Binding of Shewanella FadR to the fabA fatty acid biosynthetic gene: implications for contraction of the fad regulon

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ABSTRACT
The Escherichia coli fadR protein product, a paradigm/ prototypical FadR regulator, positively regulates fabA and fabB, the two critical genes for unsaturated fatty acid (UFA) biosynthesis. However the scenario in the other γ-proteobacteria, such as Shewanella with the marine origin, is unusual in that Rodionov and coworkers predicted that only fabA (not fabB) has a binding site for FadR protein. It raised the possibility of fab regulon contraction. Here we report that this is the case. Sequence alignment of the FadR homologs revealed that the N-terminal DNA-binding domain exhibited remarkable similarity, whereas the ligand-accepting motif at C-terminus is relatively-less conserved. The FadR homologue of S. oneidensis (referred to FadR_she) was over-expressed and purified to homogeneity. Integrative evidence obtained by FPLC (fast protein liquid chromatography) and chemical cross-linking analyses elucidated that FadR_she protein can dimerize in solution, whose identity was determined by MALDI-TOF-MS. In vitro data from electrophoretic mobility shift assays suggested that FadR_she is almost functionally-exchangeable/equivalent to E. coli FadR (FadR_ec) in the ability of binding the E. coli fabA (and fabB) promoters. In an agreement with that of E. coli fabA, S. oneidensis fabA promoter bound both FadR_she and FadR_ec, and was disassociated specifically with the FadR regulatory protein upon the addition of long-chain acyl-CoA thioesters. To monitor in vivo effect exerted by FadR on Shewanella fabA expression, the native promoter of S. oneidensis fabA was fused to a LacZ reporter gene to engineer a chromosome fabA-lacZ transcriptional fusion in E. coli. As anticipated, the removal of fabR gene gave about 2-fold decrement of Shewanella fabA expression by β-gal activity, which is almost identical to the inhibitory level by the addition of oleate. Therefore, we concluded that fabA is contracted to be the only one member of fab regulon in the context of fatty acid synthesis in the marine bacteria Shewanella genus.

KEYWORDS  FadR, fab regulon, fabA, fabB, contraction, Shewanella

INTRODUCTION
Current knowledge on the regulation of fatty acid metabolism is mostly from studies with Escherichia coli (E. coli). The E. coli FadR regulatory protein that is classified into the GntR family of transcription factors, acts as a global regulator controlling bacterial lipid metabolism (Henry & Cronan, 1992, Irám & Cronan, 2005). The two opposite roles played by this regulator include repression of fatty acid degradation (fad) system (Feng & Cronan, 2009b, Henry & Cronan, 1991, Irám & Cronan, 2005), and activation of fabA and fabB, the two genes for unsaturated fatty acid synthesis (Feng & Cronan, 2009a, Henry & Cronan, 1992, Nunn et al., 1983). In fact, the E. coli FadR also indirectly regulates transcription of the glyoxylate bypass operon (aceBAK), through direct activating the
As the paradigm FadR regulator, the E. coli fadR protein product behaves as a dimer (van Aalten et al., 2000), and consists of the N-terminal DNA-binding domain (Xu et al., 2001) and the ligand-interacting motif at C-terminus (van Aalten et al., 2001). The accumulated crystal structures of FadR alone and its complex with DNAacyl-CoA defined clearly the structural basis for FadR-mediated regulatory mechanism in the context of lipid metabolism (van Aalten et al., 2001, van Aalten et al., 2000, Xu et al., 2001). In addition to the residues directly contacting target DNA (Xu et al., 2001), we also mapped three more key residues with indirect role in FadR-DNA interplay (Zhang et al., 2014). In vitro and in vivo evidence proved that long-chain fatty acid (LCFA) acyl-CoA thioesters are small molecule effectors for the FadR regulatory system (Henry & Cronan, 1992, van Aalten et al., 2001, Cronan, 1997). The mechanism by which LCFA induces fad expression lies in the fact that the binding of LCFA acyl-CoA to FadR protein results in the alteration of protein configuration, which in turn triggers the loss of its DNA binding ability. However, it still remains unclear why the unexpected functional diversity exists amongst the FadR regulatory proteins (Iram & Cronan, 2005). Of particular note, it is mystery that in relative to the prototypical FadR with an origin of E. coli, the Vibrio cholerae (V. cholerae) FadR is strikingly superior to in the regulatory amplitude, and bound its ligands appreciably stronger (Iram & Cronan, 2005). Further sequence analyses revealed that an extra 40-aa longer region present in V. cholerae FadR might explain the excellent performance of its regulation role in the context of lipid metabolism (Zhang et al., 2014). Unlike the scenario seen with its closely-relative V. cholerae, the FadR homologue from the other marine bacterium Shewanella, is quite similar to that of the paradigm organism E. coli.

