



# The Fatty Acid Regulator FadR Influences the Expression of the Virulence Cascade in the El Tor Biotype of *Vibrio cholerae* by Modulating the Levels of ToxT via Two Different Mechanisms

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**ABSTRACT** FadR is a master regulator of fatty acid (FA) metabolism that coordinates the pathways of FA degradation and biosynthesis in enteric bacteria. We show here that a  $\Delta$ *fadR* mutation in the El Tor biotype of *Vibrio cholerae* prevents the expression of the virulence cascade by influencing both the transcription and the post-translational regulation of the master virulence regulator ToxT. FadR is a transcriptional regulator that represses the expression of genes involved in FA degradation, activates the expression of genes involved in unsaturated FA (UFA) biosynthesis, and also activates the expression of two operons involved in saturated FA (SFA) biosynthesis. Since FadR does not bind directly to the *toxT* promoter, we determined whether the regulation of any of its target genes indirectly influenced ToxT. This was accomplished by individually inserting a double point mutation into the FadR-binding site in the promoter of each target gene, thereby preventing their activation or repression. Although preventing FadR-mediated activation of *fabA*, which encodes the enzyme that carries out the first step in UFA biosynthesis, did not significantly influence either the transcription or the translation of ToxT, it reduced its levels and prevented virulence gene expression. In the mutant strain unable to carry out FadR-mediated activation of *fabA*, expressing *fabA* ectopically restored the levels of ToxT and virulence gene expression. Taken together, the results presented here indicate that *V. cholerae* FadR influences the virulence cascade in the El Tor biotype by modulating the levels of ToxT via two different mechanisms.

**IMPORTANCE** Fatty acids (FAs) play important roles in membrane lipid homeostasis and energy metabolism in all organisms. In *Vibrio cholerae*, the causative agent of the acute intestinal disease cholera, they also influence virulence by binding into an N-terminal pocket of the master virulence regulator, ToxT, and modulating its activity. FadR is a transcription factor that coordinately controls the pathways of FA degradation and biosynthesis in enteric bacteria. This study identifies a new link between FA metabolism and virulence in the El Tor biotype by showing that FadR influences both the transcription and posttranslational regulation of the master virulence regulator ToxT by two distinct mechanisms.

**KEYWORDS** FadR, fatty acid, virulence, pathogenesis, ToxT

*Vibrio cholerae* O1 is a natural inhabitant of aquatic environments and causes the diarrheal disease cholera. The expression of its two primary virulence factors, toxin-coregulated pilus (TCP) (1) and cholera toxin (CT), is activated by a transcriptional

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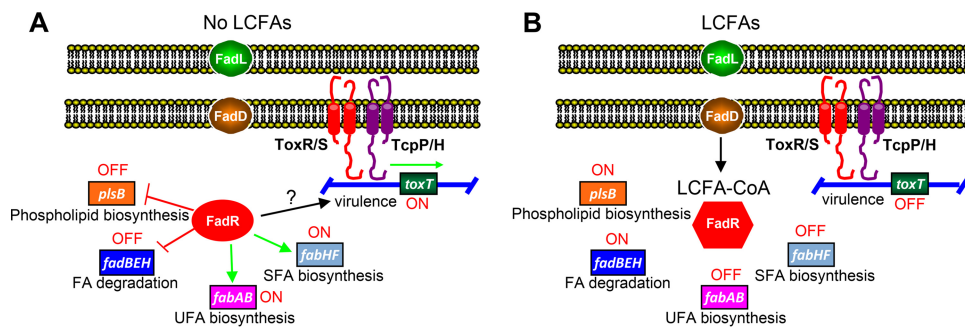
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**FIG 1** Roles of FadR in *V. cholerae*. (A) In the absence of LCFAs, FadR represses the expression of genes involved in FA degradation and phospholipid biosynthesis by binding to sites in the promoters of the *fadB*, *fadE*, *fadH*, and *plsB* genes and activates the expression of genes involved in UFA and SFA biosynthesis by binding to sites in the promoters of the *fabA*, *fabB*, *fabH*, and *fabF* genes. (B) When exogenous LCFAs are present, they diffuse across the outer membrane through FadL and are activated to LCFA-CoAs by FadD. The resulting activated LCFA-CoAs bind to FadR, causing a conformational change that releases it from DNA and results in derepression and failure to activate its regulated promoters. The mechanism by which it controls virulence is the subject of this work.

cascade involving a number of regulatory proteins (2). ToxT, an AraC family member, directly activates the expression of the *tcp*, *ctx*, and accessory colonization factor genes (3). The expression of ToxT, in turn, is activated by cooperation of two homologous transmembrane protein pairs, ToxRS and TcpPH (4–6). The induction of the virulence cascade by activation of the *tcpPH* promoter is dependent upon two additional regulators, AphA and AphB (7, 8). AphA is a winged helix transcription factor that facilitates the binding of the LysR-type regulator AphB to the promoter (9–11).

The expression of the *V. cholerae* virulence cascade is influenced by a wide variety of environmental stimuli such as pH, temperature, osmolarity, oxygen tension, bile, unsaturated fatty acids (UFAs), bicarbonate, c-di-GMP, and quorum sensing (2, 12–17). UFAs, which are a component of bile in the intestinal lumen, have been shown to inhibit virulence gene expression in *V. cholerae* (14). This occurs by direct binding of a UFA into a pocket in the N-terminal domain of ToxT, which prevents the protein from dimerizing and binding to DNA (18–20). The binding of UFAs to ToxT is thought to prevent the expression of virulence genes until the bacteria have penetrated the mucus of the intestine, where the concentrations of UFAs are presumably reduced (18). Thus, UFAs may serve as an *in vivo* signal to indicate that *V. cholerae* has progressed into the appropriate environment to initiate pathogenesis.

The pathways of fatty acid (FA) degradation and biosynthesis in enteric bacteria are coordinately controlled at the level of transcription by the master regulator FadR (21, 22). FadR from *Escherichia coli* is an extensively characterized member of the GntR family of transcriptional regulators with an N-terminal winged helix DNA binding domain and a C-terminal acyl coenzyme A (acyl-CoA) binding domain (23–25). In the absence of exogenous long-chain fatty acids (LCFAs), FadR represses transcription of the *fad* genes that encode proteins required for the transport, activation, and  $\beta$ -oxidation of LCFAs (Fig. 1A) (21). These genes include *fadL*, *fadD*, *fadBA*, *fadE*, and *fadH* (26). FadR also activates the expression of the *fabA* and *fabB* genes, which encode proteins required for the biosynthesis of UFAs (27, 28) as well as genes involved in saturated FA (SFA) biosynthesis (29, 30). When exogenous LCFAs are present (Fig. 1B), they diffuse across the outer membrane through FadL and are activated by the inner membrane-associated acyl-CoA ligase FadD (31) to produce long-chain fatty acyl-CoAs (LCFA-CoAs). These LCFA-CoAs bind directly to FadR and induce a conformational change that disrupts the FadR DNA complex (24, 32). This upregulates the expression of genes involved in FA degradation to utilize the LCFAs and downregulates the expression of genes involved in FA biosynthesis, which are no longer needed.

