A new regulatory mechanism for bacterial lipoic acid synthesis

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cAMP-receptor protein (CRP), LipA, LipB, Lipoic acid, lipoic acid synthesis, *Shewanella*

Abstract
Lipoic acid, an essential enzyme cofactor, is required in three domains of life. In the past 60 years since its discovery, most of the pathway for lipoic acid synthesis and metabolism has been elucidated. However, genetic control of lipoic acid synthesis remains unclear. Here, we report integrative evidence that bacterial cAMP-dependent signaling is linked to lipoic acid synthesis in *Shewanella* species, the certain of unique marine-borne bacteria with special ability of metal reduction. Physiological requirement of protein lipoylation in γ-proteobacteria including *Shewanella oneidensis* was detected using Western blotting with rabbit anti-lipoyl protein primary antibody. The two genes (*lipB* and *lipA*) encoding lipoic acid synthesis pathway were proved to be organized into an operon *lipBA* in *Shewanella*, and the promoter was mapped. Electrophoretic mobility shift assays confirmed that the putative CRP-recognizable site (AAGTGTGATCTATCTTACATTT) binds to cAMP-CRP protein with origins of both *Escherichia coli* and *Shewanella*. The native *lipBA* promoter of *Shewanella* was fused to a LacZ reporter gene to create a chromosome *lipBA-lacZ* transcriptional fusion in *E. coli* and *S. oneidensis*, allowing us to directly assay its expression level by β-galactosidase activity. As anticipated, the removal of *E. coli crp* gene gave above fourfold increment of *lipBA* promoter-driven β-gal expression. The similar scenario was confirmed by both the real-time quantitative PCR and the LacZ transcriptional fusion in the *crp* mutant of *Shewanella*. Furthermore, the glucose effect on the *lipBA* expression of *Shewanella* was evaluated in the alternative microorganism *E. coli*. As anticipated, an addition of glucose into media effectively induces the transcriptional level of *Shewanella lipBA* in that the lowered cAMP level relieves the repression of *lipBA* by cAMP-CRP complex. Therefore, our finding might represent a first paradigm mechanism for genetic control of bacterial lipoic acid synthesis.

Introduction
Lipoic acid (6,8-dithiooctanoic acid, thioctic acid, or R-5-(1,2-dithiolan-3-yl) pentanoic acid), is a type of two-sulfur inserted eight-carbon fatty acid derivative and acts as a coenzyme widespread in three domains of life (Perham 2000; Cronan et al. 2005). This covalently bound cofactor is required for aerobic metabolism of 2-oxoacids.
in *Escherichia coli* and C1 metabolism in plants like *Arabidopsis* (Perham 2000; Cronan et al. 2005; Engel et al. 2007). In *E. coli*, the three well-known enzymes whose activities require lipoylation, the post-translational modification, include PDH (pyruvate dehydrogenase), OGDH (2-oxoglutarate dehydrogenase), and GCV (glutamate cleavage system) system (Cronan et al. 2005; Hermes and Cronan 2009). All the three enzyme systems possess such subunits (the E2 subunits of both PDH and OGDH, and the H protein of GCV system) that contain no less one lipoyl domains (LD) featuring with a conserved structure of around 80 residues long (Reche 2000; Cronan et al. 2005). Generally, a specific/conserved lysine residue on these LDs is attached by lipoic acid via an amide bond (Perham 2000). Therefore, it seems likely that lipoic acid facilitates shuttle of the activated reaction intermediates amongst the active sites of the lipoate-dependent multienzyme systems (Perham 2000; Cronan et al. 2005).

Most of current knowledge of lipoic acid metabolisms comes from studies with *E. coli* (Zhao et al. 2003; Cronan et al. 2005). Two alternative strategies have been developed in *E. coli* to satisfy the trace physiological demand for lipoic acids. It includes de novo biosynthesis pathway and the scavenging route (Cronan et al. 2005; Hermes and Cronan 2009; Rock 2009; Christensen and Cronan 2010). The former pathway is constituted of two consecutive steps: the LipB (octanoyl-ACP: protein N-octanoyl-transferase) transfers the endogenously produced octanoyl moieties from octanoyl-ACP (an intermediate of the fatty acid biosynthesis) to lipoyl domains (Fig. 1A) (Jordan and Cronan 2003; Zhao et al. 2003, 2005); in the second step the LipA (lipoyl synthase) uses S-adenosyl-L-methionine (SAM)-dependent radical chemistry to insert two sulfur atoms at carbons 6 and (Fig. 1A) (Zhao et al. 2003; Cronan et al. 2005; Douglas et al. 2006; Christensen and Cronan 2010). The lipoyl protein ligase (LplA) plays a critical role in utilization of exogenous lipoic acids from environments in which the lipoyl-adenylate intermediate is required (Fig. 1A) (Morris et al. 1994, 1995; Reed et al. 1994). Although the metabolic mechanism of lipoic acids that was initially discovered in the early of 1940s (Reed 2001) was extensively investigated (Reed 2001; Cronan et al. 2005; Hermes and Cronan 2009; Rock 2009; Christensen and Cronan 2010), its genetic regulation/control is poorly understood (Kaleta et al. 2010; Feng and Cronan 2014).

**cAMP receptor protein** (CRP, also called catabolic activator protein, CAP) is a type of global regulator, representing a classical model for bacterial gene regulation systems (Green et al. 2014). The paradigm version of CRP in *E. coli* is a protein module that modulates expression of hundreds of genes involved in a variety of bacterial physiological aspects such as energy metabolism (e.g., galactose catabolism) (Zheng et al. 2004; Green et al. 2014).

