or action (Balleza *et al.*, 2009) and therefore function as a repressor as in the case of the *fad* regulon genes (rather than the activation seem with the *fabA*, *fabB* and *iclR* genes). However, since FadR binding sites contain only three strictly conserved base pairs (Fig. 5A), these sites must be experimentally validated.

Binding of *V. cholerae* FadR to the *plsB* promoter region and its reversal by long chain acyl-CoAs

The predicted FadR-binding site of the *plsB* promoter region was tested by electrophoretic mobility shift assays using a 39 bp probe containing the predicted 17 bp FadR-binding site (Fig. 4A and 5A) and either *V. cholerae* FadR or *E. coli* FadR. Gel shift assays showed that binding of the DNA probe to either FadR_ec or FadR_vc was dose-dependent, as previously observed with the *fadM* (Feng & Cronan, 2009b) and *fadH* (Feng & Cronan, 2010) promoter regions. As before super-shifted bands were frequently observed at high FadR concentrations (Fig. 5 B&C). Similar super-shifted bands were seen our prior analyses (Feng & Cronan, 2010). We believe that the super-shifted bands are due to a portion of the protein forming soluble aggregates (perhaps tetramers) in the buffer used in the mobility shift experiments.

We also tested the effects of fatty acyl-CoA addition on FadR-promoter binding. As we expected, long chain (but not short chain) acyl-CoAs such as oleoyl-CoA (C18:1) antagonized DNA binding by both FadRs (Fig. 6). Addition of long chain acyl-CoA thioesters to the probe-FadR_vc complex resulted in liberation of the probe (Fig. 6B and 6D) (Note, due to an unknown reason traces of shifted bands remained after addition of stearoyl-CoA, Fig. 6C). The super-shifted bands caused by FadR_vc multimers behaved similarly to the bands shifted by FadR_ec dimers in that they disappeared upon an addition of a long chain acyl-CoA (palmitoyl-CoA, stearoyl-CoA or oleoyl-CoA) (Figs. 6C & D). Our *in vitro* data therefore indicate that the *V. cholerae plsB* palindromic site predicted by (Kazakov *et al.*, 2009) is specifically recognized by FadR and the interaction is eliminated by physiologically relevant small molecule ligands.

Repression of *V. cholerae plsB* expression by FadR and its induction by oleate in vivo

We first tested the physiological role of FadR binding to the *V. cholerae plsB* promoter in *E. coli* because it is more amenable to genetic manipulation than *V. cholerae*. A *plsB*_vc-*lacZ* transcriptional fusion was constructed and inserted into the chromosome to give *E. coli* strain FYJ135. A *fadR* null mutant derivative of this strain was then constructed and called FYJ136. Assays of LacZ activity showed that the level of *plsB*_vc transcription was about 2.5 fold higher in the strain that lacked FadR. This extent of FadR regulation was similar to that of *E. coli fadD* which encodes acyl-CoA synthetase (Fig. 7A). Repression by FadR was largely reversed by addition of oleic acid to the medium which upon conversion to oleoyl-CoA provides a potent antagonist of FadR binding to its operator sites (Fig. 7B).

Given these data we had hoped to isolate *V. cholerae* strains that lack *fadR* function by their ability to grow on decanoate, the method used to isolate *fadR* mutants of *E. coli* and *Salmonella enterica* (in those bacteria decanoate is a carbon source, but cannot induce β-oxidation) (Iram & Cronan, 2005). However, decanoic acid was found to completely block *V. cholerae* growth which precluded this approach (Fig. 8 A and B). We subsequently found that other workers have reported inhibition of the growth of *Vibrio* species by medium chain length fatty acids (Immanuel *et al.*, 2011). We also attempted to delete the *fadR* gene of the *V. cholerae* chromosome by homologous recombination, but repeatedly failed presumably due to the inefficiency and strong strain-dependence of genetic manipulation of *Vibrio* species which can probably be attributed to the known high genome plasticity of these bacteria (Thompson *et al.*, 2010). Finally, we constructed a *plsB*_vc-*lacZ* transcriptional

fusion in the *V. cholerae* plasmid pTL61T and after many attempts managed to transform this plasmid into *V. cholerae* to give strain FYJ176. This allowed us to test the effects of fatty acid supplementation on *plsB*_vc transcription in its biological context and we found that *plsB*_vc transcription level increased significantly upon supplementation of RB medium with oleic acid (Fig. 8C), although the fatty acid inhibited growth. Thus, we conclude that *V. cholerae* FadR negatively regulates *plsB* expression with a regulatory strength similar to that exerted by FadR on *E. coli fadD* expression.

DISCUSSION

Our data show that the *V. cholerae* PlsB homologue is functional and its expression is regulated by FadR thereby validating the bioinformatics prediction of Kazakov *et al.* (Kazakov *et al.*, 2009). However, transcriptional regulation of *plsB* expression by FadR seems to be an uncommon mechanism among the proteobacteria. We extended the prior search for this regulatory mechanism (Kazakov *et al.*, 2009) to *Vibrionales* genome sequences (and finished contigs) that became available since the Kazakov report. From these data FadR regulation of *plsB* seems to occur in eight *Vibrionales* species (Fig. 4C). The *V. cholerae plsB* FadR-binding site (AAAAGGTTTGACCAGTT) is completely conserved in these species, although their promoter sequences vary greatly. An exception is *V. fischeri* which seems to contain a derivative FadR binding site of uncertain function (Fig. 4C). Among these *Vibrionales* species, the FadR orthologs are 76.6%–85.7% identical whereas the putative PlsB proteins are 76.7%–94.9% identical (data not shown) and thus it seems very likely that FadR regulates *plsB* expression in these bacteria. It should be noted that it is “notoriously difficult” to define a *Vibrio* species due to mobile genetic elements and horizontal gene transfer (Thompson *et al.*, 2010). However, all of the *Vibrio* FadR orthologs contain the 40-residue insertion relative to *E. coli*, the presence of which may be useful in defining *Vibrio* species especially since no obvious relics of gene horizontal transfer are seen (the GC content of *Vibrio fadR* is essentially the same as that of *V. cholerae* chromosome I). A prior attempt to remove this *Vibrio* FadR insertion by mutagenesis resulted in loss of detectable protein expression presumably due to proteolytic destruction of a misfolded protein (Iram & Cronan, 2005). In that work the *V. cholerae* orthologue was found to have the greatest dynamic regulatory range of the five FadR proteins examined suggesting that the insertion provides the protein with a gain of function (Iram & Cronan, 2005). The distribution of FadR sites in lipid synthetic genes is asymmetric in *V. cholerae and E. coli.* In both bacteria FadR regulates *fabA* expression and FadR also regulates *fabB* expression in *E. coli* but not in *V. cholerae* whereas the opposite is true of *plsB* (Cronan & Subrahmanyam, 1998, Feng & Cronan, 2009a, Feng & Cronan, 2011).

Up-regulation of *plsB* expression seems likely to be of advantage for acquiring fatty acids from a dilute environment such as seawater, the usual habitat of most *Vibrio* species. Long chain fatty acid uptake in *E. coli* requires three known proteins, the first of which is the outer membrane protein FadL (Clark, 2005). The available complete genome sequences indicate that each of the *Vibrionales* encodes three homologues of FadL rather than the single *fadL* found in *E. coli*. However, FadL does not perform active transport, it functions only a ligand-gated channel for substrate diffusion (Lepore *et al.*, 2011). Thus in order to obtain vectorial uptake, the fatty acids must be converted to their acyl-CoA esters by the FadD acyl-CoA synthetase (VC_1985 in *V. cholerae* El Tor N16961) which in turn would pass the acyl chains to PlsB (which readily accepts acyl-CoAs as acyl donors). Based on transposon mutagenesis studies, it seems probable that utilization of exogenous fatty acids for phospholipid synthesis may be the primary physiological role of *V. cholerae* PlsB. In *V. cholerae* a transposon insertion early in *plsB* is not a lethal mutation (Cameron *et al.*, 2008) whereas in *E. coli plsB* is an essential gene synthesis (Yoshimura *et al.*, 2007) (Baba *et al.*, 2006). In contrast *plsX*, which encodes a component of the other known bacterial G3P

acyltransferase system (PlsX/PlsY), is essential in *V. cholerae* (Cameron *et al.*, 2008), but not in *E. coli* (Yoshimura *et al.*, 2007) (Baba *et al.*, 2006). Therefore it may be that *V. cholerae* PlsX, together with its membrane bound partner PlsY, catalyzes acylation of G3P with *de novo* synthesized acyl chains whereas PlsB performs this task in *E. coli*. Since *in vitro E. coli* PlsX prefers acyl-ACPs rather than the CoA substrates formed upon uptake of exogenous fatty acids (Lu *et al.*, 2006), *V. cholerae* PlsX seems unlikely to play a major role in fatty acid uptake.