The genus of Shewanella is a family of Gram-negative bacteria inhabiting in marine environment/ecosystem, including no less than 50 diversified species such as S. oneidensis and S. algae (Janda & Abbott, 2014). S. oneidensis is referred to an alternative model anaerobic microorganism with the known genome sequence (~4.9 Mb) that encodes over 4700 genes (Kolker et al., 2005, Heidelberg et al., 2002). Not only do the species of Shewanella bacteria (e.g., S. putrefaciens) act as normal components of the surface flora of fish and are involved in the spoilage of aquatic products (Parlapane et al., 2013, Li et al., 2012), but also some species like S. algae is recognized to be zoonotic pathogens in that they can cause opportunistic infections via occupational exposure of workers with skin and soft tissue cuts to marine products (Janda & Abbott, 2014). Given the excellent performance of S. oneidensis in reduction of poisonous heavy metals like iron (Cheng et al., 2013), uranium (Sheng & Fein, 2014), and even ionic mercury (Wiatrowski et al., 2008), it was believed to have the robust/potential applications into environmental bioremediation targeting toxic elements and heavy metals and development of microbial fuel cells (Fredrickson et al., 2008, Hau & Gralnick, 2007). The advantage of Shewanella in biotechnology is mainly attributed to the diversified metabolic capabilities that included versatile electron-transfer systems (Hau & Gralnick, 2007, Fredrickson et al., 2008). The deep-sea environment with low temperature where the Shewanella bacteria naturally reside/inhabit determined that some special mechanism might be evolved for their survival. Wang et al. (Wang et al., 2009) found that Shewanella has appreciable ability to produce various types of low-melting-point fatty acids with monounsaturated fatty acids (MUFA) included. The similar scenario was also noted in the other marine bacterium V. cholerae, in which relatively-high percentage of unsaturated fatty acids (UFA) is present in relative to E. coli (Massengo-Tiasse & Cronan, 2008, Feng & Cronan, 2011a). The physiological explanation proposed lies in that the high percentage of UFA in the bacterial membrane incorporated with phospholipids confers the better membrane fluidity, which in turn enhances its capability of cold adaptation. Although the type II fatty acid synthesis (FAS) pathway in Shewanella was constructed using the approach of comparative genomics (Wang et al., 2009), it seemed likely that some unusual/unclear aspects are present in the regulation of this specialized Type II FAS (Rodionov et al., 2011). Of particular note, Rodionov and coworkers (Rodionov et al., 2011) predicted that only fabA (not fabB) of Shewanella has a binding site for FadR protein, posing the possibility of fab regulon contraction.

In this paper, we integrated in vitro and in vivo approaches to address this uncommon question and reported that this is the case. As expected, Shewanella FadR regulates expression of fabA (not fabB) through the direct protein-DNA physical interplay. Therefore, it is reasonable that the fabA fatty acid synthesis gene is contracted as the only one member of fab regulon in the context of fatty acid synthesis in the marine bacteria Shewanella genus.

RESULTS AND DISCUSSION

Contraction of fab regulon in Shewanella

Different from the paradigm enteric bacterium E. coli that has only one chromosome of 4.64 Mb with average GC contents of 50.8% and encodes 4498 putative genes (Blattner et al., 1997), S. oneidensis MR-1, the representative cousin with marine origin, not only has a chromosome (4.96 Mb, 46% GC percentage) encoding 4403 genes, but also contains a megaplasmid (0.16 Mb, 43.7% GC percentage) encoding 149 putative proteins (Heidelberg et al., 2002, Kolker et al., 2005). The similar scenario was also seen with V. cholerae N16961, its closely-relative with the same marine origin, in that it carries two genomes (one of which is 2.98 Mb with 47.7% GC contents and encodes 2690 genes, and other one is 1.07 Mb (46.9% GC percentage) corresponding to 1003 genes (Heidelberg et al., 2000).
In relative to *E. coli* that has no less than 12 \( \textit{fad} \) members, those genes controlled by the fatty acid-responsive FadR regulator (Fig. 1A, 1C and 1E), the \( \textit{fad} \) members seemed to be contracted in the cousin *S. oneidensis* in that only 4 well-known \( \textit{fad} \) genes/operons (\( \textit{fadE}, \textit{fadL}, \textit{fadIJ} \) and \( \textit{fabA} \)) has the putative FadR-binding sites (Fig. 1B, 1D and 1F). Also,
A

E. coli

Dimer

Monomer

B

C

D

Phosphate

OD

Elution volume (mL)

kDa

M

FadR_she

1MIAKGPAS FAEKYIVRSI WENKFPPGSI LPAERSEL1 IQVTRTLRE

VQLRRLARDGW LKIQHOKPTR VNWFETSGL NILETIADLN PEGFVLVDQ

LTSARTNVSA IIYFRGARNN PTDAMEVLAA IHQLEDTEAS FAEYDYLLHH

TLaFSSGNPL YVLINNGFKG LYSVGRYYF SSSEROQLAL NYKELLA

201 KAKNYLDVPA LMRTYGINSG KMWLQRDM PSSIAQKDSH

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S. oneidensis possesses two more new FadR-regulated genes (SO_4761 encoding the GNAT family of Acetyltransferase and SO_0572 encoding the GNAT family of Acetyltransferase) (van Aalten et al., 2000), the protein secondary structure was illustrated in cartoon (top) (Zhang et al., 2014), α: alpha-helix; β: beta-sheet; T: Turn; n: coil. The seven known DNA-binding sites (R35, T44, R45, T46, T47, R49 and 65H) are highlighted with black triangles (Xu et al., 2001), the three known ligand-binding sites are shown with grey triangles (216G, 219S and 223W) (van Aalten et al., 2000), the newly-proposed amino acids with indirect role for FadR-DNA interaction are highlighted with blue arrows (W60, F74 and W75) (Zhang et al., 2014). The extra 40-aa (138–177) longer region of V. cholerae FadR was underlined in blue. The FadR sequences are separately sampled from E. coli K12 (Accession no.: CAA30891), V. cholerae (Vibrio cholerae) (Accession no.: NP_718457) and S. oneidensis (Accession no.: NP_718457). (B) Gel exclusion chromatographic profile of the recombinant S. oneidensis FadR protein run on a Superdex 75 column (GE Healthcare). The expected peak of the target FadR was eluted at the position of 10.5 mL (highlighted with an arrow). The inset gel is the 15% SDS-PAGE photography of the collected S. oneidensis FadR protein sample. The mass of the monomeric S. oneidensis FadR is estimated to be ∼27 kDa. Abbreviations: M, protein marker; OD280, optical density at 280 nm; mAu, milli-absorbance units. The ruler on the top was given to describe the elution pattern of the standard proteins (Pharmacia). The standards used here included Ferritin (∼440 kDa), Aldolase (153 kDa), Bovine serum albumin (∼67 kDa), Ovalbumin (∼44 kDa) and ribonuclease (∼13.7 kDa), respectively. (C) Chemical cross-linking analyses for the purified S. oneidensis FadR protein. The level of EGS chemical cross-linker was illustrated with a triangle varies from 0, 0.1, 0.2, 0.5, 1.0, 1.5, to 2.0 μmol/L. (D) MS determination of the recombinant S. oneidensis FadR protein. The matched amino acid residues that exhibited 69% coverage to the native S. oneidensis FadR are given bold and underlined type.
Characterization of *Shewanella* FadR