Indeed, the activity of CRP requires the presence of its physiological ligand/effector, cyclic AMP (cAMP) (Zheng et al. 2004; Green et al. 2014). Upon the CRP protein is occupied by the cAMP small molecule, it proceeds to an allosteric alteration/structural rearrangement, allowing its acquisition of an ability to specifically bind a collection of specific target DNA sequences (Schultz et al. 1991; Green et al. 2014). As we know, the typical CRP box (cAMP-CRP binding site) is referred to the imperfect palindromic consensus sequence “N3TGTGAN4TCACAN3” (Zheng et al. 2004). In the similarity to the well-studied FadR regulator that has dual functions in fatty acid metabolism (Feng and Cronan 2009a,b, 2010, 2012), it appears that the dimeric CRP protein-mediated regulation also can exert two opposite roles, i.e., either activation (Hanamura and Aiba 1992; Ishizuka et al. 1994; Zheng et al. 2004) or repression (Aiba 1983; Hanamura and Aiba 1991; Ishizuka et al. 1994) in response to distinct external and/or internal stimuliinputs (Green et al. 2014). Recently, comparative genomics-based reconstruction of bacterial regulatory networks RegPrecise (http://regprecise.lbl.gov/RegPrecise) by Rodionov and his research group (Rodionov et al. 2013).
et al. 2011; Novichkov et al. 2013) predicted that a possible CRP box (AAGTGTGATCTATCTTACATTT) is present in front of lipBA operon (SO1162-SO1161) of Shewanella oneidensis MR-1 (a marine-borne species of γ-proteobacteria family) with considerable potential for the remediation of contaminated environments and application in microbial fuel cells (Fredrickson and Romine 2005; Fredrickson et al. 2008).

More importantly, it seemed likely that the predicted site reflects an evolutionarily conserved regulatory mechanism in that it is found in nearly all the Shewanella species with known genome sequences and similar scenario were seen even with the two human pathogens Salmonella typhimurium and Klebsiella pneumonia. This might raise a possibility that cAMP signaling is linked to bacterial lipoic acid synthesis in certain species of γ-proteobacteria. However, this hypothesis requires further in vitro and in vivo experimental verification.

In this paper, we aimed to resolve this unanswered question. As anticipated, electrophoresis mobility shift assays (EMSA), we conducted and confirmed that the two CRP proteins of E. coli and Shewanella are functionally exchangeable and the predicted CRP sites of Shewanella are functional. Using the chromosome lipBA-lacZ transcriptional fusion in E. coli, we visualize that the removal of E. coli crp gene gave above fourfold increment of lipBA promoter-driven β-gal expression, which is almost identical to the scenario seen with Shewanella. Somewhat it is unexpected, but not without precedent that an addition of glucose into media effectively induces lipBA expression in Shewanella, in that the lowered cAMP level relieves the repression of lipBA by cAMP-CRP complex (Fig. 1B and C). Therefore, our finding answered the long-term unresolved question in the field of lipoic acid metabolism and might represent a first paradigm illustrating the genetic control of bacterial lipoic acid synthesis by cAMP-dependent CRP signaling in certain species of γ-proteobacteria.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used here were derivatives of both E. coli K-12 and S. oneidensis MR-1 (Table 1) and cultivated aerobically at 37°C and 30°C, respectively. For the growth of E. coli, the following three media were utilized, including Luria-Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter; pH 7.5), rich broth (RB) medium (10 g of tryptone, 1 g of yeast extract, and 5 g of NaCl per liter), and M9 minimal medium with either 5 mmol/L sodium acetate or 0.4% glucose as the sole carbon source (Feng and Cronan 2009b, 2010). M1-defined medium containing 0.02% (w/v) of vitamin-free casamino acids and 15 mmol/L lactate as electron donor was used to cultivate S. oneidensis (Gao et al. 2008). If required, chemicals or antibiotics were added as follows: 2,6-diaminopimelic acid (DAP), 0.3 mmol/L; sodium ampicillin, 100 μg/mL; kanamycin sulfate, 25 μg/mL; and tetracycline, 15 μg/mL; gentamycin, 15 μg/mL.

Plasmids and DNA manipulations

Using polymerase chain reaction (PCR) with primers crp she-F plus crp she-R (Table 2), the S. oneidensis crp gene was amplified and inserted into the BamHI and XhoI sites of pET28a(+) expression vector, giving the recombinant plasmid pET28-crp she (Table 1). The promoter region of S. oneidensis lipBA (referred to LipBA she) covering the predicted CRP site (Table 3) was amplified with a set of specific primers LipBA-F plus LipBA-R and cloned into the two cuts SalI and EcoRI of promoter-less plasmid pAH125 to give the recombinant plasmid pAH-LipBA she. Consequently, the pAH-LipBA she plasmid was transformed into MC4100 (Δlac), resulting in the LacZ reporter strain FYJ453 with LipBA she-lacZ transcriptional fusion on chromosome (Table 1). The inserts introduced in the recombinant plasmids we generated were validated by both PCR assays and direct DNA sequencing (Feng and Cronan 2011a,b).

The lipBA promoter activity was assessed using an integrative lacZ reporter system as described recently (Fu et al. 2014). A fragment covering the sequence upstream of the lipB gene from −300 to +1 was amplified and cloned into the reporter vector pHGEl01, verified by sequencing, and the correct plasmid was then transferred into S. oneidensis strains by conjugation. Proper integration of the promoter fusion constructs was confirmed by PCR. To eliminate the antibiotic marker, the helper plasmid pBBR-Cre was transferred into the strains carrying the correct integrated construct. Colonies without the integrated antibiotic marker were screened and verified by PCR, and followed by the loss of pBBR-Cre as described previously (Fu et al. 2013).