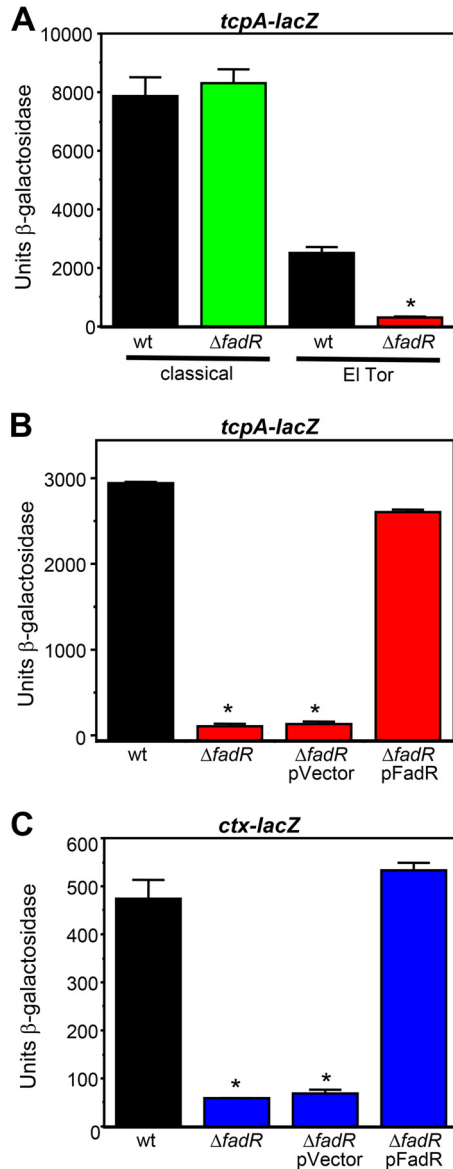
In *V. cholerae*, FadR is also involved in regulating the pathways of FA degradation and UFA biosynthesis (33). However, unlike *E. coli* FadR, which has only a single binding site for acyl-CoA, FadR from *V. cholerae* has two distinct binding sites for acyl-CoA (33).

One of these is structurally similar to the site in *E. coli* FadR, whereas the other is unique and comprised of residues from a 40-amino-acid insertion in the protein that is present only among *Vibrionaceae* (33, 34). Binding of ligand to both of these sites in *V. cholerae* FadR results in a more dramatic conformational change within the DNA binding domain than in *E. coli* FadR, which appears to more fully disrupt DNA binding (33). This is likely responsible for the enhanced expression of FA utilization genes in the presence of LCFAs in *V. cholerae* relative to other bacterial species (34). Since *Vibrionaceae* are natural inhabitants of aquatic environments, where they obtain FAs from the sediment, the acquisition of a second binding site in FadR may provide a more efficient mechanism for utilizing FAs in this environment.

In addition to its roles in FA degradation and UFA biosynthesis (33), FadR represses the expression of the *plsB* gene involved in phospholipid biosynthesis in *V. cholerae* (35) and, as shown here, activates the expression of two operons involved in SFA synthesis. Although prior studies in the classical biotype of *V. cholerae* did not reveal a role for FadR in the expression of the virulence cascade (36), the results here show that in the El Tor biotype of *V. cholerae*, FadR is also required for expression of TCP and CT. Examination of a *V. cholerae* strain C6706  $\Delta$ *fadR* mutant revealed a modest reduction in the transcription of *ToxT*. However, since FadR does not regulate expression from the *toxT* promoter directly, we assessed whether the ability of FadR to directly regulate the transcription of any of its known target genes indirectly influences *ToxT*. This was accomplished by individually preventing FadR-mediated regulation of each of its target genes by inserting a double point mutation into their FadR binding sites. Interestingly, disrupting FadR-mediated regulation of *fabA*, which encodes the enzyme that catalyzes the first step in UFA biosynthesis, decreased the levels of *ToxT* via a posttranslational mechanism. These results indicate that FadR influences the levels of *ToxT* in *V. cholerae* indirectly through two different mechanisms.

## RESULTS

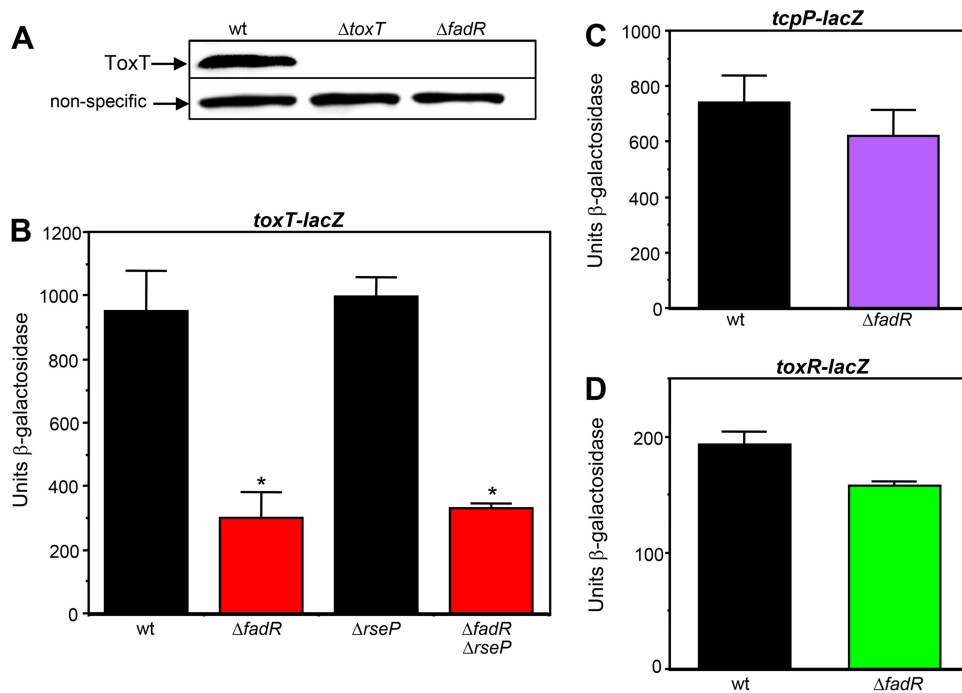
**FadR influences the expression of the virulence cascade in the El Tor, but not in the classical, biotype of *V. cholerae*.** It has previously been shown that loss of FadR does not influence the expression of the virulence cascade in the classical biotype of *V. cholerae* (36). Since the conditions that induce the expression of the virulence cascade in the El Tor biotype (static growth in a peptone-based medium, AKI [37], at 37°C in the presence of bicarbonate) are different from those that induce its expression in the classical biotype, we assessed the loss of FadR in both biotypes on the expression of the virulence cascade under AKI conditions. As shown in Fig. 2A, in the classical biotype, both the wild-type and  $\Delta$ *fadR* mutant strains showed high-level expression of a *tcpA-lacZ* fusion, indicating that FadR does not have an influence on the expression of the virulence cascade under this condition. Although the wild-type El Tor biotype fusion showed a 3-fold reduction in expression relative to the wild-type classical biotype fusion, consistent with the lowered expression of the virulence cascade in this biotype (38), the El Tor  $\Delta$ *fadR* mutant showed an 8-fold reduction in expression relative to the wild type, indicating that FadR does have an influence on the expression of the virulence cascade in this biotype. Under AKI conditions, as well as when subjected to shaking, the growth rate of the El Tor biotype  $\Delta$ *fadR* mutant (see Fig. S1 in the supplemental material) is only slightly reduced compared to that of the wild type. Introducing a plasmid expressing FadR into the El Tor  $\Delta$ *fadR* mutant restored expression of *tcpA-lacZ* to wild-type levels (Fig. 2B). The expression of an El Tor biotype *ctx-lacZ* fusion was also reduced 8-fold by the  $\Delta$ *fadR* mutation, and this effect was similarly complemented by the FadR expression plasmid (Fig. 2C). These findings indicate that FadR influences the expression of both *tcpA* and *ctx* in the El Tor biotype under AKI conditions. This effect is independent of quorum sensing, since a C6706  $\Delta$ *fadR*  $\Delta$ *hapR* mutant still showed reduced expression of the virulence cascade (data not shown). Since the amino acid sequence of FadR is identical for classical and El Tor biotypes, the above-described findings indicate that the loss of FadR influences the expression of the virulence cascade differently in the two biotypes of *V. cholerae*.



**FIG 2** Influence of a  $\Delta fadR$  mutation on the expression of *tcpA* and *ctx* promoter-*lacZ* fusions in *V. cholerae*. From left to right: MBN135, GK1536, KSK979, and GK1502 (\*,  $P = 0.006$ ) (A); KSK979, GK1502 (\*,  $P = 0.003$ ), GK1502/pKAS178 (\*,  $P = 0.005$ ), and GK1502/pWEL231 (B); KSK2325, GK1954 (\*,  $P = 0.0006$ ), GK1954/pKAS178 (\*,  $P = 0.007$ ), and GK1954/pWEL231 (C). Cultures were grown in AKI medium statically for 3.5 h.