An earlier study (Iram & Cronan, 2005) has found that the FadR lipid metabolism regulator of *V. cholerae* has an unusual insert of 40 residues. Our results (submitted) plus Shi’s observations (Shi et al., 2015) revealed an unexpected contribution of this unique inserting sequence in constituting an extra-ligand binding motif for FadR regulatory protein. The second ligand-binding site confers its excellent ability in fatty acid sensing. Given the fact that both *V. cholerae* and *S. oneidensis* are closely-related marine bacteria that shared a similar ecological niche with poor availability of fatty acids, we initially anticipated that this insert might be an indicator or relic for such kind of unparalleled regulation by FadR (Fig. 1).

In fact, it is not this case. Multiple sequence alignments of three FadR proteins (FadR_ec for *E. coli*, FadR_vc for *V. cholerae*, and FadR_she for *S. oneidensis*) showed that: 1) the N-terminal DNA-binding motifs are very conserved featuring a full set of all the known residues critical for DNA binding; 2) the C-terminal ligand-interacting domains are appreciably diversified; and 3) the so-called insert of 40 residues (138–177 aa) is only present in FadR_vc (Fig. 2A). Considered the fact combined with atypical features seemed in fatty acid transport system, we favored the anticipation that *Shewanella* somewhat retains the evolutional relic that is partially observed with *E. coli* and *Vibrio*, respectively.

To further functional analyses of the above bacterial FadR proteins, we over-expressed the three types of recombinant FadR proteins (FadR_ec FadR_vc & FadR_she) and purified them to homogeneity. As expected, SDS-PAGE profile clearly showed the purified FadR_she protein migrates at the position of ~27 kDa. The FPLC profile showed that the expected peak of purified *S. oneidensis* FadR was eluted at the position of 10.5 mL (indicated with an arrow, Fig. 2B), suggesting its apparent molecular mass is more than 44 kDa, but less than 67 kDa. Given the fact that the ideal molecular weight of recombinant *S. oneidensis* FadR in momomer is ~27 kDa, we believed that the form of FadR_she in solution might be a dimer (~54 kDa). It was generally consistent with the scenario seen with the *E. coli* FadR as a dimer. Subsequently, we used chemical cross-linking assays to further prove this speculation. As we expected, appearance/formation of the dimerization for the FadR_she protein is appreciably increased upon addition of chemical cross-linker EGS (Fig. 2C). Also, it behaves in an EGS dose-dependent manner (Fig. 2C). In particular, the dimer band was excised from the SDS-PAGE and subjected to liquid chromatography mass spectrometry. As a result, the MS results confirmed this identity in that the digested peptides matched the *S. oneidensis* FadR protein with the coverage of 69% (Fig. 2D).

The FadR proteins of *E. coli* and *S. oneidensis* are functionally-equivalent

Gel shift assay was performed to detect the binding ability of FadR_ec, FadR_vc and FadR_she to the cognate DNA binding sites. As expected, EMSA-based experiments showed that the *E. coli* FadR protein (as the positive control) binds well to its own promoters of both *fabA* (Fig. 3A) and *fabB* (Fig. 3B) promoters. The fact that the FadR_vc protein gives consistently the super-shift bands for both *fabA* and *fabB* probes in the gel shift assays, is mostly attributed to the essence of its easy-forming the protein multimer (Fig. 3). Of note, FadR_she exhibited an excellent ability of interacting with the *fabA* (Fig. 3A) and *fabB* (Fig. 3B) with the origin of *E. coli*. It seemed likely that the FadR proteins of *E. coli* and *S. oneidensis* are functionally-equivalent (and/or exchangeable). It is not surprise since the FadR/FabR orthologue from other marine bacterium, *Vibrio*, also followed this rule (Feng et al., 2005).