In-frame mutant construction and complementation

In-frame deletion strains for S. oneidensis were constructed using the att-based Fusion PCR method as described previously (Jin et al. 2013). In brief, two fragments flanking gene of interest were amplified by PCR, which were linked together by a second round of PCR. The fusion fragments were introduced into plasmid pHGM1.0 by using Gateway BP clonase II enzyme mix (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instruction, resulting in mutagenesis vec-
Table 1. Bacterial strains and plasmids in this study.

| Bacteria or plasmids | Relevant characteristics | Refs or origins |
|----------------------|--------------------------|-----------------|
| **Bacterial strains** |                         |                 |
| Escherichia coli      |                         |                 |
| BL21(DE3)            | Engineered E. coli strain as an expression host for recombinant plasmids | Lab stock |
| MG1655               | Wild type of E. coli K-12 (F-, λ-, rph-1) | CGSC¹, Lab stock |
| WM3064               | Donor strain for conjugation; ΔdapA | W. Metcalf, UIUC |
| BW25113              | A Δlac strain of E. coli K-12 (F-, λ-, ΔaraD-araB)567 ΔlacZ4787(ΔrmB-3) Δ(rhaD-rhaB)568 hsdR514 | CGSC¹, Baba et al. (2006) |
| JWS702-4             | (BW25113, Δcrp-765::kan) | CGSC¹, Baba et al. (2006), Feng and Cronan (2010) |
| MC1061               | F-, λ, ΔaraA-leu7697, [araD139]leu7697, Δ(codB-lac)3, galK16, galE15 (GalS), e14, mcrA0, relA1, rpsL150(strR), spoT1, mcrB1, hsdR2 | Lab stock, Casadaban and Cohen (1980), Feng and Cronan (2009b) |
| RH77                 | MC4100, ΔcyA, Δcrp::Tn10 | Lab stock Feng and Cronan (2010, 2012) |
| DH5a (λ-pir)         | An E. coli Δlac host for pAH125 and its derivatives | Feng and Cronan (2009a, 2012), Haldimann and Wanner (2001) |
| FYJ208               | Vibrio cholerae O395 | Jame Jun Zhu’s lab |
| FYJ239               | BL21(DE3) carrying pET28-crP_ec | Feng and Cronan (2012), Goble et al. (2013) |
| FYJ426               | Salmonella enterica serovar Typhimurium 14028s | Slauch’s lab |
| FYJ452               | DH5a(λ-pir) carrying pAH-P lipBA she | This work |
| FYJ453               | MC4100 whose chromosome was integrated with the lipBA she-lacZ transcriptional fusion at the λ phage site | This work |
| FYJ457               | MC1061, lipBA she-lacZ transcriptional fusion | P1<sub>crp</sub>(FYJ453) × MC1061<sup>2</sup>, This work |
| FYJ458               | MC4100, ΔcyA, Δcrp::Tn10, lipBA she-lacZ transcriptional fusion | P1<sub>crp</sub>(FYJ453) × RH77<sup>2</sup>, This work |
| FYJ462               | Topo carrying pET28-crP she | This work |
| FYJ463               | BL21(tuner) carrying pET28-crP she | This work |
| **S. oneidensis**    |                         |                 |
| MR-1                 | Wild-type | Gao’s lab |
| HG0624               | Δcrp derived from MR-1 | Gao et al. (2010) |
| HG1162-1             | ΔlipA derived from MR-1 | This work |
| HG0424               | ΔaceE derived from MR-1 | This work |
| HG1329               | ΔcyA derived from MR-1 | This work |
| **Plasmids**         |                         |                 |
| pET28(a)             | Commercial T7-driven expression vector, Km<sup>R</sup> | Novagen |
| pET28-crP_ec         | pET28(a) encoding E. coli crp gene, Km<sup>R</sup> | Feng and Cronan (2012), Goble et al. (2013) |
| pAH125               | A promoter-less lacZ reporter plasmid in E. coli, Km<sup>R</sup> | Haldimann and Wanner (2001) |
| pHG101               | A promoter-less broad-host Km<sup>R</sup> vector | Wu et al. (2011) |
| pHG010               | An integrative lacZ reporter vector | Fu et al. (2014) |
| pAH-PlipBA she       | A pAH125 derivative encoding Shewanella oneidensis lipBA promoter region (~350 bp) | This work |
| pET28-crP she        | pET28(a) encoding S. oneidensis crp gene, Km<sup>R</sup> | This work |

¹CGSC denotes Coli Genetic Stock Center, Yale University.
²Selection for kanamycin resistance.

Tors in *E. coli* WM3064, which were subsequently transferred into *S. oneidensis* MR-1 via conjugation. Integration of the mutational constructs into the chromosome was selected by resistance to gentamycin and confirmed by PCR. The verified transconjugants were grown in LB broth in the absence of NaCl and plated on LB supplemented with 10% sucrose. Gentamycin-sensitive and sucrose-resistant colonies were screened by PCR for deletion of the target gene. Mutants were verified by direct sequencing of the mutated regions.

Plasmids pHG101 and pHG102 were used in genetic complementation of mutants (Wu et al. 2011). For complementation of genes next to their promoter, a fragment containing the gene of interest and its native promoter was generated by PCR and cloned into pHG101. For the remaining genes, the gene of interest was amplified and inserted into MCS of pHG102 under the control of the *arcA* promoter, which is constitutively active (Gao et al. 2010). The resulting vectors were transferred into its corresponding mutant strain via conjugation and its presence...
Table 2. DNA oligonucleotide sequences used in this work.