### Loss of FadR reduces the transcription of *toxT* but not that of *tcpP* or *toxR*.

Given that ToxT directly activates the expression of both *tcpA* and *ctx* in *V. cholerae*, the observation that the  $\Delta fadR$  mutation reduced the expression of both of these genes suggested that FadR influences ToxT. Consistent with this finding, the El Tor biotype  $\Delta fadR$  mutant strain does not produce detectable levels of ToxT by Western blotting (Fig. 3A). Since the expression of the virulence cascade is highly regulated at the level of transcription, we first examined the expression of a *toxT-lacZ* transcriptional fusion in wild-type and  $\Delta fadR$  backgrounds. The  $\Delta fadR$  mutation reduced the transcription of *toxT* approximately 3-fold under static incubation conditions (Fig. 3B). In contrast, the  $\Delta fadR$  mutation did not significantly influence the transcription of either of its two activators, TcpP or ToxR, under these conditions (Fig. 3C and D). These results indicate that the expression of ToxT is the first point in the virulence cascade that is influenced by FadR. High-level CT production can be achieved in the El Tor biotype when static



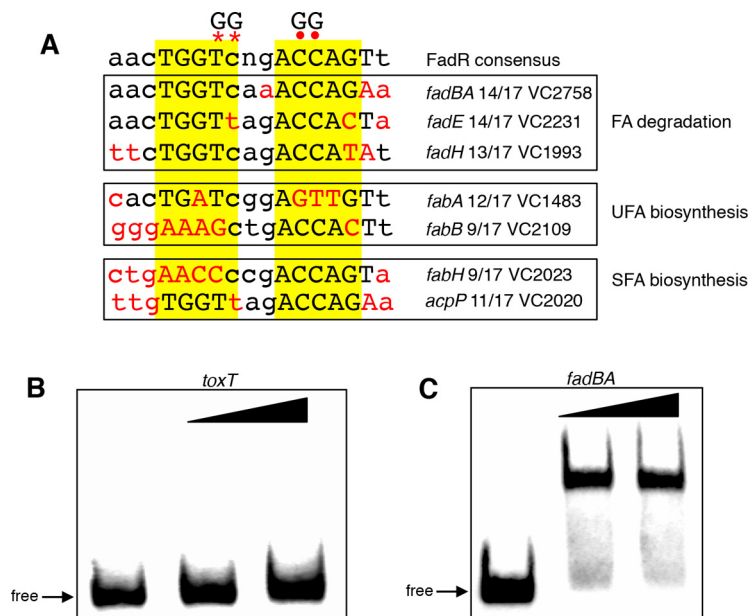
**FIG 3** A  $\Delta$ *fadR* mutation reduces the transcription of *toxT* but not that of *tcpP* or *toxR*. (A) Western blot with strains C6706 str2, KSK1184, and GK1257. (B to D) Influence of  $\Delta$ *fadR* on the transcription of *toxT*, *tcpP*, and *toxR*. From left to right: KSK1267, GK1499 (\*,  $P < 0.0001$ ), JAS273, and GK2136 (\*,  $P < 0.0001$ ) (B); KSK725 and WL982 (C); WL124 and GK1504 (D). Cultures were grown in AKI medium statically for 3.5 h.

incubation is followed by an aerobic shaking phase (37). As shown in Fig. S2 in the supplemental material, both the wild-type and  $\Delta$ *fadR* mutant strains showed a reduction in *toxT* expression after the aerobic phase of growth. However, the  $\Delta$ *fadR* mutant still showed a 2-fold decrease in expression relative to the wild type that is statistically significant.

It has recently been shown that the fatty acyl-CoA ligase FadD (Fig. 1) influences the virulence cascade in *V. cholerae* by promoting the localization of TcpP into the membrane (36). In the absence of FadD, induction of the  $\sigma^E$ -dependent extracytoplasmic stress response results in proteolysis of membrane-localized TcpP by the integral membrane protease RseP and reduces expression from the *toxT* promoter (39). To determine whether the effects of FadR on the virulence cascade shown here are independent of FadD and TcpP, we introduced a  $\Delta$ *rseP* mutation into both the wild-type and  $\Delta$ *fadR* *toxT-lacZ* fusions. As shown in Fig. 3B, the absence of RseP alone did not influence the expression of *toxT* and in the presence of the  $\Delta$ *fadR* mutation did not restore the expression of *toxT* as it did in a  $\Delta$ *fadD* mutant (39). These results suggest that FadR influences the virulence cascade in a manner independent of FadD and the  $\sigma^E$ -dependent extracytoplasmic stress response.

**FadR directly regulates the expression of genes involved in UFA biosynthesis and FA degradation but not *toxT*.** We have previously shown that in *V. cholerae*, similar to the situation in *E. coli*, FadR represses the expression of the *fadBA* (VC2758-59), *fadE* (VC2231), and *fadH* (VC1993) genes, involved in FA degradation, and activates the expression of the *fabA* (VC1483) and *fabB* (VC2109) genes, involved in UFA biosynthesis (33). Each of these promoters contains a 17-bp palindromic motif (Fig. 4A) that matches the consensus sequence established for *E. coli* FadR binding (40–42). In contrast, a similar motif was not detected in the *toxT* promoter, and consistent with this, purified FadR was unable to bind to the *toxT* promoter (Fig. 4B), although it bound to the *fadBA* promoter (Fig. 4C). These findings suggest that the influence of FadR on *toxT* expression is indirect and may be due to its effects on genes that it directly regulates.

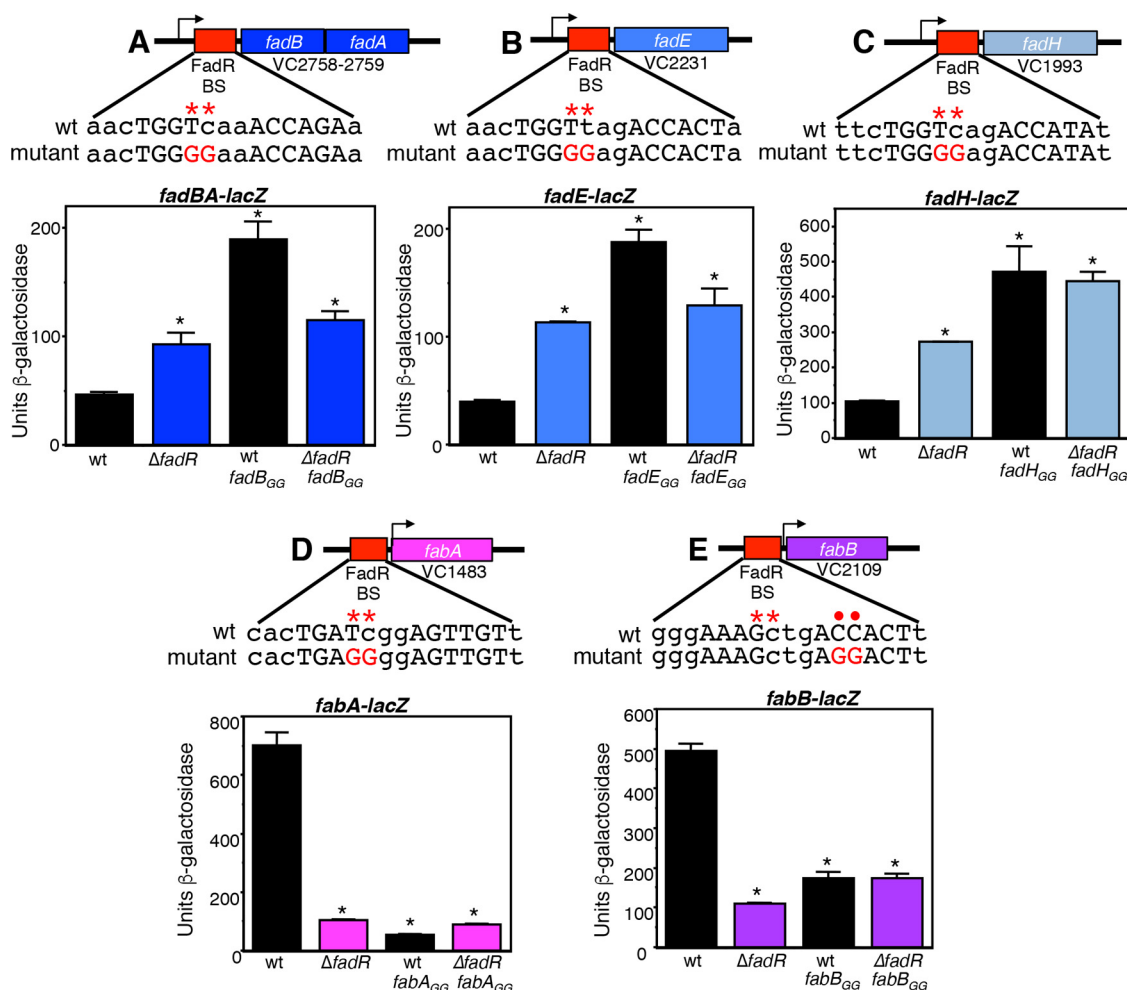
In an attempt to understand how FadR influences the expression of *toxT*, we wanted to determine whether the ability of FadR to directly regulate the transcription of any of