Figure 3. *Shewanella* FadR protein is functionally-exchangeable to the paradigm *E. coli* version. (A) EMSA-based evidence for binding of *E. coli* *fabA* promoter to FadR protein of three origins. (B) EMSA analyses for crosstalk of *E. coli* *fabA* promoter with three kinds of bacterial FadR proteins. A representative photograph was given here, which were from no less than three independent EMSA experiments (7% native PAGE). Three versions of FadR protein here are FadR_ec, FadR_vc and FadR_she, respectively. In gel shift assays, the FadR protein (5 pmol) is incubated with DIG-labeled *fabAec* (or *fabBec*) probe (0.2 pmol). Note: An unexpected but interesting scenario “super-shift” is consistently observed in our trials with *V. cholerae* FadR.
& Cronan, 2011b, Feng & Cronan, 2011a). To our knowledge, the cases of similar functional exchange of transcriptional regulators can be extended to BioR, the other GntR-type regulators implicated into the metabolism of biotin, a sulfur-containing fatty acid (Feng et al., 2013a, Feng et al., 2013b). Thereby, it makes sense that the atypical regulation by FadR in UFA synthesis of *Shewanella* is due to the cryptic site in front of *fabB* (Fig. 1), not FadR_she (Fig. 3).

*S. oneidensis* fabA has a functional FadR-binding site, and this binding is specifically reversed by long-chain acyl-CoA

Through sequence comparison of the *fabA* and *fabB* promoter regions of *E. coli*, *V. cholerae* and *S. oneidensis*, we found that the cognate FadR-specific binding site in front of the *fabA* promoter regions of these three bacterial species are much more conservative (Fig. S1A), but that of *fabB* promoter region is not (Fig. S1B). This observation is generally consistent with the prediction by Rodionov and coworkers (Rodionov et al., 2011) that only *fabA* (not *fabB*) of *Shewanella* has a binding site for the FadR regulator. To further prove the function of this predicted site, termed *fabA* probe, we synthesized it using the approach of annealing the two complementary DNA strand. This DNA probe is digoxigenin-labeled DNA fragment of 56 bp that overlaps the candidate FadR_she binding site (Fig. S1A and Table 2). Gel shift assays confirmed that FadR_ec (Fig. 4A) and FadR_she (Fig. 4B) both can efficiently bind the *S. oneidensis* fabA promoter. In much similarity to the scenario seen with FadR_ec here (Fig. 4A), plus our former observations with FadR regulators of *E. coli* (Feng & Cronan, 2009b, Feng

Figure 4. Evidence that *Shewanella* fabA promoter has a functional FadR-recognizable palindrome. (A) Binding of *Shewanella* fabA promoter to *E. coli* FadR protein. (B) Interplay between *Shewanella* fabA promoter and *Shewanella* FadR protein. The gel shift tests were conducted using 7% native PAGE, and a representative result is shown here. In these assays, levels of FadR protein (FadR_ec and FadR_she) added are denoted with a triangle on right hand (0.1, 0.5, 2, and 5 pmol), whereas the DIG-labeled fabBshe probe is added to 0.2 pmol. Minus sign denotes no addition of FadR protein.

![Figure 4](image)

Figure 5. SPR-based dynamic analyses for binding of fabA to *Shewanella* FadR. (A) SPR assay for interaction between fabA and FadR_she. (B) Measurement of the KD value for fabA-FadR_she.

![Figure 5](image)
and Cronan, 2010) and *V. cholerae* (Feng & Cronan, 2011b), we also found that the fabA_she probe binds FadR_she protein in a dose-dependent manner (Fig. 4B). To preliminarily elucidate the kinetics of FadR_she/fabA_she interaction, we conducted surface plasmon resonance (SPR)-based measurements. SPR results revealed that the binding affinity \((K_D)\) of fabA_she to FadR_she is roughly 436 nmol/L (Fig. 5A and 5B), and the binding mode is 2:1 (a dimeric FadR protein is bound to a target DNA fragment (not shown).

Given the fact that long-chain (but not short chain) fatty acyl-CoA species can antagonize the DNA-binding activity of FadR with origins of *E. coli* (Henry & Cronan, 1992, Cronan, 1997) and *Vibrio* (Iram & Cronan, 2005, Feng & Cronan, 2011b), it is of much interest to test the behaviors of these ligands in the case of *S. oneidensis* FadR. Therefore, we tested six acyl-CoA species of different acyl chain lengths. The EMSA-based competition assays showed that medium-chain acyl-CoA (C9:0, C10:0) don’t interfere with the fabA_she binding to either FadR_ec (Fig. 6A) or FadR_she (Fig. 6B). In contrast, the long-chain acyl-CoA species (C16:0, C16:1, C18:0 and C18:1) strongly impaired DNA binding (Fig. 6A and 6B). We believed that long-chain but not...
medium-chain acyl-CoA can specifically interact with FadR_she ligand-binding site and release FadR_she from the S. oneidensis fabA promoter. In summary, the in vitro data accumulated suggest that long-chain acyl-CoAs regulate the Shewanella fabA transcription via their interaction with the FadR protein.

**Expression of Shewanella fabA is activated by FadR, but repressed by oleate in E. coli**

It is well known that: 1) FadR acts as an activator for expression of fabA and fabB, the two genes required for E. coli UFA synthesis, 2) whereas FabR is a repressor for expression of fabA and fabB. We construct three E. coli strains including FYJ241 (WT), FYJ246 (∆fadR) and FYJ247 (∆fabR). The three strains all carried a single copy of fabAshe-lacZ transcriptional fusion on chromosomes which allows us to detect whether the E. coli counterpart of FadR regulatory proteins has the in vivo role in modulating the Shewanella fabA expression and to monitor the physiological effect on fabA_she transcription exerted by exogenous fatty acids. Measurement of the β-Gal levels of these strains showed that deletion of fabR eliminate its activation to fabA_she expression (Fig. 6C). In comparison with the wild-type strain, appreciable lower β-gal activity was seen in the ∆fadR mutant (Fig. 6C). In contrast, the removal of the opposite regulator, FabR repressor, gave significant increment of fabA_she expression (Fig. 6C). As expected, the activity of fabA_she promoter is inhibited by the addition of oleate and this down-regulation is dependent on the presence of FadR regulator (Fig. 6C). Thus, our results represent in vivo evidence that expression of Shewanella fabA is activated by FadR, but repressed by oleate.