Vibrio species have diverse sources of fatty acids. The luminescent bacterium *V. fischeri*, incorporates unusual fatty acids derived from the lipids of its squid symbiont host (Wier *et al.*, 2010). Moreover, Giles and coworkers (Giles *et al.*, 2011) reported that five *Vibrio* species including *V. cholerae* efficiently incorporate exogenous fatty acids from both bile and from marine sediments into their membrane phospholipids. It might be argued that for efficient fatty acid utilization *plsC*, the gene that encodes the acyltransferase that attaches the second acyl chain, should be regulated in concert with *plsB*. However, in *V. cholerae* PlsC seems to be in functional excess over PlsB because no singly acylated G3P (lysophosphatidic acid) species accumulate (Giles *et al.*, 2011).

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used here were *E. coli* K-12 derivatives (Table 1) and ATCC 14547, an avirulent strain of *V. cholerae* (Feng & Cronan, 2011, Massengo-Tiasse & Cronan, 2008). The media used for *E. coli* included Luria-Bertani (LB) medium (tryptone, 10g/L; yeast extract, 5 g/L; NaCl, 10 g/L; pH 7.5), rich broth (RB medium; 10 g of tryptone, 1 g of yeast extract, and 5 g of NaCl per liter) and the minimal medium M9 supplemented with 0.4% glucose or another carbon source, 0.1% Vitamin-Free Casamino Acids and 0.001% thiamine. Although LB and RB media both can support *V. cholerae* growth, Mueller-Hinton broth (Difico) (MH medium, 300 g of beef infusion, 17.5 g of Casamino Acids, and 1.5 g of starch per liter) was required to obtain electroporation competent V. cholerae cells (Hamashima *et al.*, 1995). Fatty acids were neutralized with potassium hydroxide, solubilized with Tergitol NP-40 and used for induction experiments at the final concentrations of 5 mM (*E. coli*) or 2 mM (*V. cholera*e) (Feng & Cronan, 2009b, Feng & Cronan, 2010). For assays of β-galactosidase activities, either 5 mM acetate or 5 mM fatty acid were the carbon sources used (Feng & Cronan, 2009b, Feng & Cronan, 2010, Feng & Cronan, 2011). When necessary, antibiotics were used as at the following concentrations (in mg/liter): sodium ampicillin, 100; kanamycin sulfate, 50; and tetracycline HCl, 15.

Plasmids and DNA manipulations

The pCR2.1-TOPO vector (Invitrogen) was applied for PCR cloning, and Topo 10 strain is the corresponding recipient host (Table 1). Recombinant plasmid pCR-*plsB*vc that carries *V. cholerae plsB* gene along with its promoter region was introduced into strain BB26-36, which is an *E. coli* G3P auxotroph encoding a defective PlsB that results in an elevated Km for G3P, to (Table 2). Strain BL21 (DE3) carrying either pET28-*fadR*ec or pET16-*fadR*vc plasmids was used to prepare FadR_ec or FadR_vc proteins (Feng & Cronan, 2009b, Feng & Cronan, 2010, Iram & Cronan, 2005).

To quantify *V. cholerae plsB* transcription two versions of *plsB*_vc-*lacZ* fusion constructs were made using two pairs of specific primers, *PplsB*vc-F1 plus *PplsB*vc-R1 and *PplsB*vc-F2 plus *PplsB*vc-R2 (Table 2) to amplify the *V. cholerae* promoter region. These products were directly inserted into the promoter-less *lacZ* reporter plasmids pAH125 and pTL61T, respectively (Table 1). The pAH-P*plsB*_vc plasmid requires *pir* protein for replication and

replicates in DH5α λ-*pir*, but to impart antibiotic resistance the plasmid must specifically integrate into the *att*λ site of the chromosome of *E. coli* MC4100 (a *lacZ* strain lacking *pir*) in a reaction catalyzed by the pINT-ts helper plasmid.

Plasmid pTL61T-P*plsB*_vc, replicates in *V. cholerae* species (Withey & Dirita, 2005) (Table 1). Using a similar strategy, *E. coli* strain FYJ159 which has a *fadD*_ec-*lacZ* transcriptional fusion specifically integrated into the *att*λ site of the *E. coli* MC4100 chromosome was made (Table 1). PCR assays with specific primers *plsB*vc_P-F (*fadD*-check2) plus *lacZ*-R (Table 2) were conducted to determine the *plsB*_vc-*lacZ* (or *fadD*_ec-*lacZ*) junction. X-Gal plates were used to test function of the *plsB*_vc-*lacZ* transcriptional fusion.

Transformation of *V. cholera*

Electroporation was utilized to transform the *lacZ* reporter plasmid pTL61T-P*plsB*_vc into *V. cholerae*. Electro-competent cells were prepared according to Hamashima *et al*. (Hamashima *et al.*, 1995). In brief, a single colony of *V. cholerae* was cultivated overnight in 3 ml of MH medium and then 1 ml of overnight culture was transferred into 100 ml of MH medium in a 500 ml of flask with vigorous shaking at 37 °C. Once the OD_{600} reached about 0.6, bacterial cultures were chilled on ice for 30 min and then collected by spinning at 2,000×*g* for 15 min at 4 °C. The collected pellets in each tube were resuspended in 30 ml of iced-chilled EP washing buffer (272 mM sucrose, 1 mM $MgCl_2$, 7 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4) and then centrifuged at 2,000×*g* for 15 min at 4 °C. Following two more washes the cells were suspended in 1 ml of ice-cold EP buffer and this was divided into small aliquots (200 µl each tube) which were frozen on dry ice and kept at −80 °C until use. Prior to electroporation, cells (200 µl) were thawed quickly at 37 °C, mixed with about 1 µg of plasmid DNA and then placed on ice for about 20 min prior to transfer into a chilled 2 cm electrode gap cuvette. Electroporation was a done with a time constant of 25 ms (25 µF capacitance, 1000 Ω) at 1.6 kV (8.0 kV/cm). The pulsed cells were quickly diluted with 600 µl of pre-warmed MH medium. The cultures were cultivated at 37 °C for 60 min and the cells were plated on MH agar plates containing ampicillin at 50 µg/ml. The plates were incubated at 37 °C for about 36 h. Colonies containing the reporter plasmid were confirmed by colony PCR, assay of β-galactosidase activity plus DNA sequencing of the isolated plasmid.

$P1_{vir}$ phage transduction

$P1_{vir}$ transductions were performed as described by Miller (Miller, 1992) with little modifications. Strain FYJ136 (*plsB*vc-*lacZ*, Δ*fadR*) was generated by *$P1_{vir}$* transduction of strain FYJ135 (*plsB*vc-*lacZ*) using a lysate grown on strain MFH8 (*fadR*::Tn*10*) with selection for tetracycline resistance. Strain FYJ161 was obtained by *$P1_{vir}$* transduction of strain FYJ57 (Δ*fadR*) with a lysate grown on strain FYJ159 (*fadD*ec-*lacZ*) and selection for kanamycin resistance (Table 1).