**FIG 4** FadR binding sites in *V. cholerae*. (A) The FadR consensus determined from *E. coli* (40) is shown at the top. Within the consensus sequence, upper- and lowercase letters represent nucleotides found more and less frequently, respectively. Mismatches from the consensus are in red. Asterisks show the position of the GG mutations in the *fadBA*, *fadE*, *fadH*, *fabA*, *fabH*, and *acpP* promoters. Dots show the position of the GG mutation in the *fabB* promoter. (B, C) Binding of purified FadR to *toxT* and *fadBA* promoter fragments. The first lane in each set has no protein added, the second lane has 80 ng FadR, and the third lane has 160 ng FadR.

its known target genes indirectly influences the *toxT* promoter. This was accomplished by individually preventing FadR-mediated regulation of each of its regulated promoters by inserting a double point mutation into their FadR binding sites (GG at positions 7 and 8 of the consensus, shown by asterisks in Fig. 4). This mutation has previously been shown in *E. coli* to eliminate FadR binding *in vitro* and prevent transcriptional regulation *in vivo* (40). Since the putative site at the *fabB* promoter, which shows a very poor match to the consensus (42), naturally contains a G at position 7 of the consensus, a different double point mutation (GG at positions 12 and 13 of the consensus, shown by dots in Fig. 4) was used to inactivate this binding site. As shown in Fig. 5, the ability of FadR to repress expression from the *fadBA*, *fadE*, and *fadH* promoters, as well as its ability to activate expression from the *fabA* and *fabB* promoters, was lost in the presence of the GG mutations. In addition, introduction of the  $\Delta$ *fadR* mutation into each GG mutant strain did not further alter expression from the promoters, indicating that the FadR binding sites were no longer functional. These results indicate that FadR uses these binding sites to directly regulate the expression of these genes in *V. cholerae*.

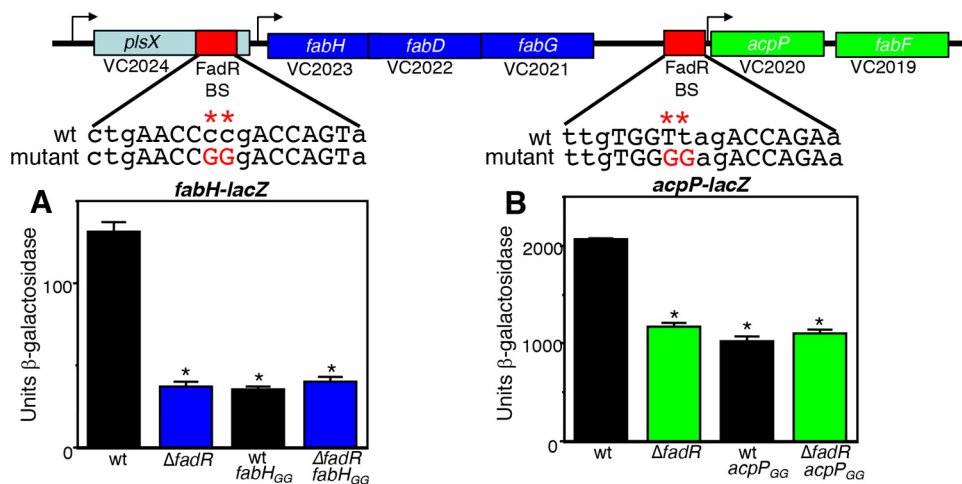
**FadR activates the expression of SFA biosynthesis genes in *V. cholerae*.** It has been shown that in addition to its roles in FA degradation and UFA biosynthesis, FadR activates the expression of genes involved in SFA biosynthesis in *E. coli* (29, 30). Unlike in mammals, where FAs are synthesized by a large multifunctional protein known as type I synthase (22, 43), bacteria produce a type II synthase composed of individual enzymes that catalyze discrete steps in the process, with each intermediate attached to the universal and highly conserved acyl carrier protein (ACP) (22, 43). Specifically, it has been shown that FadR activates the expression of the *fabHDG* operon, encoding three enzymes involved in SFA biosynthesis, and shows a modest activation of the *acpP* promoter, which controls the expression of the gene encoding ACP, *acpP*, coexpressed with another SFA biosynthetic enzyme encoded by *fabF* (29). Since the arrangement of the SFA genes in *V. cholerae* is similar to that in *E. coli* (Fig. 6), we assessed whether these genes were also regulated by FadR. We identified a putative FadR binding site (Fig. 4) upstream of *fabH* (VC2023) within the



**FIG 5** FadR regulates the expression of genes involved in FA degradation and UFA biosynthesis. The genetic organization of each of the genes is shown. The red boxes indicate the positions of the FadR binding sites (shown in Fig. 4), and the arrows show the putative transcriptional start sites. The positions of the GG mutations are shown by asterisks and dots. From left to right: WL1031, WL1035 (\*,  $P = 0.003$ ), WL1060 (\*,  $P = 0.005$ ), and WL1062 (\*,  $P = 0.005$ ) (A); WL1027, WL1029 (\*,  $P = 0.002$ ), WL1056 (\*,  $P = 0.0001$ ), and WL1058 (\*,  $P = 0.002$ ) (B); WL1040, WL1042 (\*,  $P = 0.005$ ), WL1064 (\*,  $P = 0.01$ ), and WL1066 (\*,  $P = 0.0001$ ) (C); WL1005, WL1007 (\*,  $P = 0.0007$ ), WL1020 (\*,  $P = 0.002$ ), and WL1022 (\*,  $P = 0.002$ ) (D); GK1609, GK1610 (\*,  $P = 0.002$ ), GK1669 (\*,  $P = 0.02$ ), and GK1672 (\*,  $P = 0.02$ ) (E). Cultures were grown in tryptone broth for 5 h with aeration.

*V. cholerae* *plsX* gene and, as shown in Fig. 6A, FadR activated a *fabH-lacZ* promoter fusion 3.6-fold. Inserting a GG mutation into the putative binding site reduced this activation to a level similar to that of the  $\Delta$ *fadR* mutant. We also identified another putative FadR binding site (Fig. 4) upstream of the *acpP* and *fabF* (VC2020-VC2019) genes (Fig. 6B). FadR activated the expression of this promoter nearly 2-fold, and inserting a GG mutation into the putative binding site also reduced this activation to a level similar to that of the  $\Delta$ *fadR* mutant. These results indicate that in addition to its roles in regulating the expression of genes involved in FA degradation and UFA biosynthesis, FadR activates the expression of two SFA biosynthesis operons in *V. cholerae*.