| Primers             | Primer sequences                                                                 | Purposes                                      |
|---------------------|----------------------------------------------------------------------------------|----------------------------------------------|
| crp_she-F (BamHI)   | 5'-CG GGAATCC ATG GCT CTG ATT GGT AAG CC-3'                                      | Gene cloning                                 |
| crp_she-R (Xhol)    | 5'-CTCGAG TTA AGC GGT ACC ATA TAC CAC-3'                                        | PCR detection for the crp mutant of Shevannella |
| crp_she-cK2         | 5'-GTG AAT CCA GTG AGT TGG ACA-3'                                               | PCR amplification and cloning of the lipBA promoter |
| FpIPaB-F (SalI)     | 5'-CG TGGCA GAT GAA CTG AGT GAG TTC CCC-3'                                      | Synthesis of the predicted CRP site of Shewanella lipBA |
| FpIPaB-R (EcoRI)    | 5'-AAACC GAATTCA AAG GGA CAA CCT CCC TCC TA-3'                                  | Synthesis of the CRP site of E. coli lipA     |
| LipA_she CRP site-F | (43 bp) 5'-CAA GGT CAT AAA GTG TGA TCT ATC TTCAATTTA TGG CCA AGA G-3'             | Synthesis of the known CRP site of E. coli fadD |
| LipA_she CRP site-R | (43 bp) 5'-CTC TTG GCC ATA AATGTA AGA TAG ATC ACACCT TAT GAC CTT G-3'             | Synthesis of the CRP site of E. coli ybeD      |
| Lipa_ec CRP site-R  | (42 bp) 5'-ACG GAG TAA TAGATG TTA TCC GTAATG CATTTT GAA AAA GTA-3'                | Synthesis of the CRP site of S. oneidensis lipD |
| Lipa_ec CRP site-R  | (42 bp) 5'-TAC TTT TTC AAA ATG CAT TAC GAA TAA CAT CTA TTA CTC CGT-3'             | Synthesis of the CRP site of S. oneidensis ybeD |
| FadD_ec CRP site-F  | (48 bp) 5'-GTA AAG ATA AAA ATA AAT AGT GAC GCC CTTCGCAACCTT TTC GTT GGG-3'         | Synthesis of the CRP site of S. oneidensis ybeD |
| FadD_ec CRP site-R  | (48 bp) 5'-CCC AAC GAA GGT GCG AAG GCC GTC ACTATT TAT TTT TAT CCT TAC-3'          | Synthesis of the CRP site of S. oneidensis ybeD |
| YbeD_ec CRP site-F  | (42 bp) 5'-AAA CAC TTA AAGAATG TAA TTC TCC CCG TCC CATAT ACT AAG CTT-3'           | Synthesis of the CRP site of S. enterica ybeD |
| YbeD_ec CRP site-R  | (42 bp) 5'-ATG CTT AGT ATATGG GGA CGG AAA TTA CACCTT CAA GTG TTT-3'               | Synthesis of the CRP site of S. enterica ybeD |
| YbeD_es CRP site-F  | (42 bp) 5'-GAA CAC TTA AAA GTG TGA TTT CCA TCC CCA TAT ACT AGG TAT-3'             | Synthesis of the CRP site of S. enterica ybeD |
| YbeD_es CRP site-R  | (42 bp) 5'-ATA CCT AGT ATA TGG GGA TGG AAA TCA CAC TTT CAA GTG TTC-3'             | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-F  | (48 bp) 5'-GAACACTTGAAG ATG TTA TTC CCA TCC CCA TATACATATTTCAT-3'                 | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-R  | (48 bp) 5'-ATG AAT AGT ATATGG GGA TGG AAA TCA CAC TTT CAA GTG TTC-3'             | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-F  | (42 bp) 5'-GAA CGCTTGGAA GTG TGA TTT TCG TCC CCA TAT ACTATGCAT-3'                 | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-R  | (42 bp) 5'-ATG CTT AGT ATA TGG GGA CGA AAA TTA CACCTT CAA GTG TTC-3'             | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-F  | (42 bp) 5'-CTG TGG CGG GAC TTA TTT TTT TTA CGT AAT GCC GGA GCT-3'                 | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-R  | (42 bp) 5'-AGC TCC GGC ATTACG TAA AAA AAA TAAACACCT CCG CCA CAG-3'                | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-F  | (42 bp) 5'-ATT GGC CCC ATATTTG TTA ATTA TCA TGG AAA TAA GCT-3'                    | Synthesis of the CRP site of S. enterica ybeD |
| YbeD Es CRP site-R  | (42 bp) 5'-AGC TTA TTT GCA ATA TAA GAT TAA TCA CAA TAT GCC GGC AAT-3'            | Synthesis of the CRP site of S. enterica ybeD |
| LacZ-R              | 5'-CAT GGA CTT CAT GTG AAT CAT GGT C-3'                                         | PCR assay for the lipBA-lacZ junction         |
| crp_ec-F            | 5'-CAG GTA GCG GGA AGA ATG AAG TAA ATG CAC TAC-3'                                | PCR assay for the E. coli crp                 |
| crp_ec-R            | 5'-CAG CTA GCG CAG AAT TGG ACC AAC CAT G-3'                                     | PCR (RT-PCR) assay for the S. oneidensis ybeD |
| ybeD-F (19-39)      | 5'-GAT GAA CTG ATG GAG TTC CCC-3'                                               | PCR (RT-PCR) assay for the S. oneidensis lipD |
| ybeD-D (223-243)    | 5'-GAT GTG GCC GAG CTG TGT GTA-3'                                               | PCR (RT-PCR) assay for the S. oneidensis lipA |
| lipB-F (471-491)    | 5'-CTG TGG ATC GTG GAA CAT CCA-3'                                              | PCR (RT-PCR) assay for the S. oneidensis lipA |
| lipA-D (1253-1273)  | 5'-CAT AAA GGT TGC TGT GCC GTG-3'                                              | PCR (RT-PCR) assay for the S. oneidensis lipA |

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was confirmed by plasmid purification and restriction enzyme digestion.

P1vir phage transductions

Following the protocol described by Miller (1992), we conducted the experiment of P1vir transduction. Transduction of strain MC1061 with a lysate grown on FYJ453 (PlipBA_she-lacZ) with selection for kanamycin resistance gave strain FYJ457 (MC1061, PlipBA_she-lacZ). Strain FYJ458 was constructed by transduction of strain RH77 (MC4100, Δcrp::Tn10) with a P1vir lysate grown on FYJ457 (MC1061, PlipBA_she-lacZ) with selection for kanamycin resistance (Table 1). All the relevant genotypes were determined using PCR with a primer set (e.g., PlipBA_she-F plus LacZ-R, Table 2), and the PCR products were confirmed by direct DNA sequencing (Feng and Cronan 2012).