β-Galactosidase assays

Mid-log phase cultures grown in either RB or minimal medium were collected for assay of β-galactosidase (Feng & Cronan, 2009b, Feng & Cronan, 2009a, Miller, 1992). The data were recorded in triplicate in more than three independent experiments.

RNA isolation and 5'-RACE

As documented previously (Feng & Cronan, 2009b, Feng & Cronan, 2009a, Feng & Cronan, 2010, Feng & Cronan, 2011) bacterial RNAs were isolated from cells of *V. cholerae* ATCC14547 grown to mid-log phase in (either LB or rich broth medium) using the RNeasy bacterial RNA isolation kit (Qiagen). RNA quality was determined by agarose gel

electrophoresis, and PCR detection (using total RNA as template with primers *16S*vc-F plus *16S*vc-R, in Table 2) to test possible DNA contamination of the RNA samples (Feng & Cronan, 2009b, Feng & Cronan, 2009a, Feng & Cronan, 2010). The qualified RNA preparations then were used for subsequent 5'-RACE experiments.

RLM-RACE (Ambicon), an improved version of the customary 5'-RACE methods was used to determine the putative transcription start site of *V. cholerae plsB* gene as recently described (Feng & Cronan, 2009b, Feng & Cronan, 2009a, Feng & Cronan, 2010). Following three steps of customary treatments (calf intestinal alkaline phosphatase digestion, tobacco acid pyrophosphatase treatment and ligation of a 5'-RACE adaptor to the pyrophosphatase-treated mRNAs), the reverse-transcription reaction was performed. Subsequently, a set of nested PCR reactions were performed in which combinations of Outer Primer plus *plsB*vc-GSP and Inner Primer plus *plsB*vc-Nested (Table 2) were used. The PCR program was a denaturing cycle at 95 °C for 5 min followed by 35 cycles comprising 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The purified PCR products were cloned into the pCR2.1 TOPO vector (Invitrogen) for direct DNA sequencing. The transcriptional start site was taken to be the first nucleotide adjacent to the RLM-RACE adaptor (Feng & Cronan, 2009a, Feng & Cronan, 2010, Feng & Cronan, 2011)}.

Expression and purification of *V. cholerae* FadR

Recombinant hexahistidine-tagged FadR_vc protein was expressed in *E. coli* BL21 (DE3) carrying the pET16-*fadR*vc plasmid (Table 1). Induction with 0.3 mM isopropyl β-D-1-thiogalactopyranoside at 30 °C for 3 h gave soluble FadR_vc protein. The clarified bacterial supernatant obtained by lysis in a French pressure cell and removal of bacterial debris by centrifugation was loaded onto a nickel chelate column (Qiagen). The column was washed with buffer containing 20 mM imidazole and then the protein was eluted in elution buffer containing 150 mM imidazole. The protein was concentrated by ultrafiltration (30 kDa cutoff) and exchanged into 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl (Feng & Cronan, 2010)

Liquid chromatography quadrupole time-of-flight mass spectrometry

A Waters Q-Tof API-US Quad-ToF mass spectrometer connected to a Waters nano Acquity UPLC) was applied to determine identify the *in vitro* prepared FadR_vc protein (Feng & Cronan, 2011). The protein band of interest was cut from SDS-PAGE gel. The gel slices were de-stained and the proteins digested with 25 μl of Sequencing Grade Trypsin (G-Biosciences St. Louis, MO, 12.5 ng/μl in 25 mM ammonium bicarbonate) using a CEM Discover Microwave Digestor (Mathews, NC) for 15 min at 55 °C and 50W. Subsequently, the resulting peptides were extracted using 50% acetonitrile containing 5% formic acid, dried using a Savant SpeedVac and suspended in 13 μl of 5% acetonitrile containing 0.1% formic acid. Samples (10 μl) were loaded on a Waters Atlantis C-18 column (0.03 mm particle, 0.075 mm × 150 mm) and eluted at a flow rate of 250 nl per min using a linear gradient of water/acetonitrile containing 0.1% formic acid 0–60% B in 60 min. The mass spectrometer was set for data dependent acquisition and ms/ms analysis was carried out on the most abundant four peaks at any given time. Data collected were processed using the Waters Protein Lynx Global Server 2.2.5, Mascot (Matrix Sciences) and BLAST against NCBI nr database (Feng & Cronan, 2011).

Size exclusion chromatography

Relative to FadR_ec, FadR_vc contains a 40 residue insertion into the center of the protein sequence (Iram & Cronan, 2005). To test if this insertion affected the solution structure of the protein the purified FadR_vc protein was subjected to gel filtration analyses using a Superdex 200 column (Pharmacia) run on an Äkta fast protein liquid chromatography

system (GE Healthcare) essentially as recently described (Feng & Cronan, 2010, Feng & Cronan, 2011). The column effluent was monitored at a flow rate of 0.5 ml/min in running buffer (20 mM Tris-HCl,100 mM NaCl, pH 8.0). When necessary, samples of interest were separated by 15% SDS-PAGE and stained with Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO).

Chemical cross-linking assays

To further test the solution structure of *V. cholerae* FadR, chemical cross-linking with ethylene glycol bis-succinimidylsuccinate (Pierce) was performed (Feng & Cronan, 2010). In each chemical cross-linking reaction (20 μl in total), the purified FadR protein (~10 mg/ml) was separately mixed with cross-linker at different concentrations (0, 1.0, 2.5, 5.0, 7.5 and 10 μM), and kept 30 min at room temperature before analysis. All the reaction products were assayed using SDS-PAGE as above.

Electrophoretic mobility shift assays

To address whether FadR can bind to the *V. cholerae plsB* promoter region, gel shift assays were conducted as we recently reported (Feng & Cronan, 2009b, Feng & Cronan, 2010, Feng & Cronan, 2011) with minor modifications. Two DNA probes were used, one of which is about 100 bp of a PCR product obtained by amplification with specific primers *plsB*vc_P-F plus *plsB*vc_P-R (Table 2). The second DNA probe containing the predicted FadR binding palindrome was generated by annealing two complementary oligonucleotides (*plsB*vc_FadR_BS-F plus *plsB*vc_FadR_BS-R, Table 2) by incubated at 95 °C in TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) for 5 min followed by slow cooling to 25 °C. The probes were labeled with digoxigenin by terminal transferase incorporation of digoxigenin-ddUTP (Roche). The digoxigenin-labeled DNA probes (~1 pmol) were mixed with purified FadR (in appropriate concentrations) in the binding buffer (Roche) and incubated 15–20 min at room temperature. The DNA/protein mixtures were then analyzed by native PAGE (6% PAGE for the ~100 bp PCR probe, and 8% PAGE for the 39 bp synthetic probe). Contact blotting-aided gel transfer was conducted,followed by UV cross-linking (120 mJ for 180 s), 1 h of blocking of the nylon membrane in 50 ml blocking buffer, and 1 h of incubation with an anti-digoxigenin antibody solution (1:10,000) at room temperature. Subsequently the nylon membrane was washed 5 times (15 min each) and equilibrated with detection buffer for 15 min before 1 h of development of the luminescent reaction in CSPD working solution (Roche) at 37 °C. Finally the membrane was exposed to ECL film (Amersham) for signal capture.

Bioinformatic analyses

The known DNA binding sites by FadR_ec were all from literature compiled in the *E. coli* Regulon Data Base (http://regulondb.cs.purdue.edu/), whereas those putative DNA binding sites recognized by FadR_vc were searched by BLAST using known FadR_ec binding sites as probes.