**Altered FA content of FA biosynthesis FadR binding site mutants.** Only two enzymes, encoded by the *fabA* and *fabB* genes, are required for the biosynthesis of UFAs (44, 45). Since the expression of both of these genes is dependent on FadR,  $\Delta$ *fadR* mutants in both *E. coli* and *Vibrio vulnificus* contain lower levels of UFAs (46, 47). To determine whether the levels of UFAs are reduced in the *V. cholerae*  $\Delta$ *fadR*, *fabA<sub>GG</sub>*, and *fabB<sub>GG</sub>* mutants, these strains were analyzed for total FA content. As shown in Table 1, the levels of UFAs in wild-type C6706 were reduced from 59% to 43% by the  $\Delta$ *fadR*



**FIG 6** FadR activates the expression of genes involved in SFA biosynthesis. Genetic organization of the *fab-acp* locus. The red boxes indicate the positions of the FadR binding sites (shown in Fig. 4) and the arrows show the putative transcriptional start sites. The positions of the GG mutations are shown by asterisks. From left to right: GK2017, GK2020 (\*,  $P = 0.01$ ), GK2039 (\*,  $P = 0.009$ ), and GK2040 (\*,  $P = 0.01$ ) (A); GK1630, GK1632 (\*,  $P = 0.02$ ), GK2035 (\*,  $P = 0.02$ ), and GK2037 (\*,  $P = 0.02$ ) (B). Cultures were grown in tryptone broth for 5 h with aeration.

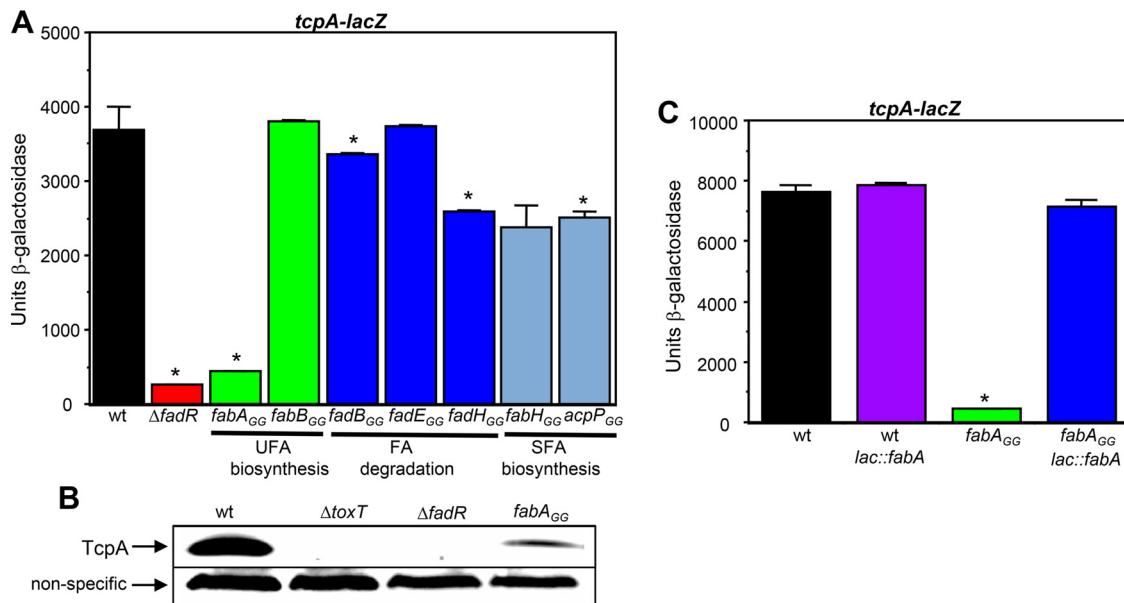
mutation. In the *fabA*<sub>GG</sub> mutant, they were reduced to 39%, and in the *fabB*<sub>GG</sub> mutant, they were reduced to 23%. In addition, for each of these mutants, the levels of SFAs correspondingly increased (Table 1). These findings confirm that in *V. cholerae*, as in *E. coli* and *V. vulnificus*, the intracellular levels of UFAs are dependent upon FadR. In contrast, the intracellular levels of SFAs in *V. cholerae* do not appear to depend on FadR since the FA content of the *fabH*<sub>GG</sub> and *acpP*<sub>GG</sub> mutants was not appreciably different from that of the wild type (Table 1).

**Preventing FadR-mediated activation of UFA biosynthesis decreases the expression of the virulence cascade.** The above findings show that FadR directly regulates the expression of genes involved in FA degradation, UFA biosynthesis, and SFA biosynthesis in *V. cholerae*. To determine whether disruption of FadR-mediated regulation of any of these genes is responsible for the altered expression of the virulence cascade in the  $\Delta$ *fadR* mutant, the GG-disrupted FadR binding sites in the promoters of these genes were individually introduced into a *tcpA-lacZ* fusion strain. As shown in Fig. 7A, the GG mutation in the *fabA* promoter strongly reduced the transcription of *tcpA* (approximately 8-fold) to a level similar to that of the  $\Delta$ *fadR* mutant, whereas the GG mutations in the promoters of the genes involved in FA degradation or SFA biosynthesis did not. In addition, introduction of the *fabA*<sub>GG</sub> mutation into wild-type C6706 significantly reduced the levels of TcpA as observed by Western blotting (Fig. 7B). To confirm that a functional *fabA* gene is required for the expression of *tcpA*, a merodiploid *fabA*<sub>GG</sub> *tcpA-lacZ* mutant strain that contains a wild-type copy of the *fabA* gene and its promoter inserted at the *lac* locus was constructed. As shown in Fig. 7C, the wild-type copy of *fabA* in this strain fully restored the expression of *tcpA*. These results indicate that FadR-mediated activation of UFA biosynthesis influences the expression of the virulence cascade in *V. cholerae*.

**TABLE 1** FA content of strains with GG mutations in FA biosynthesis promoters

Strain	% unsaturated	% saturated	Ratio
C6706 str2 (wild type)	59 ± 2	40 ± 2	1.4
GK1257 ( $\Delta$ <i>fadR</i> )	43 ± 0.5	56 ± 0.8	0.8
GK1689 ( <i>fabA</i> <sub>GG</sub> )	39 ± 0.2	60 ± 0.4	0.7
GK1691 ( <i>fabB</i> <sub>GG</sub> )	23 ± 0.6	77 ± 0.6	0.3
GK2043 ( <i>fabH</i> <sub>GG</sub> )	59 ± 0.2	40 ± 0.2	1.4
GK2041 ( <i>acpP</i> <sub>GG</sub> )	60 ± 0	39 ± 0	1.5