**CONCLUSIONS**

The data reported here defined that the Shewanella FadR homologue is a functional regulator with the involvement of fatty acid metabolism. Also, we experimentally proved the proposal by Rodionov et al. (Rodionov et al., 2011) that fab regulon is contracted in Shewanella. Retrospectively, Wang

### Table 1. Strains and plasmids used in this study

| Bacteria or plasmids | Relevant characteristics | Sources |
|----------------------|--------------------------|---------|
| **Bacterial strains** |                          |         |
| *Shewanella* oneidensis MR-1 | A Gram-negative anaerobic bacteria which is predominantly found in deep sea anaerobic habitats | Heidelberg et al. (2002), CGSC^a |
| BL21(DE3) | An expression host for recombinant plasmids | Lab stock |
| MFH8 | UB1005, ΔfadR::Tn10 | Henry & Cronan (1992) |
| SI91 | UB1005, ΔfabR::Cm | Feng & Cronan (2009b) |
| FYJ187 | MC4100/pNTts | Feng & Cronan (2011b) |
| FYJ189 | BL21 carrying pET28a-fabRshe | This work |
| FYJ214 | BL21 carrying pET28a-fadRshe | This work |
| FYJ193 | DH5a (∆pir) | Lab stock, (Feng & Cronan, 2009a, Feng & Cronan, 2011b) |
| FYJ236 | DH5a (∆pir) carrying pAH-PfabAshe | This work |
| FYJ241 | MC4100 with a single copy of fabAshe-lacZ fusion integrated at the λ-site | This work |
| FYJ246 | FYJ241, ΔfadR : Tn10 | FYJ241/P1vir(MFH8) |
| FYJ247 | FYJ241, ΔfabR : Cm | FYJ241/P1vir(SI91) |
| **Plasmids** |                          |         |
| pET28(a) | T7 promoter-driven expression vector, Kan^R | Novagen |
| pAH125 | Promoter-less lacZ reporter plasmid in E. coli, Kan^R | Feng & Cronan (2009a), Haldimann & Wanner (2001) |
| pET28-fadRec | Recombinant plasmid carrying E. coli fadR, Kan^R | Cherepanov & Wackernagel (1995), Feng & Cronan (2009b), Feng & Cronan (2009a) |
| pET16-fadRvc | Recombinant plasmid carrying V. cholerae fadR, Kan^R | Feng & Cronan (2011b), Iram & Cronan (2005) |
| pET28-fadRshe | Recombinant plasmid carrying Shewalle fadR, Kan^R | This work |
| pAH-PfabAshe | Recombinant plasmid carrying Shewalle fabA promoter region, Kan^R | This work |

^a CGSC denotes Coli Genetic Stock Center, Yale University.
et al. (Wang et al., 2009) reported the pilot exploration of fatty acid metabolism in Shewanella piezotolerans with concentration on its relevance to different temperatures and pressures.

Very recently, Gao’s research group also provided genetic evidence in aiming to pose its role of fadR into the UFA pathway in S. oneidensis MR-1 (Luo et al., 2014). Right now, the picture of Shewanella FadR seemed to be much more complete in the context of lipid metabolism. Unlike the paradigm E. coli in which both fabA and fabB have the FadR-binding sites, Shewanella only rendered the fabA fatty acid synthesis gene under the control of the FadR activator (Figs. 1 and 6C) and the FabR repressor (Fig. 6C). Moreover, it is in rational to functionally assign this unparalleled regulation to its unique evolutional selection and even adaptation to its growing (neighboring) environmental/ ecological niches with the availability of limited fatty acids. Taken together, we provided integrative evidence that FadR binds to the fabA Shewanella binding sites, (Figs. 1 and 6C) and the FabR repressor (Fig. 6C). More-}

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

With an exception of Shewanella oneidensis MR-1 (S. oneidensis), all of the bacterial strains used here were E. coli K-12 derivatives (Table 1). The media are separately LB medium (10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter), and rich broth (RBO medium; 10 g of tryptone, 1 g of yeast extract and 5 g of NaCl per liter). To measure the activity of β-galactosidase in induction assays, olate was solubilized with Tergitol NP-40 and used at a 5 mmol/L final concentration. Antibiotics were supplemented as follows (in mg/L): sodium ampicillin, 100; kanamycin sulfate, 50; tetra-cycline HCl, 15; and chloramphenicol, 20.

**Plasmids and DNA manipulations**

The fabA promoter region of S. oneidensis was PCR amplified and directly cloned into pAH125, giving the recombinant plasmid pAH125-P fabAshe (Table 1). Similarly, the fadR gene amplified from S. oneidensis was inserted into expression vector pET28(a), generating the chimeric plasmid pET28-fadRshe (Table 2). To prepare three different versions of FadR proteins, the corresponding expression plasmids (pET28-fadRec, pET28-fadRshe and pET16-fadRvc, in Table 2) were transformed into the strain BL21 (DE3) (Feng & Cronan, 2009b). The acquired plasmids were verified by direct DNA sequencing.