Acknowledgments

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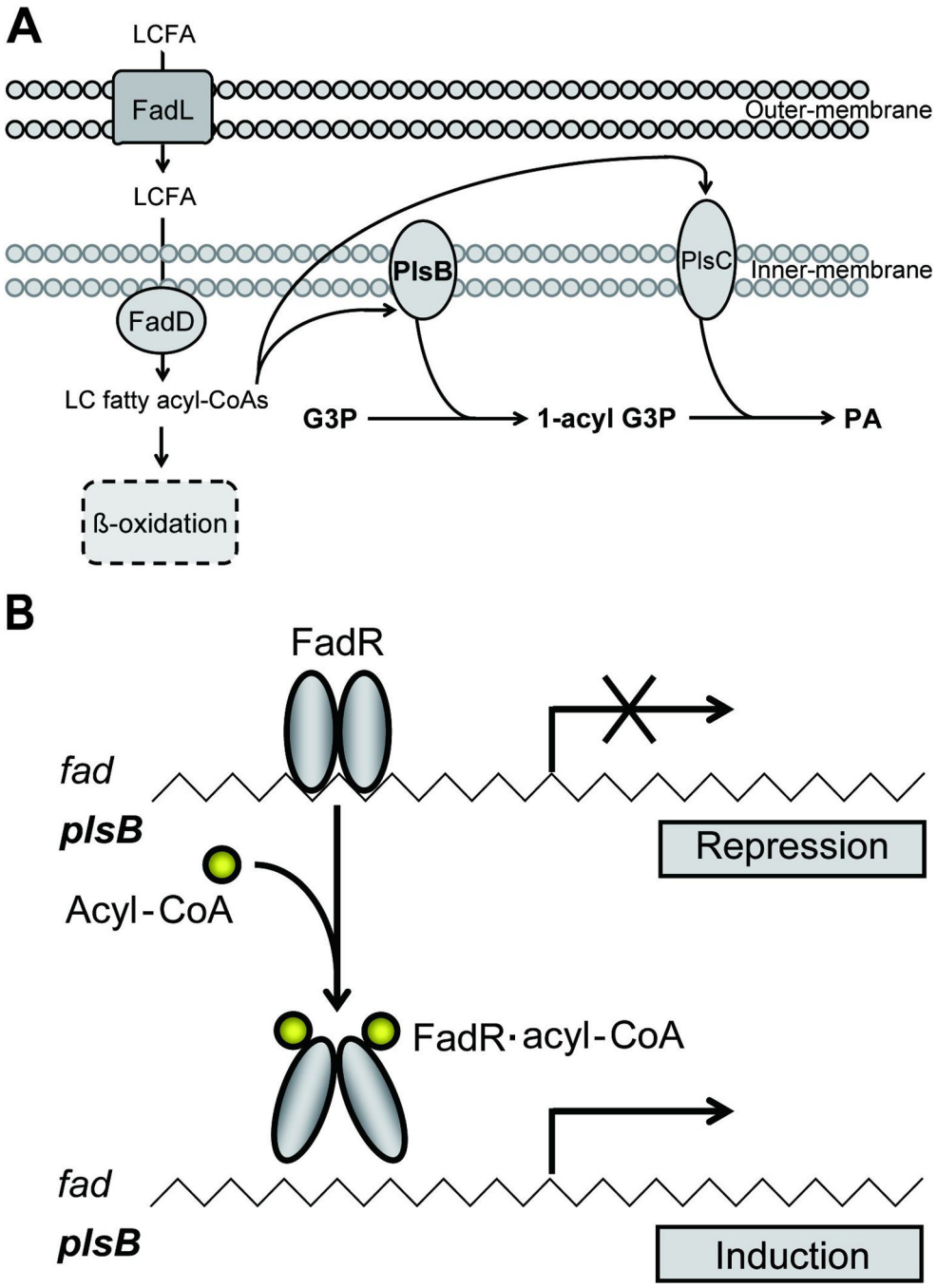

Fig. 1. Working model for the regulation of membrane phospholipid biosynthesis in *V. cholerae*

A. Phosphatidic acid biosynthesis from exogenous acyl chains in *V. cholerae*. Phosphatidic acid synthesis is initiated by the PlsB-catalyzed transfer of fatty acyl chains to G3P from acyl-CoA, and subsequent PlsC-catalyzed acylation of acyl-G3P. LCFA: Long chain fatty acids; G3P: *sn*-glycerol-3-phosphate; LPA: 1-acylG3P; PA: phosphatidic acid.

B. Cartoon of the model for regulation of *plsB* expression in *V. cholera*e (the *fad* regulon would be similarly regulated). Binding of acyl-CoA results in dramatic rearrangement of the FadR C-terminal domain which drives the N-terminal DNA binding domains into a conformation which precludes cooperative DNA binding (van Aalten *et al.*, 2001).

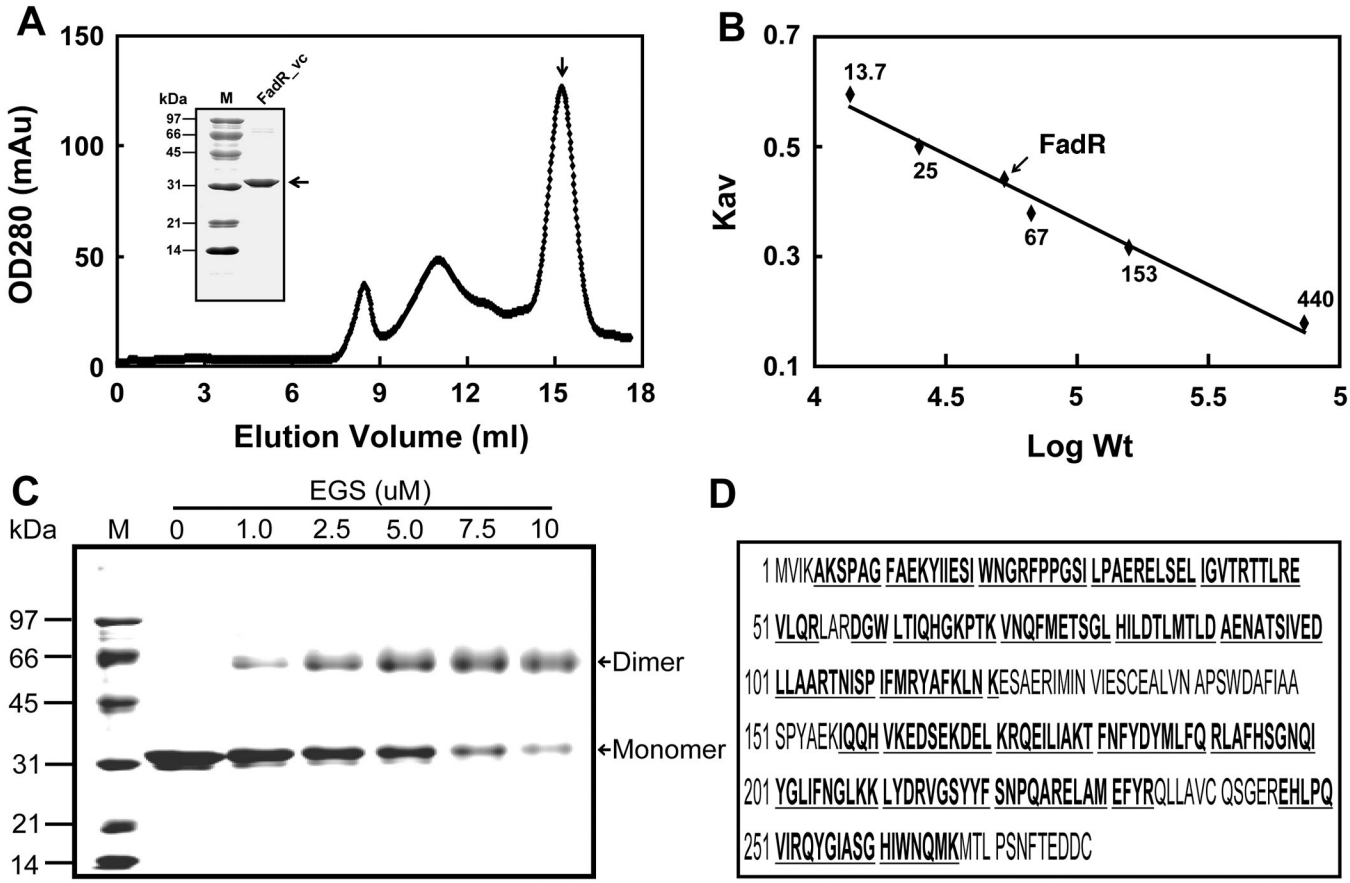

Fig. 2. Biochemical and structural characterization of *V. cholerae* FadR

A. Gel exclusion chromatographic profile of recombinant *V. cholerae* FadR run on a Superdex 200HR 10/30 column (GE Healthcare). The expected peak of purified *V. cholerae* FadR was eluted at the position of 15.2 ml (indicated with an arrow). The inset gel is the SDS-PAGE analysis of the purified *V. cholerae* FadR. The apparent molecular weight of recombinant *V. cholerae* FadR is about 31 kDa. OD280, optical density at 280 nm; mAu, milli-absorbance units.