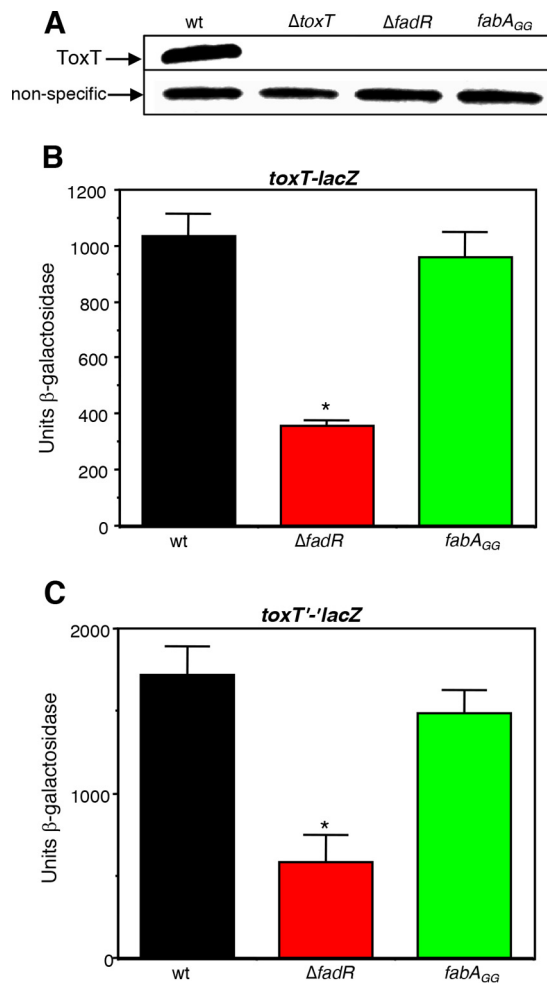


**FIG 7** A *fabA<sub>GG</sub>* mutation reduces the expression of *tcpA*. (A) Influence of GG mutations on the expression of *tcpA*. From left to right: KSK979, GK1502 (\*,  $P = 0.001$ ), GK1675 (\*,  $P = 0.0006$ ), GK1657, GK1982 (\*,  $P = 0.02$ ), GK1687, GK1981 (\*,  $P = 0.003$ ), GK2022, and GK2016 (\*,  $P = 0.03$ ). (B) Western blot with strains C6706 str2, KSK1184, GK1257, and GK1689. (C) Ectopic expression of wild-type *fabA* restores the expression of *tcpA* to the *fabA<sub>GG</sub>* mutant. From left to right: KSK979, GK2207, GK1675 (\*,  $P = 0.0008$ ), and GK2209. Cultures were grown in AKI medium statically for 3.5 h.

We next determined whether the *fabA<sub>GG</sub>* mutation influences the expression of the virulence cascade in a manner similar to that of the  $\Delta fadR$  mutant. Consistent with the reduced level of *tcpA* expression observed in the *fabA<sub>GG</sub>* mutant as described above, introduction of this mutation into wild-type C6706 prevented the production of ToxT (Fig. 8A), but unlike the  $\Delta fadR$  mutation, it did not influence the transcription of *toxT* (Fig. 8B). This finding suggests that the *fabA<sub>GG</sub>* mutation influences ToxT posttranscriptionally, by reducing its translation and/or by decreasing its stability. To address the former possibility first, a translational fusion of ToxT to  $\beta$ -galactosidase (*toxT-lacZ*) in the chromosome was made by fusing the first 44 amino acids of ToxT to amino acid 9 of  $\beta$ -galactosidase. Since this reporter is expressed under the control of the native *toxT* promoter, it measures transcription as well as translation. As shown in Fig. 8C, the  $\Delta fadR$  mutation reduced the transcription of the *toxT-lacZ* fusion similar to what is shown in Fig. 8B, whereas the *fabA<sub>GG</sub>* mutation did not influence either the transcription or the translation of ToxT. These findings suggest that preventing FadR from activating *fabA* expression reduces the levels of ToxT via a posttranslational mechanism. Taken together, the results shown here indicate that loss of FadR reduces the expression of the virulence cascade by two distinct mechanisms that influence ToxT. One is a reduction in the transcription of *toxT*, which occurs via an unknown process, and the second is a posttranslational reduction in the levels of ToxT due to an inability to activate *fabA* expression and UFA biosynthesis.

## DISCUSSION

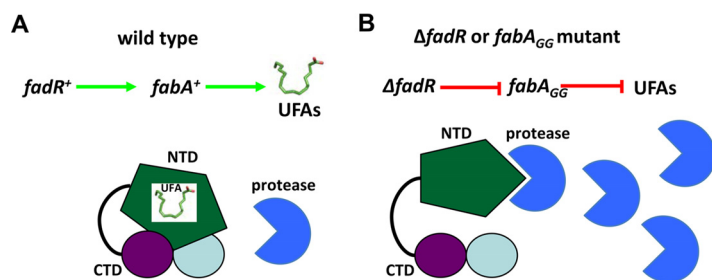
FadR is present in a variety of bacterial species, where it plays a role in the transcriptional regulation of genes involved in FA metabolism (41, 42). Although no role for FadR in the regulation of virulence was observed in the classical biotype of *V. cholerae* (36), we show here that FadR is required for the expression of the cascade in the El Tor biotype. The influence of FadR on the expression of both the *tcpA* and *ctx* genes in this biotype suggested a possible effect on ToxT, the regulator that directly activates both of these genes. Since it has previously been shown that UFAs are capable of binding directly to ToxT and inhibiting its activity (18), it seemed possible that the altered regulation of UFAs in the  $\Delta fadR$  mutant influences the activity of ToxT. However,



**FIG 8** The  $fabA_{GG}$  mutation reduces the levels of ToxT but does not influence either its transcription or its translation. (A) Western blot with strains C6706 str2, KSK1184, GK1257, and GK1689. (B and C) Left to right: transcriptional *toxT-lacZ* fusions in KSK1267, GK1499 (\*,  $P = 0.0004$ ), and WL1231 (B) and translational *toxT'-lacZ* fusions in WL1268, WL1271 (\*,  $P < 0.0001$ ), and GK1790 (C). Cultures were grown in AKI medium statically for 3.5 h.

as we show here, the  $\Delta fadR$  mutation appears to influence ToxT at two different levels: (i) by reducing its transcription by an unknown mechanism and (ii) by reducing its levels posttranslationally due to an inability to activate the expression of *fabA*, encoding the enzyme that catalyzes the first step in the biosynthesis of UFAs.

FadR was originally identified in *E. coli* as a transcriptional repressor controlling the genes of FA metabolism (48) and later shown to also function as a transcriptional activator of genes involved in UFA biosynthesis (27, 28). We previously demonstrated that FadR also regulates both of these processes in *V. cholerae* (33) and have now extended these studies to show that sequences with similarity to the *E. coli* FadR binding site consensus are necessary for the FadR-dependent expression of these genes in *V. cholerae*. FadR has recently been shown to activate the expression of two operons involved in SFA biosynthesis in *E. coli* (29). We have similarly found that FadR also plays a role in activating the expression of these operons in *V. cholerae*. In *E. coli*, FabH is essential for the initiation of FA synthesis and is a regulated step thought to play a key role in determining the amount of FAs produced by the pathway (49). Despite the dependence of FadR on expression from the *fabH* and *acpP* promoters in *V. cholerae*, the GG mutations in these promoters did not strongly influence the intracellular levels of SFAs. One explanation to account for this is that the *fabH* operon (*fabH*HDG) is transcribed from two different promoters; in addition to the FadR-



**FIG 9** Model for the influence of FadR on the expression of the virulence cascade. (A) In wild-type *V. cholerae*, FadR activates the expression of *fabA*, encoding the enzyme that catalyzes the first step in UFA biosynthesis. In the presence of UFAs, ToxT forms a “closed complex” that is unable to activate gene expression or to be proteolyzed. (B) In a  $\Delta$ *fadR* or *fabA*<sub>GG</sub> mutant, the levels of certain UFAs are reduced and ToxT is locked into the “open complex” that is capable of activating gene expression but is also able to be proteolyzed. In the  $\Delta$ *fadR* and *fabA*<sub>GG</sub> mutants, there may also be an increase in the expression and/or activity of the proteases. Model based on that previously described (56). CTD, C-terminal domain; NTD, N-terminal domain.

dependent promoter within *plsX*, *fabH* is expressed from a FadR-independent promoter that is located further upstream (29, 50). A similar situation appears to occur with *acpP*. In addition to the FadR-dependent promoter immediately upstream of its gene, *acpP* has been shown to be coexpressed with *fabG* (51). Thus, other factors in addition to FA availability appear to be involved in regulating the levels of SFAs in bacteria (45).