RNA isolation and RT-PCR

Mid-log phase cultures of S. oneidensis MR-1 grown in RB media were collected for total bacterial RNA preparations. As we did before, the RNeasy bacterial RNA isolation kit (Qiagen, Hilden, Germany) was adopted (Feng and Cronan 2009b; Feng et al. 2013b). The quality of the acquired RNA samples was visualized using agarose gel

Table 2. CRP binding sites in front of potential lipBA operons from a variety of species amongst γ-proteobacteria.

| Organisms | Gene | Loci | CRP site | Position1 | Score |
|-----------|------|------|----------|-----------|-------|
| Enterobacter sp. 638 | ybeD | Ent638_1166 | AAGTGTGATTTCCATCCCCATA | –90 | 4.4 |
| Escherichia coli MG1655 | ybeD | b0631 | AAGTGTGATTTCCATCCCCATA | –94 | 3.6 |
| Cibroacter koseri | ybeD | CKO_02527 | AAGTGTGATTTCCATCCCCATA | –91 | 4.4 |
| Klebsiella pneumonia | ybeD | KPN_00663 | AAGTGTGATTTCCATCCCCATA | –97 | 4.4 |
| Salmonella typhimurium LT2 | ybeD | STM0636 | AAGTGTGATTTCCATCCCCATA | –35 | 3.9 |
| Yersinia pestis | ybeD | y1174 | TATGTGTATTAATCTTATATTG | –146 | 4.2 |
| Shewanella baltica | lipB | Sba_3281 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. halifaxensis | lipB | Shal_3240 | AAATGTGATCTGTCTTACATTT | –76 | 5.2 |
| S. loehlica | lipB | Shew_2941 | AAATGTGATCTGTCTTACATTT | –70 | 5.3 |
| S. oneidensis | lipB | SO1162 | AAATGTGATCTGTCTTACATTT | –68 | 5.1 |
| S. pealeana | lipB | Spea_3155 | AAATGTGATCTGTCTTACATTT | –76 | 5.2 |
| S. piezotolerans | lipB | swp_3928 | AAATGTGATCTGTCTTACATTT | –69 | 5.2 |
| S. putrefaciens | lipB | Sputnc2_2875 | AAATGTGATCTGTCTTACATTT | –69 | 5.3 |
| S. sediminis | lipB | Ssed_3491 | AAATGTGATCTGTCTTACATTT | –75 | 5.3 |
| S. woodii | lipB | Swoo_3714 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp ANA-3 | lipB | Shewanan3_0989 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp MR-4 | lipB | Shewmr4_0985 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp MR-7 | lipB | Shewmr7_1050 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp W3-18-1 | lipB | Sputw3181_1028 | AAATGTGATCTGTCTTACATTT | –75 | 5.3 |
| S. sp W3-18-1 | lipB | Sputw3181_1028 | AAATGTGATCTGTCTTACATTT | –75 | 5.3 |

CRP, cAMP-receptor protein.

1The position is relative to the translation initiation site. All the information is sampled from the RegPrecise database (http://regprecise.lbl.gov/RegPrecise/search.jsp).

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|-----------|------|------|----------|-----------|-------|
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| Escherichia coli MG1655 | ybeD | b0631 | AAGTGTGATTTCCATCCCCATA | –94 | 3.6 |
| Cibroacter koseri | ybeD | CKO_02527 | AAGTGTGATTTCCATCCCCATA | –91 | 4.4 |
| Klebsiella pneumonia | ybeD | KPN_00663 | AAGTGTGATTTCCATCCCCATA | –97 | 4.4 |
| Salmonella typhimurium LT2 | ybeD | STM0636 | AAGTGTGATTTCCATCCCCATA | –35 | 3.9 |
| Yersinia pestis | ybeD | y1174 | TATGTGTATTAATCTTATATTG | –146 | 4.2 |
| Shewanella baltica | lipB | Sba_3281 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. halifaxensis | lipB | Shal_3240 | AAATGTGATCTGTCTTACATTT | –76 | 5.2 |
| S. loehlica | lipB | Shew_2941 | AAATGTGATCTGTCTTACATTT | –70 | 5.3 |
| S. oneidensis | lipB | SO1162 | AAATGTGATCTGTCTTACATTT | –68 | 5.1 |
| S. pealeana | lipB | Spea_3155 | AAATGTGATCTGTCTTACATTT | –76 | 5.2 |
| S. piezotolerans | lipB | swp_3928 | AAATGTGATCTGTCTTACATTT | –69 | 5.2 |
| S. putrefaciens | lipB | Sputnc2_2875 | AAATGTGATCTGTCTTACATTT | –69 | 5.3 |
| S. sediminis | lipB | Ssed_3491 | AAATGTGATCTGTCTTACATTT | –75 | 5.3 |
| S. woodii | lipB | Swoo_3714 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp ANA-3 | lipB | Shewanan3_0989 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp MR-4 | lipB | Shewmr4_0985 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp MR-7 | lipB | Shewmr7_1050 | AAATGTGATCTGTCTTACATTT | –74 | 5.2 |
| S. sp W3-18-1 | lipB | Sputw3181_1028 | AAATGTGATCTGTCTTACATTT | –75 | 5.3 |

CRP, cAMP-receptor protein.