B. Determination of *V. cholerae* FadR solution structure according to elution patterns of a series of standard proteins (Pharmacia). The standard proteins were ribonuclease (~13.7 kDa), chymotrypsinogen (~25 kDa), bovine serum albumin, 67 kDa), aldolase (153 kDa), and ferritin (~440 kDa). The elution position of *V. cholerae* FadR is indicated with an arrow. K_{AV}, partition coefficient; M, molecular weight.

C. Chemical cross-linking assay of *V. cholerae* FadR solution structure.

D. MS identification of *V. cholerae* FadR. The matched amino acid residues are given bold and underlined type.

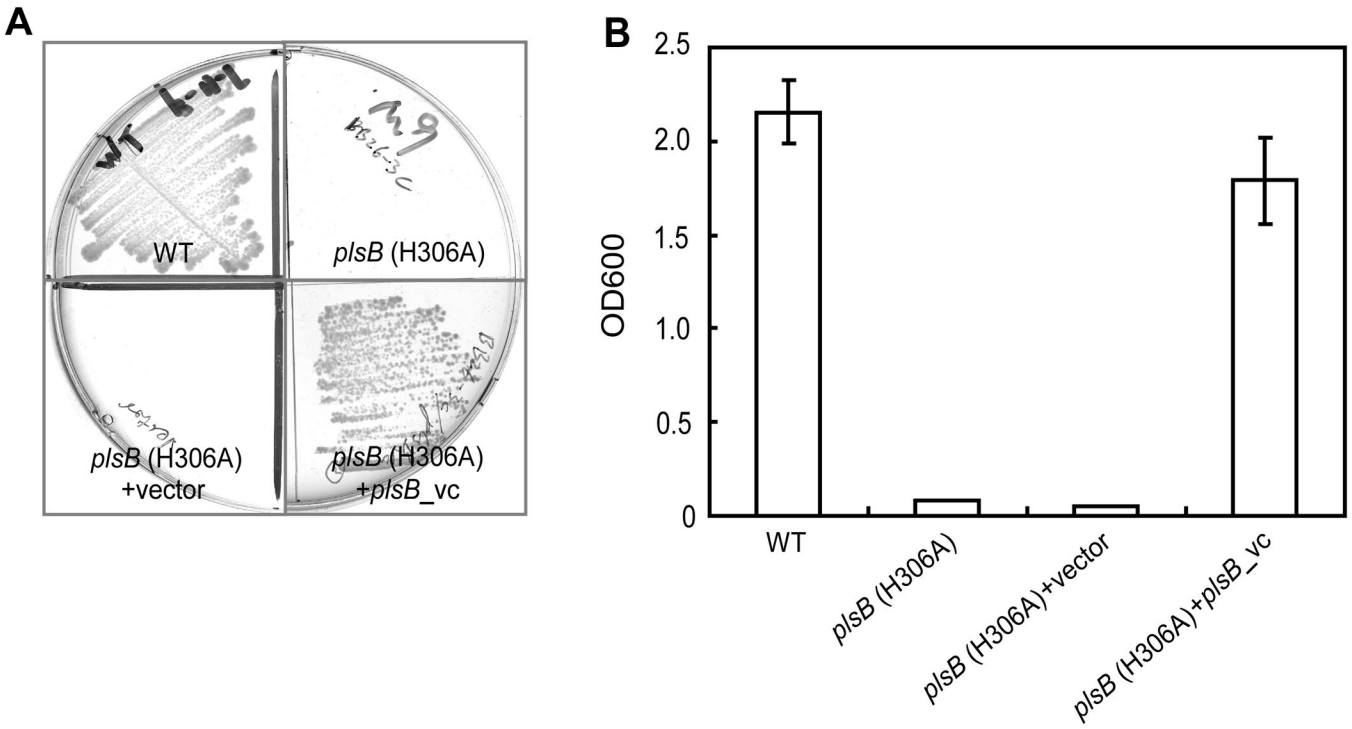

Fig. 3. Functional identification of *V. cholerae plsB*

A & B. Growth of *E. coli plsB* mutant strain BB26-36 (which encodes a mutant H306A PlsB acyltransferase) on minimal medium in the presence or absence of *V. cholerae plsB* gene expression on either solid (**A**) or liquid medium (**B**). The vector was pCR2.1.