To shed light on how FadR influences the virulence cascade in *V. cholerae*, the FadR binding site GG mutations that abolished regulation of its target genes were individually assessed in a *tcpA-lacZ* reporter strain. Only one of these, the *fabA*<sub>GG</sub> mutation, strongly reduced *tcpA* expression. The *fabA* gene encodes the enzyme  $\beta$ -hydroxydecanoyl-ACP dehydratase, which introduces a double bond at the C-10 level and is an essential step in the formation of UFAs (52, 53). The second step in the pathway, carried out by the *fabB* gene, encodes  $\beta$ -ketoacyl-ACP synthase I, which is capable of elongating the product synthesized by FabA (54). Null mutations in either one of these genes are lethal in *E. coli*, as they both encode enzymes that are essential for UFA biosynthesis (54). This appears to be the case in *V. cholerae* as well, since we were not able to isolate deletions of these genes. In view of the fact that both the *V. cholerae* *fabA*<sub>GG</sub> and *fabB*<sub>GG</sub> mutants have reduced levels of UFAs relative to the wild type, the finding that the former, but not the latter, exhibits reduced *tcpA* expression suggests that it is not the absolute level of UFAs in these mutants that influences the expression of the virulence cascade. Since the profiles of FA species produced in these two strains are not identical, it is possible that one or more of these differences are responsible for the effects on the virulence cascade. Experiments to assess this are currently in progress.

Since the *fabA*<sub>GG</sub> mutation reduced the expression of *tcpA* to a level similar to that of the  $\Delta$ *fadR* mutation, it was surprising to find that, unlike the  $\Delta$ *fadR* mutation, it did not influence the transcription of *toxT*. Since the *fabA*<sub>GG</sub> mutation also did not influence the translation of *toxT*, this suggests that the inability to activate *fabA* expression reduces the levels of certain UFAs in the cell that, in turn, reduce the levels of ToxT. ToxT has previously been shown to undergo proteolysis when *V. cholerae* is shifted to conditions that do not support virulence (i.e., from 30°C to 37°C, or when the pH increases from pH 6.5 to pH 8), but the specific proteases involved in this process are unknown (55). ToxT appears to be specifically cleaved in a recently structured region of the protein that lies in the N-terminal domain of the protein between amino acids 100 and 109 (56, 57). Moreover, it has also been shown that the addition of the UFA linoleic acid to ToxT prevents its proteolysis (56), possibly by promoting the “closed” conformation of ToxT that is unable to bind to DNA or to activate gene expression (18). These findings raise the possibility that in the absence of UFA biosynthesis in the  $\Delta$ *fadR* and *fabA*<sub>GG</sub> mutants there is more ToxT present in the “open” conformation that lacks UFAs and this increases its susceptibility to proteolysis (see the model in Fig. 9). It is also possible that in the  $\Delta$ *fadR* and *fabA*<sub>GG</sub> mutants there is an increase in the expression

and/or activity of proteases that facilitates the degradation of ToxT. Further work is necessary in order to elucidate the specific mechanism involved in reducing the levels of ToxT in the *fabA<sub>GG</sub>* mutant.

It is still unclear how FadR influences the transcription of ToxT. The *toxT* promoter does not contain a FadR binding site that matches the consensus sequence observed in the promoter of its other regulated genes, and the purified protein does not appear to bind to it. The most likely explanation is that the influence of FadR on *toxT* transcription is indirect. However, none of the genes involved in FA degradation or UFA or SFA biosynthesis examined in this study strongly influenced the transcription of *toxT* when their expression was altered by FadR binding site mutations. Although we were unable to demonstrate repression of *plsB* by FadR in *V. cholerae* C6706, we ruled out a possible effect of this gene on virulence gene expression in this strain by inserting a GG mutation into the FadR binding site (35) and determined that it did not affect the expression of *tcpA* (data not shown). However, FadR clearly regulates the expression of additional genes in *V. cholerae* and one or more of these may influence the transcription of *toxT*. Since the *toxT* promoter is responsive to changes in central metabolism (58), it is also possible that global metabolic changes that occur in the  $\Delta$ *fadR* mutant result in altered transcription of ToxT.

The strategy that we used here to try to elucidate why the  $\Delta$ *fadR* mutant shows reduced expression from the *toxT* promoter in the El Tor biotype unexpectedly led to the discovery of a second route by which FadR impacts ToxT, via the activation of *fabA* expression. Since both ToxT and FadR are responsive to the presence of UFAs, it is possible that the intracellular levels of certain UFAs need to be optimal to promote the expression of the virulence cascade. High levels of exogenous UFAs bind directly to ToxT and inhibit its activity. In contrast, low levels of intracellular UFAs caused by the downregulation of *fabA* expression may promote the proteolysis of ToxT. In *V. vulnificus*, FadR was found to be essential for the organism to cause disease and the addition of the UFA oleate restored virulence to the  $\Delta$ *fadR* mutant (47). However, the addition of oleate or other UFAs to either the *V. cholerae*  $\Delta$ *fadR* or *fabA<sub>GG</sub>* mutants do not restore the levels of ToxT in these strains. We are currently in the process of trying to understand the reasons for this.

The work presented here identifies a new link between the expression of genes involved in UFA biosynthesis that are regulated by FadR and virulence in *V. cholerae*. Since the amino acid sequences of FadR from the classical and El Tor biotypes are identical, it is not yet clear why FadR influences the expression of the virulence cascade in the latter but not in the former. Previous transcriptome analyses have revealed a large number of differences in gene expression between the classical and El Tor biotypes (59). Thus, a novel way of regulating the expression of the virulence cascade in response to UFAs appears to have evolved in the El Tor biotype, and future work will be directed toward elucidating these mechanisms.

## MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains used in this study are described in Table 2. Strains were maintained at  $-70^{\circ}\text{C}$  in Luria-Bertani (LB) medium (60) containing 30% (vol/vol) glycerol. Cultures were grown in either AKI medium (37) or tryptone broth (60). Antibiotics were used at the following concentrations in LB medium: ampicillin, 100  $\mu\text{g/ml}$ ; kanamycin, 45  $\mu\text{g/ml}$ ; polymyxin B, 50 units/ml; streptomycin, 1 mg/ml. X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) was used in LB agar at 40  $\mu\text{g/ml}$ .

**Construction of *lacZ* fusion strains.** The various *lacZ* fusions were constructed by amplifying DNA fragments upstream and downstream of each gene from C6706 str2 chromosomal DNA using primers as follows (see Table S1 in the supplemental material): for *ctx*, CTX1a/CTX1b and CTX3/CTX4; for *toxT*, RT19/TX5 and TX6/TX7; and for *toxR*, MN27/TXR21 and MN30/TXR22. The fragments, together with a promoterless *lacZ* fragment from pVC200 (61), were inserted into either pKAS46 (62) or pKAS154 (63), and the resulting plasmids were used for allelic exchange (62) into *V. cholerae* strain KSK262 (8). The *tcpA-lacZ* and *tcpP-lacZ* fusions were previously described (8, 64). The *toxT-lacZ* translational fusion was constructed by amplifying a DNA fragment from the *toxT* gene using RT12ET with TX14 and a DNA fragment from plasmid pVC200 (61) using T-LacZ10 with T-LacZ11. The fragments were ligated into pKAS154 (63), and the resulting plasmid was used for allelic exchange into *V. cholerae* strain KSK1267. The wild-type *fabH-lacZ* and *acpP-lacZ* fusions were constructed using primers VC2023FabH8/VC2023FabH2 and FabF1/FabF2, respectively. The resulting fragments were inserted into pWEL236 (33), and the fusions