Given that the replication of pAH-P fabAshe plasmid requires the presence of pir protein, it thus was maintained in strain DH5α λ-pir (Table 1). To impart antibiotic resistance in E. coli MC4100 (a lacZ strain lacking pir), this plasmid must specifically integrate into the attL site of bacterial chromosome in a reaction catalyzed by the plINT-ts helper plasmid, giving strain FYJ241 carrying fabAshe-lacZ transcriptional fusion (Table 1). PCR assay was applied to confirm the fabAshe-lacZ junction.

**P1vir phage transductions**

P1vir transductions were conducted as described by Miller (Miller, 1992) with little changes. The strain FYJ246 was constructed by P1vir transduction of strain FYJ241 using a lysate grown on strain MFH8 (ΔfadR::Tn10) with selection under a variety of limited fatty acid concentrations. 

**Table 2. Primers used in this study**

| Primers   | Primer sequences |
|-----------|------------------|
| fadR_she-F | 5′-CG GATTC ATG ATT ATC AAT GCC AAA GGA CC-3' |
| fadR_she-R | 5′-CGG CTCGAG CTA ATG GGA GTC CTG CTG TG-3' |
| fabR_she-F | 5′-GGA TAT CCT GTG ATG GGA GTC CTG CTG TG-3' |
| fabR_she-R | 5′-CGG CTCGAG CTA CCG ATG TTC AAC TTT ATT T-3' |
| fabA_she-BD-F | 5′-GAC ATT AAT TAG CGT ATC GGA GTT TGT TAG CTT ACA CGT GGT TG-3' |
| fabA_she-BD-R | 5′-CGG CAA GAT TAG CAA ACA GTG GTA AGC TAA ACA ACT CCG ATC AGC TAA TTA ATG TC-3' |
| fabA_ec-BD-F | 5′-TTT ATT CCG AAC TGA TCG TAC TTG TCC AGC GTA CAT GTC GTG TTA GCT ATC CTG CTG GC-3' |
| fabA_ec-BD-R | 5′-GCA CGG AGG ATA GCT AAC ACG TGT ACG CTG AAC AAG TCC GAT CAG TCC GGA ATA AA-3' |
| fabB_ec-BD-F | 5′-TCT ATT AAA TGG CTG ATC GGA GTC CTG TTG CGG GTG ACA AGT GTA CGC TAT TGT GCA TTC-3' |
| fabB_ec-BD-R | 5′-GAA TGC ACA ATG GCC TAC ACT TGT AGC GCG ACG TAC AAG TCC GAT CAG CCA TAT AAT AGA -3' |
| PfabAshe-F | 5′-CCG GTCCGAC GAG GGT TAA CGG GTA AAC AAG-3' |
| PfabAshe-R | 5′-AACG GAAATC GTC GAT CAT CAG CAT GAT TCT GTC-3' |
| LacZ-R | 5′-GAC GAT CAT GAT TAC GGA TTC ACT G-3' |

The sequences underlined are restriction sites. Putative FadR binding sites are in bold letters, and the predicted FabR palindromes are indicated in bold and italic.
for tetracycline resistance. Similarly, strain FYJ241 was transduced by \textit{P.\textit{titer}} lyse obtained from strain SI91 (\textit{ΔfadR::Cm}) with selection for kanamycin, giving strain FYJ247 (Table 1). The relevant genotypes of the acquired strains were proved by PCR analyses.

\textbf{β-Galactosidase assays}

Mid-log phase cultures grown in either LB or RB were collected by spinning, washed with Z-buffer and suspended in Z-buffer for further measurement of β-galactosidase activity (Feng & Cronan, 2009a, 2009b, Miller, 1992). The data were recorded in triplicate in no less than three independent assays.

\textbf{Expression and purification of three different FadR proteins}

In addition to the FadR protein with origins of both \textit{E.\textit{coli}} and \textit{V.\textit{cholerae}}, the \textit{S. oneidensis} FadR protein was produced in solubility via the induced expression with 0.2 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 3.5 h. The bacterial lysis by two rounds of sonication treatment was clarified by centrifugation, and the resultant supernatant was loaded onto a nickel-ion affinity column (Qiagen). The contaminant proteins were removed with wash buffer containing 50 mmol/L imidazole, and subsequently the 6× His-tagged FadR proteins in three versions (FadR\textsubscript{she}, FadR\textsubscript{ec} and FadR\textsubscript{vc}) were eluted in elution buffer containing 100 mmol/L imidazole. The protein was concentrated by ultra-filtration (30 kDa cutoff) and exchanged into 1× PBS buffer (pH 7.4) containing 10% glycerol. The purified proteins were visualized by 15% SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO).

\textbf{Size exclusion chromatography}

Given the fact that both FadR\textsubscript{ec} and FadR\textsubscript{vc} can form dimer, we aimed to check the solution structure of FadR\textsubscript{she}. Therefore, we used a Superdex 75 column (Pharmacia) run on an Åkta fast protein liquid chromatography system (GE Healthcare) (Feng & Cronan, 2010, Feng & Cronan, 2011a) to perform gel filtration analyses for the purified FadR\textsubscript{she} protein. In our trials, the column effluent was monitored at a flow rate of 0.35 mL/min in running buffer (20 mmol/L Tris-HCl, 50 mmol/L NaCl, pH 7.9). The peaks of interest were collected and confirmed with 15% SDS-PAGE.