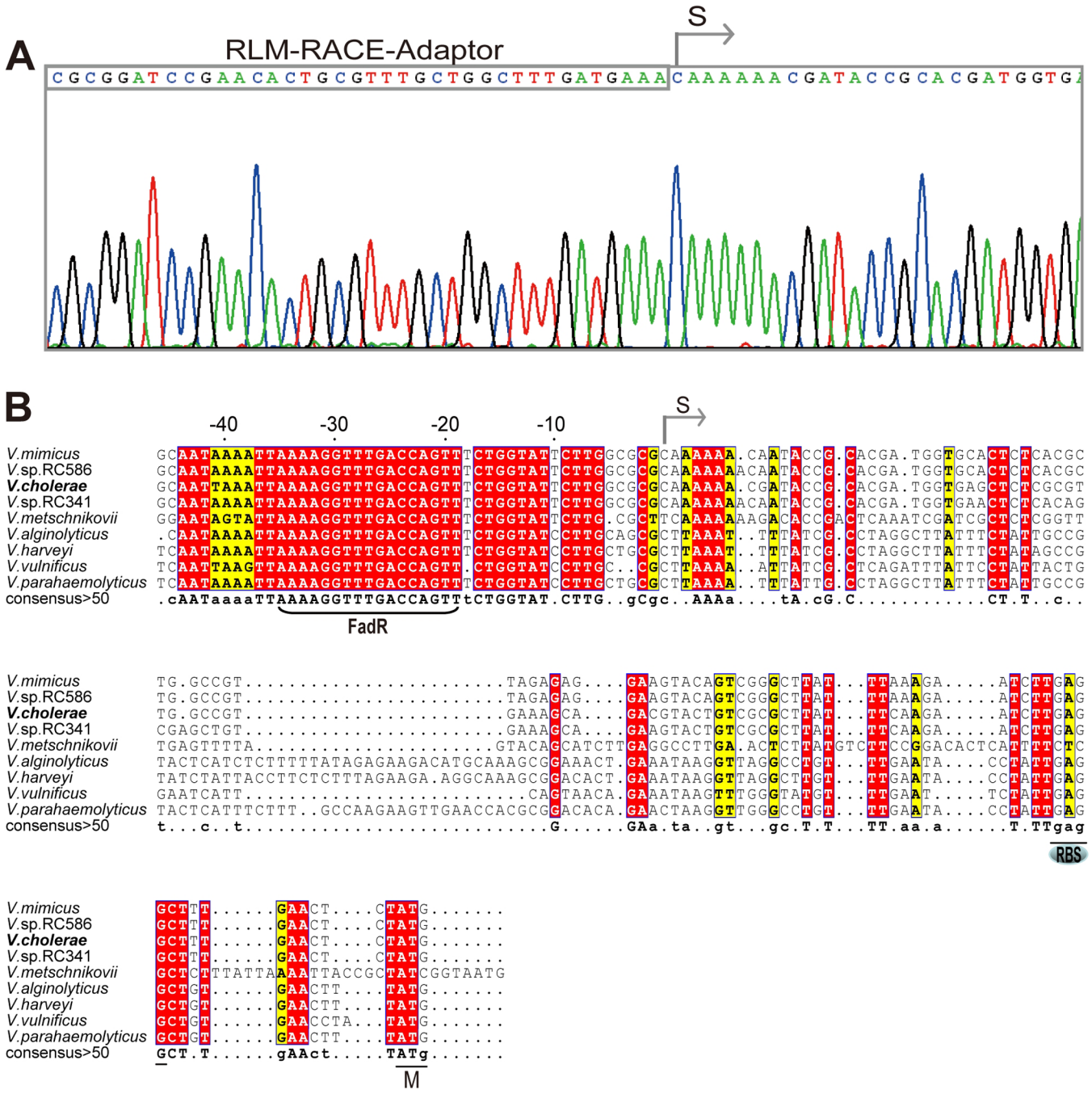

Fig. 4. Mapping of the *V. cholerae plsB* promoter

A. 5'-RACE-based mapping of the *V. cholerae plsB* transcriptional start site

B. Multiple sequence alignments of *V. cholerae plsB* promoter region with those of some other *Vibrio* species. The predicted FadR-binding site is bracketed. Designations: S, transcriptional start site; RBS, ribosome binding site; M: initiator methionine. Identical residues are in given as white letters with red background, similar residues are in black letters with yellow background, varied residues are in grey letters and dots denote gaps. The *plsB* promoter region sequences are from *V. mimicus* (ACYU01000137.1, only one of four isolates with available contig sequences is given), *V. vulnificus* YJ016 (NC_005139.1), *V. parahaemolyticus* (BA000031), V. *alginolyticus* 40B (ACZB01000122.1), *V. harveyi*

(CP000789), *V. metschnikovii* CIP (ACZO01000002.1), *V.* sp. RC586 (ADBD01000014.1), *V.* sp. RC341 (ACZT01000013.1), and *V. cholerae* ATCC14547 (results of direct sequencing in this study), respectively.

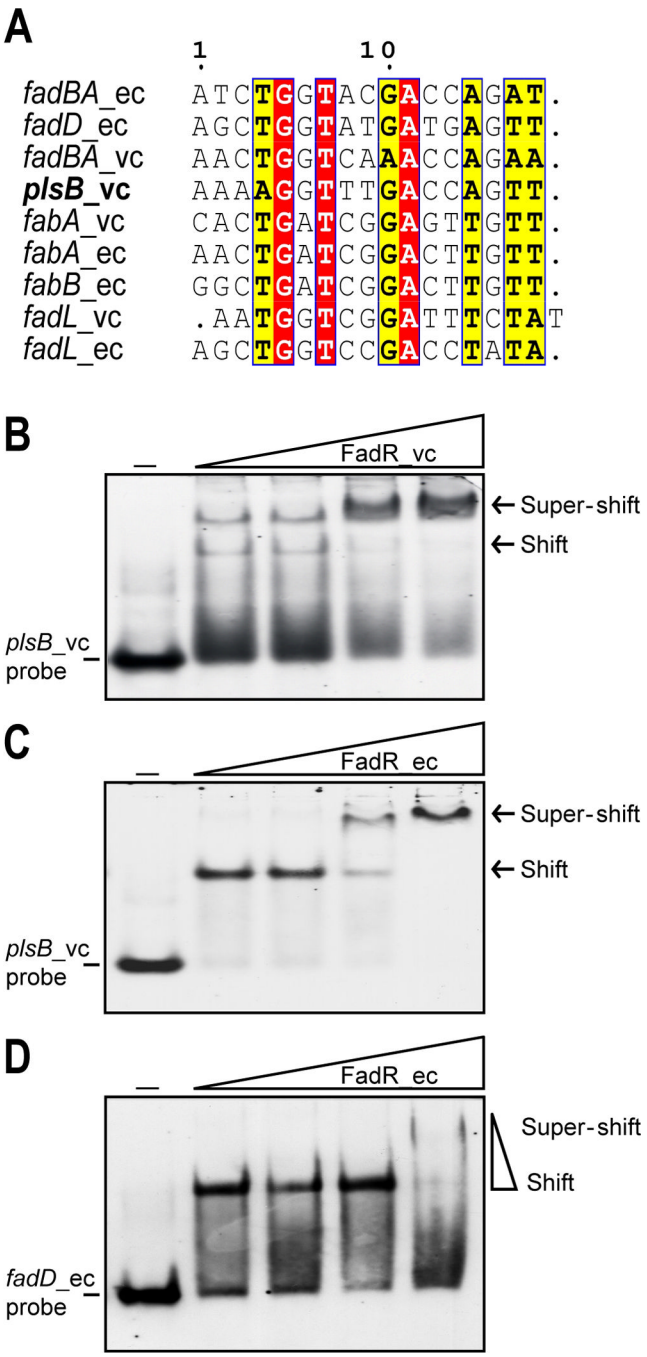

Fig. 5. The *V. cholerae plsB* promoter region binds both *V. cholerae* FadR and *E. coli* FadR

A. Sequence alignments of the putative *plsB* FadR-binding site with known sites. Identical residues are black letters with red background, similar residues are in black letters in yellow background and differing residues are in grey letters.

B & C. Electrophoretic mobility shift assays of the binding of *V. cholera* FadR **(B)** or *E. coli* FadR **(C)** to the *V. cholera plsB* promoter region.

D. Binding of *E. coli FadD* promoter region to FadR protein.

The minus sign denotes no addition of FabR protein whereas the DIG-labeled probe shifted by FadR protein is indicated with an arrow. The FadR levels in the right hand four lanes of each panel were (from left to right) 5, 10, 20 and 50 pmol. The protein samples were

incubated with 1 pmol of DIG-labeled probe in a total volume of 20 μl. Designations ec and vc denote *E. coli* and *V. cholerae*, respectively. FadR_ec and FadR_vc denote the FadRs of *E. coli* and *V. cholerae*, respectively. Representative gels (8% native PAGE) from at least three independent gel shift assays are given.

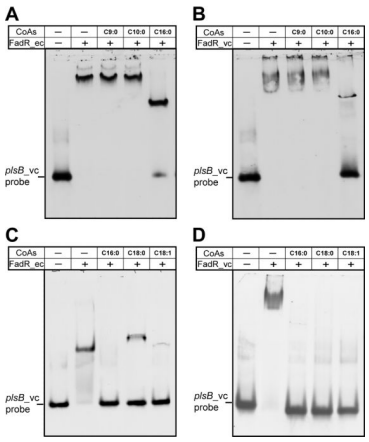

Fig. 6. Acyl-CoA species having long chain but not medium chain length acyl chains impair interaction between FadR and the *plsB*_vc promoter region

Panels **A & B**. Effects of medium and a long chain acyl-CoA species on binding of *E. coli* FadR **(A)** or *V. cholerae* FadR **(B)** to the *V. cholerae plsB* probe. Panels **C & D**. Effects of different long chain acyl-CoA species on binding of *E. coli* FadR **(C)** or *V. cholerae* FadR **(D)** to the *V. cholerae plsB* probe. Designations ec and vc denote *E. coli* and *V. cholerae*, respectively. Minus sign denotes no addition, whereas plus sign denotes addition. Designations C9:0, nonanoyl-CoA; C10:0, decanoyl-CoA; C16:0, palmitoyl-CoA; C18:0, stearoyl-CoA; C18:1, oleoyl-CoA. Note the quantitative discrepancy between the right lane of **A** and the center lane of **C** (the C16:0 lanes). The discrepancy correlates with the puzzling tendency of FadR binding to occasionally give super-shifted bands that seem more refractory to dissociation by long chain acyl-CoAs. In the binding reaction mixtures (20 μl total), the FadR (~20 pmol) was incubated with 1 pmol of DIG-labeled *plsB*_vc probe. When required, acyl-CoA (~50 pmol) was added as we recently described (Feng & Cronan, 2011). The gel shift assays were conducted for more than three times using 8% native PAGE, and the representative result is given.