1The position is relative to the translation initiation site. All the information is sampled from the RegPrecise database (http://regprecise.lbl.gov/RegPrecise/search.jsp).
were calculated with the 2
of various cDNA samples. The relative expression levels
acted as blank control to monitor cross-contamination
the16
stranded DNA products. The reference gene was
temperature from 60
72
(Feng and Cronan 2011a,b). The nested PCR reactions
the transcription start site of
S. oneidensis lipBA
improved version of 5
RLM-RACE (Ambicon, Grand Island, NY, USA), an
5
eprealplex (Eppendorf, Hauppauge, NY, USA), using the
employed to evaluate the altered expression profile of
previously mentioned (Feng and Cronan 2009b, 2010), real-
protein assay reagent (Pierce Chemical Co., Rockford, IL,
concentrations were determined by the bicinchoninic acid
under experimental settings) were pelleted for assaying its
low by centrifugation (10 min at 12,000 g and
4°C), and the supernatants were used for assays at 25°C
as described previously (Reed and Cronan 1993). Protein
concentrations were determined by the bicinchoninic acid
protein assay reagent (Pierce Chemical Co., Rockford, IL,
USA).
To measure the β-galactosidase activities in E. coli, bacte-
rial lysates from mid-log phase cultures grown in LB
or (M9) media were prepared by treatment with sodium
dodecyl sulfate-chloroform (Miller 1972; Feng and Cro-
nan 2009b). Similarly, cells of S. oneidensis (mid-log phase
under experimental settings) were pelleted for assaysing its
β-galactosidase activity with an assay kit as described pre-
viously (Wu et al. 2011).
Measurement of intracellular cAMP levels
Cells in mid-log phase cultures (~0.3 of OD600) were col-
ceted by centrifugation and washed twice with charcoal-
treated phosphate-buffered saline (PBS; pH 7.0). Both su-
pernatant and pellet fractions were applied to the cAMP
assay using Cyclic AMP EIA kit (Cayman Chemical Co.,
Ann Arbor, Michigan, USA) according to the manufac-
turer’s instruction.
Expression, purification and identification
of two CRP proteins
To prepare the recombinant CRP protein in two versions
(CRP_ec and CRP_she), the engineered E. coli strains car-
eying either pET28-crp_ec or pET28-crp_she (Table 1)
electrophoresis. Using the general PCR assay in which the
total RNA samples function as templates with primers
16S_she-F plus 16S_she-R (Table 2), the possible contami-
ation of trace genomic DNA in the RNA samples was
routinely figured out as we described earlier (Feng and
Cronan 2009b, 2010).
On the basis of above qualified RNA samples, we per-
formed the reverse transcription (RT)-PCR experiments
(Feng and Cronan 2009b, 2010). Briefly, 1 μg of total
RNA was mixed with 0.5 μg of random primers (11 μL
in total), denatured (70°C for 5 min), and then chilled
on ice (5 min). The RT reaction mixture (20 μL total
volume) comprised 10 μL of denatured RNA template,
1 μL of random primers, 4 μL of ImProm-II 5X reaction
buffer, 2.5 μL of 1 mol/L MgCl2, 1 μL of deoxynucleo-
side triphosphate mix, 0.5 μL of the recombinant RNase
inhibitor, and 1 μL of ImProm-II reverse transcript-
ase (Feng and Cronan 2009b, 2011a). The program for
RT reaction included the equilibration at 25°C for 5 min,
an extension at 42°C for 60 min, and the inactivation of
enzyme at 70°C for 15 min. As a result, the cDNA pool
(1 μL) was used as the template to PCR-amplify the lipBA
operon-related genes/DNA fragments.

Real-time quantitative RT-PCR
On the basis of SYBR Green dye method as we previ-
ously mentioned (Feng and Cronan 2009b, 2010), real-
time quantitative RT-PCR (qRT-PCR) experiments were
employed to evaluate the altered expression profile of S.
oneidensis lipBA operon in the Δcrp mutant. qPCR reac-
tion system (20 μL) contained 12.5 μL of iQ™ SYBR
Green Supermix, 1 μL of each primer, 1 μL of the
diluted cDNA sample, and 4.5 μL of sterile water. All the
data were collected in triplicate on a Mastercycler
programming system (20
°
C for 20 sec, and a final step featuring with gradient
temperature from 60°C to 90°C for dissociating double
stranded DNA products. The reference gene was the16S_she
rRNA-encoding gene (Table 2) and water acted as blank control to monitor cross-contamination of various cDNA samples. The relative expression levels were calculated with the 2
ΔΔC
T
method developed by Livak and Schmittgen (2001).

5’-RACE
RLM-RACE (Ambicon, Grand Island, NY, USA), an
improved version of 5’-RACE kit, was applied in mapping
the transcription start site of S. oneidensis lipBA operon
(Feng and Cronan 2011a,b). The nested PCR reactions
were established using two sets of combined primers
(Outer Primer plus lipBA-GSP and Inner Primer plus lip-
BA-Nest primer) (Table 2). The PCR program was
described with a denaturing cycle at 95°C for 5 min fol-
lowed by 35 cycles comprising 95°C for 30 sec, 55°C for
30 sec, and 72°C for 30 sec. The purified PCR products
were sent for direct DNA sequencing. The transcriptional
start site was assigned to first nucleotide adjacent to the
RLM-start RACE adaptor (Feng and Cronan 2009a,b; Feng
et al. 2013b).

Enzymatic assays
For PDH assay, cells were grown at 30°C in 25 mL of LB
containing the appropriate antibiotics until the beginning
of the stationary phase, harvested, and washed twice with
a 0.04 mol/L potassium phosphate buffer (pH 7.5). The
resulting pellets were frozen rapidly and stored at −80°C.
Cell extracts were prepared by resuspending the thawed
pellets in 2 mL of the same buffer prior to sonication
with a microtip in a Branson model 200 Sonifier (2 min
total, with 40-sec pulses at 20-sec intervals). Cell debris
was removed by centrifugation (10 min at 12,000 g and
4°C), and the supernatants were used for assays at 25°C
as described previously (Reed and Cronan 1993). Protein
concentrations were determined by the bicinchoninic acid
protein assay reagent (Pierce Chemical Co., Rockford, IL,
USA).