\textbf{Liquid chromatography quadrupole time-of-flight mass spectrometry}

The identity of the recombinant FadR\textsubscript{she} protein we produced was confirmed using a Waters Q-Tof API-US Quadrupole mass spectrometer connected to a Waters nano Acquity UPLC (Feng & Cronan, 2011a). In brief, the protein band of interest was cut from 15% SDS-PAGE gel, de-stained and digested with Sequencing Grade Trypsin (G-Biosciences St. Louis, MO, 12.5 ng/μL in 25 mmol/L ammonium bicarbonate); Second, the resulting peptides were loaded on a Waters Atlantis C-18 column (0.03 mm particle, 0.075 mm × 150 mm), following the further cleaning treatment. The data dependent acquisition combined with ms/ms analysis was routinely performed (Feng & Cronan, 2011a).

\textbf{Chemical cross-linking assays}

To further test the solution structure of \textit{S. oneidensis} FadR, we carried out the experiments of chemical cross-linking with ethylene glycol bis-succinimidylsuccinate (Pierce) as we described before (Feng & Cronan, 2010). In each chemical cross-linking reaction (15 μL in total), the purified FadR protein (~10 mg/mL) was separately mixed with cross-linker at different concentrations (0, 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 mmol/L), and kept 30 min at room temperature before analysis. Note: the only protein without EGS addition serves as the negative control. All the reaction products were detected using 15% SDS-PAGE.

\textbf{Electrophoretic mobility shift assays}

To document the function of FadR-binding site located in the \textit{S. oneidensis} \textit{fabA} promoter, gel shift assays were performed as we earlier described (Feng & Cronan, 2009b, Feng & Cronan, 2010, Feng & Cronan, 2011a) with minor modifications. Totally, three sets of DNA probes (Table 2) corresponded to \textit{fabA\textsubscript{ec}}, \textit{fabB\textsubscript{ec}} and \textit{fabA\textsubscript{she}}, respectively. They were all generated by annealing two complementary oligonucleotides (e.g., \textit{fabA\textsubscript{she-BD-F}} plus \textit{fabA\textsubscript{she-BD-R}}, in Table 2) through the incubation at 95°C in TEN buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L NaCl, pH 8.0) for 5 min followed by slow cooling to 25°C. The digoxigenin-labeled DNA probes (~0.2 pmol) were mixed with purified FadR (in appropriate concentrations) in the binding buffer (Roche) and incubated 20 min at room temperature. The DNA/protein mixtures were then analyzed by the native 7% PAGE, and directly transferred onto nylon membrane by contact blotting-aided gel transfer. Following appropriate treatments, the chemical-luminescence signals were captured by an exposure of the membrane to ECL films (Amersham).

\textbf{Surface plasmon resonance}

Biacore3000 instrument (GE Healthcare) was utilized to carry out the surface plasmon resonance (SPR)-based measurement. The biotinylated \textit{fabA\textsubscript{she}} DNA probe was immobilized by streptavidin on the chip surface. The SPR assay was run in the running buffer (20 mmol/L Tris-HCl, pH 7.5, 200 mmol/L NaCl and 0.005% Tween 20) at the flow rate of 30 μL/min. FadR\textsubscript{she} protein in a series of dilution was injected and passed over the chip surface for 2 min. Kinetic parameters were analyzed using a global data
analysis program (BIA evaluation software), and final graph was given with the Origin software.

Bioinformatic analyses
The amino acid sequences of FadR regulator are derived from E. coli, V. cholerae and S. oneidensis MR-1. The FadR-binding sites were all sampled from RegPrecise database (http://regprecise.lbl.gov/RegPrecise/regulon.jsp?). The multiple alignments were conducted using the program ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the resultant output was further processed by the program ESPript 2.2 (http://esprpit.ibcp.fr/ESPrcript/cgi-bin/ESPrcript.cgi), giving the final version of BLAST photography.

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ABBREVIATIONS
FAS, fatty acid synthesis; FPLC, fast protein liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; LCFA, long-chain fatty acid; UFA, unsaturated fatty acid.

COMPLIANCE WITH ETHICS GUIDELINES
Huimin Zhang, Beiwen Zheng, Rongsui Gao and Youjun Feng declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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REFERENCES
Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of Escherichia coli K-12. Science 277:1453–1462
Cheng YY, Li BB, Li DB, Chen JJ, Li WW, Tong ZH, Wu C, Yu HQ (2013) Promotion of iron oxide reduction and extracellular electron transfer in Shewanella oneidensis by DMSO. PloS one 8:e78466
Cherepanov PP, Wackernagel W (1995) Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158:9–14
Cronan JE Jr (1997) In vivo evidence that acyl coenzyme A regulates DNA binding by the Escherichia coli FadR global transcription factor. J Bacteriol 179:1819–1823
Feng Y, Cronan JE (2009a) Escherichia coli unsaturated fatty acid synthesis: complex transcription of the fabA gene and in vivo identification of the essential reaction catalyzed by FabB. J Biol Chem 284:29526–29535
Feng Y, Cronan JE (2009b) A new member of the Escherichia coli fab regulon: transcriptional regulation of fabM (ybaW). J Bacteriol 191:6320–6328
Feng Y, Cronan JE (2010) Overlapping repressor binding sites result in additive regulation of Escherichia coli FadH by FadR and ArcA. J Bacteriol 192:4289–4299
Feng Y, Cronan JE (2011a) Complex binding of the FabR repressor of bacterial unsaturated fatty acid biosynthesis to its cognate promoters. Mol Microbiol 80:195–218
Feng Y, Cronan JE (2011b) The Vibrio cholerae fatty acid regulatory protein, FadR, represses transcription of plsB, the gene encoding the first enzyme of membrane phospholipid biosynthesis. Mol Microbiol 81:1020–1033
Feng Y, Xu J, Zhang H, Chen Z, Srinivas S (2013a) Brucella BioR regulator defines a complex regulatory mechanism for bacterial biotin metabolism. J bacteriol 195:3451–3467
Feng Y, Zhang H, Cronan JE (2013b) Proligating biotin synthesis in alpha-proteobacteria—a developing or degenerating regulatory system? Mol Microbiol 88:692–703
Fredrickson JK, Romine MF, Beliaev AS, Auchtung JM, Driscoll ME, Gardner TS, Nealon KH, Osterman AL, Pinchuk G, Reed JL, Rodionov DA, Rodrigues JL, Saffarini DA, Serres MH, Spormann AM, Zhulin IB, Tiedje JM (2008) Towards environmental systems biology of Shewanella. Nature reviews. Microbiology 6:592–603
Giles DK, Hankins JV, Guan Z, Trent MS (2011) Remodelling of the Vibrio cholerae membrane by incorporation of exogenous fatty acids from host and aquatic environments. Mol Microbiol 79:716–728
Gui L, Sunnarborg A, LaPorte DC (1996) Regulated expression of a repressor protein: FadR activates iclR. J Bacteriol 178:4704–4709
Haldimann A, Wanner BL (2001) Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J Bacteriol 183:6384–6393
Hau HH, Gralnick JA (2007) Ecology and biotechnology of the genus Shewanella. Annu Rev Microbiol 61:237–258
Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM (2000) DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. Nature 406:477–483
Heidelberg JF, Paulsen IT, Nelson KE, Gaidos EJ, Nelson WC, Read TD, Eisen JA, Seshadri R, Ward N, Methe B, Clayton RA, Meyer...
Contraction of the fad regulon in *Shewanella*.