**TABLE 2** Bacterial strains used in this study

<i>V. cholerae</i> strain	Description/relevant genotype	Reference or source
C6706 str2	El Tor Inaba; streptomycin resistant	Lab collection
GK1257	C6706 $\Delta fadR$	This work
GK1499	KSK1267 $\Delta fadR$	This work
GK1502	KSK979 $\Delta fadR$	This work
GK1504	WL124 $\Delta fadR$	This work
GK1536	MBN135 $\Delta fadR$	This work
GK1545	KSK262 with <i>lacZ</i> from <i>E. coli</i>	33
GK1609	GK1545 $\Delta lacZ::fabB-lacZ$	33
GK1610	GK1609 $\Delta fadR$	33
GK1630	GK1545 $\Delta lacZ::acpP-lacZ$	This work
GK1632	GK1630 $\Delta fadR$	This work
GK1657	KSK979 <i>fabB</i> <sub>GG</sub>	This work
GK1669	GK1545 $\Delta lacZ::fabB_{GG}-lacZ$	This work
GK1672	GK1669 $\Delta fadR$	This work
GK1675	KSK979 <i>fabA</i> <sub>GG</sub>	This work
GK1687	KSK979 <i>fadE</i> <sub>GG</sub>	This work
GK1689	C6706 <i>fabA</i> <sub>GG</sub>	This work
GK1691	C6706 <i>fabB</i> <sub>GG</sub>	This work
GK1790	WL1268 <i>fabA</i> <sub>GG</sub>	This work
GK1954	KSK2325 $\Delta fadR$	This work
GK1981	KSK979 <i>fadH</i> <sub>GG</sub>	This work
GK1982	KSK979 <i>fadB</i> <sub>GG</sub>	This work
GK2016	KSK979 <i>acpP</i> <sub>GG</sub>	This work
GK2017	GK1545 $\Delta lacZ::fabH-lacZ$	This work
GK2020	GK2017 $\Delta fadR$	This work
GK2022	KSK979 <i>fabH</i> <sub>GG</sub>	This work
GK2035	GK1545 $\Delta lacZ::acpP_{GG}-lacZ$	This work
GK2037	GK2035 $\Delta fadR$	This work
GK2039	GK1545 $\Delta lacZ::fabH_{GG}-lacZ$	This work
GK2040	GK2039 $\Delta fadR$	This work
GK2041	C6706 <i>acpP</i> <sub>GG</sub>	This work
GK2043	C6706 <i>fabH</i> <sub>GG</sub>	This work
GK2136	JAS273 $\Delta fadR$	This work
GK2207	KSK979 $\Delta lacZ::fabA$	This work
GK2209	GK1675 $\Delta lacZ::fabA$	This work
JAS273	KSK1267 $\Delta rseP$	This work
KSK262	C6706 str2 $\Delta lacZ3$	8
KSK725	KSK262 <i>tcpA-lacZ</i>	8
KSK979	KSK262 <i>tcpA-lacZ</i>	This work
KSK1184	C6706 str2 $\Delta toxT$	This work
KSK1267	KSK262 <i>toxT-lacZ</i>	This work
KSK2325	KSK262 <i>ctx-lacZ</i>	This work
MBN135	$\Delta tcpA-lacZ$ classical	68
WL124	KSK262 <i>toxR-lacZ</i>	This work
WL982	KSK725 $\Delta fadR$	This work
WL1005	KSK262 $\Delta lacZ::fabA-lacZ$	33
WL1007	WL1005 $\Delta fadR$	33
WL1020	KSK262 $\Delta lacZ::fabA_{GG}-lacZ$	This work
WL1022	WL1020 $\Delta fadR$	This work
WL1027	GK1545 $\Delta lacZ::fadE-lacZ$	33
WL1029	WL1027 $\Delta fadR$	33
WL1031	GK1545 $\Delta lacZ::fadBA-lacZ$	33
WL1035	WL1031 $\Delta fadR$	33
WL1040	GK1545 $\Delta lacZ::fadH-lacZ$	33
WL1042	WL1040 $\Delta fadR$	33
WL1056	GK1545 $\Delta lacZ::fadE_{GG}-lacZ$	This work
WL1058	WL1056 $\Delta fadR$	This work
WL1060	GK1545 $\Delta lacZ::fadBA_{GG}-lacZ$	This work
WL1062	WL1060 $\Delta fadR$	This work
WL1064	GK1545 $\Delta lacZ::fadH_{GG}-lacZ$	This work
WL1066	WL1064 $\Delta fadR$	This work
WL1231	KSK1267 <i>fabA</i> <sub>GG</sub>	This work
WL1268	KSK1267 <i>toxT-lacZ</i>	This work
WL1271	WL1268 $\Delta fadR$	This work

were introduced into the *lacZ* locus of KSK262 by allelic exchange. The construction of the wild-type *fadBA*, *fadE*, *fadH*, *fabA*, and *fabB* fusions were previously described (33).

**Construction of FadR binding site mutations.** The various mutations in the FadR-regulated promoters were constructed by PCR amplifying two DNA fragments from C6706 str2 using primers (Table S1) as follows: *fabA*, FabA3/FabA6 and FabA4/FabA5; *fabB*, FabB15/FabB13 and FabB12/FabB14; *fadBA*, FadB4/FadB7 and FadB8/FadB9; *fadE*, FadE3/FadE4 and FadE5/FadE6; *fadH*, FadH6/FadH4 and FadH5/FadH7; *fabH*, FabH4/FabH5 and FabH6/FabH7; and *acpP*, FabF4/FabF5 and FabF6/FabF7. The fragments were inserted into pKAS154. The resulting GG mutant promoter plasmids were then used as a source of DNA for PCR amplification using the same primers that were initially used to construct the wild-type fusions (33): *fabA*, FabA1/FabA3; *fabB*, FabB3/FabB4; *fadBA*, FadB1/FadB4; *fadE*, FadE1/FadE3; *fadH*, FadH1/FadH3; *fabH*, VC2023FabH8/VC2023FabH2; and *acpP*, FabF1/FabF2. The fragments were inserted into either pKAS180 (65) or pWEL236 (33), and the resulting fusions were introduced into the *lacZ* locus of KSK262 by allelic exchange.

**Construction of deletion mutations, expression plasmids, and merodiploid.** The  $\Delta$ *toxT* and  $\Delta$ *rseP* mutations were constructed by amplifying DNA fragments upstream and downstream of the genes from C6706 str2 using RT19/TX5 and TX6/TX7 for the former and YaelA/YaelB and YaelC/YaelD for the latter (Table S1). The fragments were inserted into pKAS46 (62), and the resulting deletions were introduced into *V. cholerae* by allelic exchange. The  $\Delta$ *fadR* mutation was constructed as previously described (33). The FadR overexpressing plasmid pWEL231 was constructed by amplifying *fadR* from C6706 str2 chromosomal DNA with primers FadR7 and FadR8 and ligating the resulting product into pKAS178 (66). The *fabA* merodiploid strain was constructed by PCR amplifying a DNA fragment containing the *fabA* gene along with upstream and downstream sequences using primers FabA9 and FabA10 and inserting this fragment into pGKK344, constructed similarly to pKAS180 (65) except that the fragments were ligated into pKAS154 (63). It was then introduced into *V. cholerae* by allelic exchange.

**Fatty acid analysis.** Strains were grown in tryptone broth at 37°C with aeration for 5 h. Cells were pelleted by centrifugation, washed with fresh tryptone broth, and frozen on dry ice. Fatty acid content was determined by Microbial ID, Inc. (Newark, DE).

**Immunoblot analysis.** Whole-cell extracts from the various cultures were prepared, and equivalent amounts of total protein as determined by the bicinchoninic acid (BCA) protein assay (Pierce) were analyzed on a 16% SDS-PAGE gel. Proteins were visualized by transferring to nitrocellulose and probing with either anti-ToxT antibody or anti-TcpA antibody using the ECL detection system (Amersham).

**Gel mobility shift assays.** FadR was purified using the Impact-CN protein fusion and purification system (New England Biolabs) as previously described (33). The DNA fragments for the assays were amplified by PCR as follows: for *fadBA*, FadB1/FadB2; for *toxT*, TX11/TX12. The fragments were gel purified and end labeled with digoxigenin as described previously (67). Binding reactions for FadR were carried out with 20 mM Tris (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 1  $\mu$ g poly(dI-dC). The samples were applied to a 5% polyacrylamide gel and subjected to electrophoresis. The DNA was transferred to nylon membranes by electroblotting, probed with anti-digoxigenin-alkaline phosphatase antibody (Amersham Pharmacia), and visualized using chemiluminescence.

**$\beta$ -Galactosidase assays.**  $\beta$ -Galactosidase assays (60) were carried out by growing cultures either in AKI medium statically for 3.5 h at 37°C or in tryptone broth with aeration for 5 h at 37°C. Assays were done in duplicate for each culture, and the data are representative results from at least two separate experiments.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00762-16>.

**TEXT S1**, PDF file, 0.2 MB.

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