Measurement of intracellular cAMP levels
Cells in mid-log phase cultures (~0.3 of OD600) were col-
ceted by centrifugation and washed twice with charcoal-
treated phosphate-buffered saline (PBS; pH 7.0). Both su-
pernatant and pellet fractions were applied to the cAMP
assay using Cyclic AMP EIA kit (Cayman Chemical Co.,
Ann Arbor, Michigan, USA) according to the manufac-
turer’s instruction.
were induced with 0.3 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 5 h (Feng and Cronan 2012). Following bacterial lysis by a French pressure cell, the clarified supernatants by centrifugation (30,966 g, 30 min) were loaded onto a nickel chelate column (Qiagen). After removal of the contaminated protein by washing with 1× phosphate buffered saline (PBS) with 50 mmol/L imidazole, the interested CRP proteins (CRP_ec or CRP_she) were eluted using elution buffer containing 150 mmol/L imidazole. Finally, the protein was concentrated by ultrafiltration (30 kDa cut-off) and exchanged into 1× PBS (pH 7.4) containing 10% glycerol. The purity of the recombinant CRP proteins was judged by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Feng and Cronan 2009b, 2011b). To verify the identity of the acquired proteins, the de-stained (SDS-PAGE) gel slices were subjected to liquid chromatography quadrupole time-of-flight mass spectrometry using a Waters Q-Tof API-US Quad-ToF mass spectrometer linked to a Waters nanoAcquity UPLC (Feng and Cronan 2011a; Feng et al. 2013a,b).

**Electrophoretic mobility shift assays**

The function of the predicted CRP-binding site of *Shewanella lipBA* operon was assessed in vitro using electrophoretic mobility shift assays (EMSA) with little improvements (Feng and Cronan 2011a; Goble et al. 2013; Feng et al. 2014). In the EMSA tests, nine pieces of DNA probes were composed of seven suspected probes (*lipBA_she, ybeD_ec, ybeD_es, ybeD_kp, ybeD_st1, ybeD_st2, and ybeD_yp*) and the two control probes, the *fadD_ec* site with known function (the positive control) and the *lipA_ec* without any function (the negative control) (Table 3). The digoxigenin (DIG)-labeled DNA probes were prepared in vitro through annealing two complementary oligonucleotides in TEN buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L NaCl; pH 8.0) and then labeled by the terminal transferase with DIG-ddUTP (Roche, Indianapolis, IN, USA) (Feng et al. 2014).

In the presence/absence of cAMP (20 pmol), the various DIG-labeled DNA probes (0.2 pmol) were incubated with or without CRP protein in the binding buffer (Roche) at room temperature for around 20 min. Following the separation of the DNA-protein complexes with a Waters Q-Tof API-US Quad-ToF mass spectrometer linked to a Waters nanoAcquity UPLC (Feng and Cronan 2011b, 2012).

**Bioinformatic analyses**

The alignments of DNA (and/or protein) sequences were conducted using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and final output was processed by the ESPript 2.2 server (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The *lipBA* regulons and the possible CRP-recognizable sites of γ-proteobacteria were collected from the RegPrecise database (Novichkov et al. 2010a) and were analyzed (Feng et al. 2013a) using RegPredict software (Novichkov et al. 2010b). The sequence logo for the CRP consensus palindrome was generated by WebLogo (http://weblogo.berkeley.edu/logo.cgi). The software of SPDBV_4.01 (http://spdbv.vital-it.ch/) was used for structure modeling.

![Figure 2. Genomic context of the lipBA operon GENES in the selected γ-proteobacteria. Blue arrows represent the lipA genes that encode the lipoic acid synthase catalyzing the last committed reaction of lipoic acid biosynthesis pathway, whereas green arrows indicate the octanoyl-protein ligase-encoding genes (lipB). The gray arrow upstream of the lipB gene refers to the ybeD gene of unknown function. In some cases, the tatE gene (Sec-independent protein translocase) downstream of lipA is shown with yellow arrow. In the four species (Escherichia coli, Enterobacter sp. 638, Klebsiella pneumoniae, and Salmonella typhimurium LT2), the ybeF gene encoding an LysR-type transcription factor (in orange) is located between lipA and lipB. The predicted CRP-binding palindromes are highlighted with dots (red dots represent the experimentally verified sites, whereas the gray ones are not experimentally validated). CRP, cAMP-receptor protein.](image)
Results

Shewanella lipBA is an operon

The paradigm pathway of lipoic acid synthesis is encoded by two genes lipB and lipA of E. coli. The two sequential steps of this pathway included LipB-catalyzed transfer of octanoyl moiety from octanoyl-ACP to lipoyl domains of the cognate enzymes and LipA-mediated insertion of sulfur atoms at C6 and C8 of LD-bound octanoyl moiety to give lipoate (Fig. 1). Therefore, we are interested in examining the genetic context of the lipB and/or lipA in γ-proteobacteria using RegPredict software (Novichkov et al. 2010b) (Fig. 2). In addition to the reference strains (e.g., E. coli, Salmonella enterica, Yersinia pestitis, etc.), all the other samples are focused on Shewanella species from the RegPrecise database (Novichkov et al. 2010a). We noted that the ybeD (SO1163) gene is constantly present upstream of the lipB gene (Fig. 2), and YbeD protein of E. coli origin exhibits a striking structural homology to the allosteric regulatory domain of 3-phosphoglycerate dehydrogenase (Kozlov et al. 2004).

Unlike the scenario seen with E. coli that lipB and lipA are separated by a gene (ybeF) encoding a LysR-family transcription factor of unknown function (Feng and Cronnan 2014) (Fig. 2), it seemed likely that lipB and lipA constitutes an operon in most of species of Shewanella (Fig. 1). Although physiological advantages for the co-transcription of these two genes are expected, experimental evidence is lacking. To address this hypothesis, the strain of S. oneidensis MR-1 was selected for our experiments. We established the combined PCR and RT-PCR assays using five pairs of specific primer pairs (Table 2 and Fig. 3A). The positive amplifications (1, 3 and 5) were obtained by both PCR and RT-PCR showed that all three genes (ybeD, lipB and lipA) are transcribed (Fig. 3A). The fact that the primed amplicon (designated to 2) was observed only by PCR, but not by RT-PCR suggested that ybeD is not co-transcribed together with lipB (Fig. 3A). As anticipated, the designed amplicon covering both lipB and lipA was positive in both PCR and RT-PCR assays, validating that lipB and lipA act as an operon (transcriptional unit) (Fig. 3A).