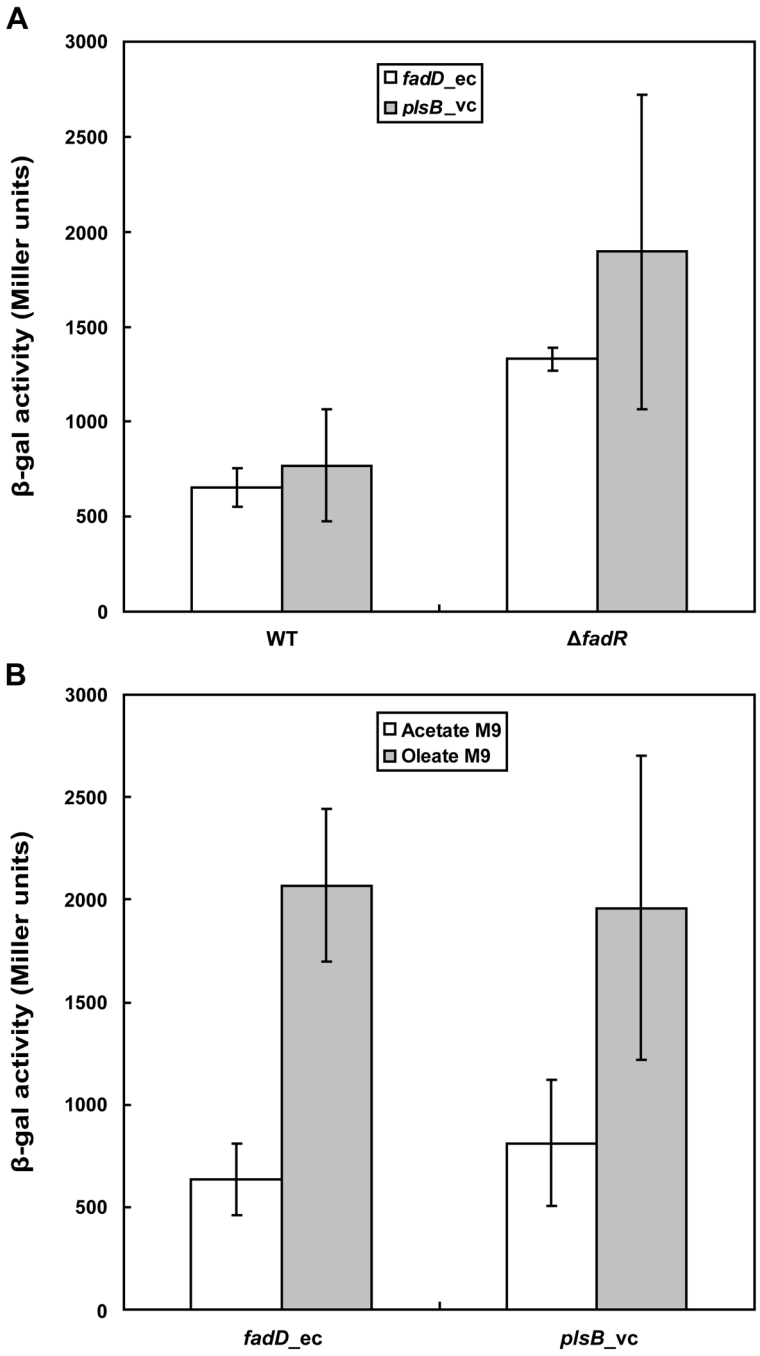

Fig. 7. Transcription of *plsB*_vc is repressed by FadR in *E. coli* and induced upon oleic acid supplementation

A. The LacZ (β-gal) activity of plsB_vc-lacZ transcriptional fusion integrated onto the *E. coli* chromosome was assayed. Strains FYJ135 (wild type *fadR*) and FYJ136 (Δ*fadR*) were assayed for the levels of *plsB*_vc transcription whereas strains FYJ159 (wild type *fadR*) and FYJ161 (Δ*fadR*) were assayed for the levels of *fadD*_ec expression.

B. Effects of oleate supplementation on transcription of *plsB_vc-lacZ* and *fadD_ec-lacZ* in *E. coli*. The lacZ-fusion constructs were *fadD*_ec-*lacZ* (FYJ159) and *plsB*_vc-*lacZ* (FYJ135), respectively. The data are expressed in average ± standard deviation and were derived from more than six independent experiments.

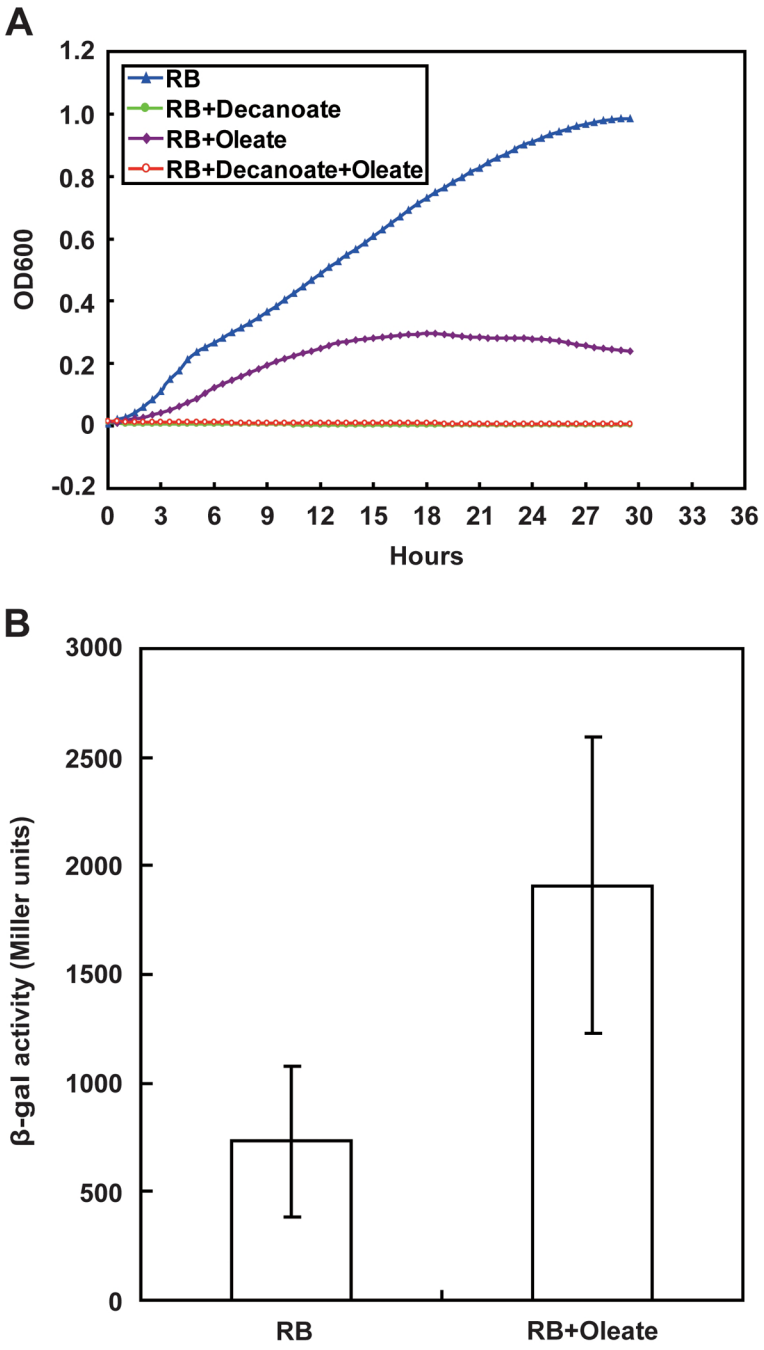

Fig. 8. Oleate addition slows growth of *V. cholerae* and induces *plsB* gene expression

A. Growth curve of *V. cholerae* in RB medium with or without fatty acid supplementation (decanoate and/or oleate). The growth curve was recorded automatically with a BioScreen C instrument in which each time point was measured in five parallel wells. A representative result of three independent trails is shown.