T, Tsapin A, Scott J, Beanan M, Brinkac L, Daugherty S, DeBoy RT, Dodson RJ, Durkin AS, Haft DH, Kolonay JF, Madupu R, Peterson JD, Umayam LA, White O, Wolf AM, Vamathevan J, Weidman J, Impraim M, Lee K, Berry K, Lee C, Mueller J, Khouri H, Gill J, Utterback TR, McDonald LA, Feldbyrum TV, Smith HO, Venter JC, Nealsom KH, Fraser CM (2002) Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nat Biotechnol 20:1118–1123

Henry MF, Cronan JE Jr (1991) *Escherichia coli* transcription factor that both activates fatty acid synthesis and represses fatty acid degradation. *J Mol Biol* 222:843–849

Henry MF, Cronan JE Jr (1992) A new mechanism of transcriptional regulation: release of an activator triggered by small molecule binding. Cell 70:671–679

Iram SH, Cronan JE (2005) Unexpected functional diversity among FadR fatty acid transcriptional regulatory proteins. *J Biol Chem* 280:32148–32156

Janda JM, Abbott SL (2014) The genus *Shewanella*. Crit Rev Microbiol 40:293–312

Kolker E, Picone AF, Galperin MY, Romine MF, Higdon R, Makarova KS, Kolker N, Anderson GA, Qiux Aubbey KJ, Babnigg G, Belaev AS, Edlefsen P, Elias DA, Gorby YA, Holzman T, Klappenbach JA, Konstantinidis KT, Land ML, Lipton MS, McCue LA, Monroe M, Pasa-Tolic L, Pinhchuk G, Purvine S, Serres MH, Tsapin S, Zakrajsek BA, Zhu W, Zhou J, Larimce FW, Lawrence CE, Riley M, Collart FR, Yates JR 3rd, Smith RD, Giometti CS, Tsapin A, Zakrajsek BA, Zhu W, Zhou J, Osterman AL, Gelfand MS (2011) Comparative genomic reconstruction of transcriptional networks controlling central metabolism in the *Shewanella* genus. *BMC Genom* 12(Suppl 1):S3

Sheng L, Fein JB (2014) Uranium reduction by *Shewanella oneidensis* MR-1 as a function of NaHCO3 concentration: surface complexation control of reduction kinetics. *Environ Sci Technol* 48:3768–3775

Shi W, Kovacikova G, Lin W, Taylor RK, Skorupski K, Voll FJ (2015) The 40-residue insertion in *Vibrio cholerae* FadR facilitates binding of an additional fatty acyl-CoA ligand. *Nat Commun* 6:6032

van Aalten DM, DiRusso CC, Knudsen J, Wierenga RK (2000) Crystal structure of FadR, a fatty acid-responsive transcription factor with a novel acyl coenzyme A-binding fold. *EMBO J* 19:5167–5177

van Aalten DM, DiRusso CC, Knudsen J (2001) The structural basis of acyl coenzyme A-dependent regulation of the transcription factor FadR. *EMBO J* 20:2041–2050

Wang F, Xiao O, Ouy HY, Gai Y, Wang F (2009) Role and regulation of fatty acid biosynthesis in the response of *Shewanella piezotolerans* WP3 to different temperatures and pressures. *J Bacteriol* 191:2574–2584

Wiatrowski HA, Ward PM, Barkay T (2006) Novel reduction of mercury (II) by mercury-sensitive dissimilatory metal reducing bacteria. *Environ Sci Technol* 40:6690–6696

Xu Y, Heath RJ, Li Z, Rock CO, White SW (2001) The FadR-DNA complex. Transcriptional control of fatty acid metabolism in *Escherichia coli*. *J Biol Chem* 276:17373–17379

Zhang Y, Gao R, Ye H, Wang Q, Feng Y (2014) A new glimpse of FadR-DNA crosstalk revealed by deep dissection of the *E. coli* FadR regulatory protein. *Protein Cell* 5:928–939