B. Induction of *V. cholerae plsB* expression by oleate.

The LacZ (β-gal) data are expressed as the mean ± standard deviation and are from more than nine independent experiments. Strain FYJ176 (*V. cholerae* carrying pTL61T-P*plsB*_vc, a plasmid encoding a *plsB-lacZ* transcriptional fusion) was assayed.

Table 1

Bacteria and plasmids in this study

***E. coli* strain or plasmid**	**Relevant characteristics**	**Sources/References**
Strains		
MG1655	Wild type stain of *E. coli* K-12	Lab stock
JW1176-1	Δ(*araD-araB*)*567* Δ*lacZ4787*(*::*rrnB-3) Δ*fadR776::kan, rph-1* Δ(*rhaD-rhaB*)*568*, *hsdR514*	CGSC[a], (Baba *et al.*, 2006)
Topo10	Δ*lac*X74, cloning strain	Invitrogen
DH5α	Cloning strain	Lab stock
BL21(DE3)	*E. coli* protein expression strain	Lab stock
MFH8	UB1005, *fadR::*Tn*10*	(Henry & Cronan, 1992, Feng & Cronan, 2010, Feng & Cronan, 2011)
BB26-36	*plsB26 plsX50 fadL701 glpR2(glp^c) glpD3 glpK14*(fb^R) Encodes a defective G3Pacyltransferase, G3P auxotroph	(Cronan & Bell, 1974)
MC4100	*araD*139, Δ(*argF-lac*)169	(Feng & Cronan, 2009b)
DH5α (λ-*pir*)	Δ*lac* host for pAH125 and its derivatives	(Feng & Cronan, 2009a)
FYJ1	DH5α (λ-*pir*) carrying pAH125	(Feng & Cronan, 2009a)
FYJ57	JW1176-1, Δ*fadR*	(Feng & Cronan, 2010, Feng & Cronan, 2011)
FYJ135	MC4100 with a *plsB*_vc-*lacZ* fusion integrated into the chromosomal attλ site	This work
FYJ136	*plsB*_vc-*lacZ* fusion, Δ*fadR::*Tn*10*	*P1vir*(MFH8)×FYJ135
FYJ158	DH5α (λ-*pir*) carrying pAH125-P*fadD*_ec	This work
FYJ159	MC4100 with a *fadD*_ec-lacZ fusion integrated into the chromosomal attλ site	This work
FYJ161	FYJ57, Δ*fadR*, *fadD-lacZ* transcriptional fusion	P1*vir*(FYJ159)×FYJ57
FYJ168	Topo 10 carrying pTL61T-P*plsB*_vc	This work
FYJ176	*Vibrio cholerae* carrying pTL61T-P*plsB*_vc	This work
FYJ187	MC4100 pINT-ts	This work
Plasmids		
pCR2.1-TOPO	Topo-cloning vector, Amp^R, Kan^R,	Invitrogen
pET28a	T7 promoter-driven expression vector, Kan^R	Novagen
pAH125	A promoter-less *lacZ* reporter plasmid used in *E. coli*, Kan^R	(Feng & Cronan, 2009a, Haldimann & Wanner, 2001)
pCR-*plsB*vc	pCR2.1 carrying *V. cholerae plsB* gene, Amp^R, Kan^R	This work
pAH-*P plsB*vc	pAH125 carrying the promoter region of *V. cholerae plsB* gene, Kan^R	This work
pINT-ts	Temperature sensitive plasmid expressing phage λ Int.	(Haldimann & Wanner, 2001)
pTL61T	A promoter-less *lacZ* fusion plasmid used for *V. cholerae*	(Bellair & Withey, 2008, Linn & St Pierre, 1990, Withey & Dirita, 2005)
pTL61T-P*plsB*vc	pTL61T carrying the promoter region of the *V. cholerae plsB* gene.	This work
pET28-*fadR*ec	Recombinant plasmid carrying the *E. coli fadR* gene, Kan^R	(Feng & Cronan, 2009b, Feng & Cronan, 2009a, Iram & Cronan, 2005, Feng & Cronan, 2010)
pET16-*fadR*vc	Recombinant plasmid carrying the *V. cholerae fadR* gene	Lab stock

[a]CGSC denotes Coli Genetic Stock Center, Yale University.

Table 2

Primers used in this study

Primers	Primer sequences
plsBvc-F[a]	5'-CG *GGATCC* CTG GAT TGC GTC CTC CAT AAT G-3'
plsBvc-R[a]	5'-CCG *CTCGAG* GTT TGG TAA TCC CAG CTT AAG GC-3'
*plsBvc*_FadR_BS-F[b]	5'-TTA AAT T**AA AAG GTT TGA CCA GTT** TCT GGT ATT CTT GGC-3'
*plsBvc*_FadR_BS-R[b]	5'-GCC AAG AAT ACC AGA **AAC TGG TCA AAC CTT TT**A ATT TAA-3'
*fadD*ec_FadR_F2	5'-GTA ATT ATC A**AG CTG GTA TGA TGA GTT** AAT ATT ATG-3'
*fadD*ec_FadR_R2	5'-CAT AAT ATT **AAC TCA TCA TAC CAG CT**T GAT AAT TAC-3'
*plsBvc*_P-F	5'-GTC CTC CAT AAT GGC TTC AAA G-3'
*plsBvc*_P-R	5'-GTT CAA AGC CTC AAG ATT CTT G-3'
PplsBvc-F1[a]	5'-CCG *GTCGAC* CAG TTG ACT GTG AGT ATA TCC AG-3'
PplsBvc-R1[a]	5'-AACC *GAATTC* AGA GTT CAA AGC CTC AAG ATT C-3'
PplsBvc-F2[a]	5'-CCG *CTCGAG*CAG TTG ACT GTG AGT ATA TCC AG-3'
PplsBvc-R2[a]	5'-CG *GGATCC* AGA GTT CAA AGC CTC AAG ATT C-3'
plsBvc-Nested (212–235)	5'-CAT TCA GCA TCA AAG GCT CTA AAG-3'
plsBvc-GSP (212–235)	5'-GAA TCA CCT GCA CAT CCA ACT C-3'
RLM-RACE Adaptor	5'-GCU GAU GGC GAU GAA UGA ACA CUG CGU UUG CUG GCU UUG AUG AAA-3'
RLM-RACE Outer	5'-GCT GAT GGC GAT GAA TGA ACA CTG-3'
RLM-RACE Inner	5'-CGC GGA TCC GAA CAC TGC GTT TGC TGG CTT TGA TGA AA-3'
fadD-promoter-F[a]	5'-CCG *GTCGAC* GTT GCG GTA CAA AAC CAG CA-3'
fadD-promoter-R[a]	5'-AACC *GAATTC* CTC TAA AAT GCG TGT TCG TCG-3'
fadD-check2	5'-GTC TGA CGA CTG ACT TAA CGC-3'
lacZ-R	5'-GAC CAT GAT TAC GGA TTC ACT G-3'
*16S*vc-F	5'-GGA AAC GAT GGC TAA TAC CGC A-3'
*16S*vc-R	5'-AGT GTG GCT GAT CAT CCT CTC A-3'

[a]The underlined italic sequences are the introduced restriction sites.

[b]The bold letters are the predicted core FadR binding palindromes.