S. oneidensis lipBA promoter

DNA sequences recognized by CRP proteins of E. coli and S. oneidensis are predicted to be similar and the interaction depends on cAMP (Gao et al. 2010; Fu et al. 2013; Zhou et al. 2013). Given the fact that a predicted CRP-binding site (AAGTGTGATCTATCTTACATTT) is located in the intergenic region between the ybeD gene and the lipBA operon of S. oneidensis (Fig. 2), we thus mapped the promoter by employing an improved method of 5′-RACE (RLM-RACE). As a result, we acquired the 5′-RACE products of approximately 450 bp in length (Fig. 3B). The result of the direct DNA sequencing showed the 5′-end of the S. oneidensis lipBA transcript (i.e., transcription start site, A) is located 20 nucleotides upstream its translation initiation codon TTG (Fig. 4C and D). Apparently, the assumed CRP-recognizable site appears to be 25 bp upstream of the transcription start site (Fig. 4D). Furthermore, the multiple sequence alignment clearly indicated that the CRP binding sites of different origins are extremely conserved, in that 16 of 22 nucleotides are identical at least (if not all) in the examined Shewanella species (Fig. 6A and B). However, the function of these putative sites needs further experimental validation.

Physiological requirement of protein lipoylation

It is reasonable that co-expression of LipB octanoyltransferase and LipA lipoate synthase assures the economical production of lipoic acid (an energy-expansive molecule) to effectively satisfy the metabolic/physiological requirement of protein lipoylation in organisms. Given the fact that both PDH and OGDH are proceeded such kind of post-translational modification, we thereby developed the anti-LA Western blot to detect this metabolic requirement in four γ-proteobacteria species (E. coli, S. enterica, V. cholerae, and S. oneidensis). As expected, we did observe
that lipoylation occurs in PDH and OGDH of *Shewanella*, which is in much similarity to the scenario seen with *E. coli* (Fig. 5A). Because lipoylation is essential for the function of all characterized PDH and OGDH proteins, the result points out the metabolic significance of this common enzyme cofactor in *S. oneidensis*.

Subsequently, we constructed a *lipBA* null mutant from the *S. oneidensis* wild-type strain. The mutant was unable to grow on minimal medium unless lipoic acid was supplemented (Fig. 5B), a phenotype observed from *E. coli* *lip* mutants (Reed and Cronan 1993). Additionally, the PDH assay revealed that this Δ*lipBA* strain contained no detectable dehydrogenase activities (Fig. 5C). Importantly, the phenotypes resulting from the *lipBA* deletion were restored by their expression in *trans*, indicating that they are due to the intended mutation. These data, collectively, conclude that the *lipBA* genes are the only enzyme accountable for protein lipoylation in *S. oneidensis*.

**Characterization of *S. oneidensis* CRP protein**

*S. oneidensis* CRP and its counterpart of *E. coli* are highly homologous (Fig. S1A), and have been shown to be func-
tionally equivalent/exchangeable in vivo (Saffarini et al. 2003). However, whether this is the case in vitro remains undefined. In addition to the *E. coli* CRP protein, an N-terminal hexahistidine fused *S. oneidensis* CRP protein was over-expressed, purified to homogeneity and gave a single protein band with an estimated molecular mass (~24 kDa) (Fig. S2B). The tertiary structure of *S. oneidensis* CRP protein was modeled using SPDBV_4.01 software, which is highly similar to that of *E. coli* (Fig. S3C). Liquid chromatography mass spectrometry analyses of tryptic peptides of the recombinant CRP protein band excised from an SDS-PAGE gel validated its identity in that the peptides matched *S. oneidensis* CRP with 70% coverage of the expected peptides (Fig. S4D). The two versions of CRP proteins we prepared were subsequently used for functional analyses of the above predicted CRP-specific palindromic sites.

**Shewanella lipBA binds the cAMP-CRP complex**  
To test the activity of the DNA probe derived from the *S. oneidensis* lipBA promoter (Fig. 6A and B), we conducted EMSA assays. First, the positive control *fadD_ec* probe with a known function (Feng and Cronan 2012) binds well to *E. coli* CRP protein in the presence of cAMP effector molecule, whereas the negative control *lipA_ec* probe with a nonfunctional CRP site did not (Fig. 6C). As expected, the *lipBA_she* probe exhibited the appreciably comparable activity of binding cAMP-CRP complex relative to the positive control. Apparently, our result is much consistent with previous observations with the CRP regulatory protein in the context of other metabolisms (Gao et al. 2010; Fu et al. 2013; Zhou et al. 2013), proving the prediction of Novichkov et al. (2013) is correct. Additionally, the specific binding of *lipBA_she* to cAMP-CRP complex seemed to be in a protein dose-dependent manner (Fig. 6D). Not only does the CRP protein of *E. coli* origin interacts with *E. coli fadD* probe (Figs. 6C and 7A) and *Shewanella lipBA* probe (Figs. 6C and 7C), but also the CRP protein encoded by *Shewanella* binds to *E. coli fadD* probe (Fig. 7B) and *Shewanella lipBA* probe (Fig. 7D). It thus fully demonstrated that the two versions of CRP protein are functionally exchangeable in vitro.

Similarly, we also tested a series of predicted CRP-binding sites located upstream of *ybeD-lipB* loci (Fig. 2 and Table 3) using EMSA tests with *E. coli* CRP protein. Unlike the *lipBA_she* probe (Fig. S2A), neither the *E. coli ybeD* probe (*ybeD_ec*, Fig. S2B) nor the *Y. pestis ybeD* probe (*ybeD_yp*, Fig. S2G) are functional for the cAMP-CRP complex. By contrast, the prediction in CRP-recognizable sites (*ybeD_es* and *ybeD kp*) in front of the *ybeD* gene of both *Enterobacter* sp. 638 and *Klebsiella* pneumo-
nia are correct in that both bind to the CRP protein (Fig. S2C and D). Of particular note, among the two CRP sites (ybeD_st1 and ybeD_st2) proposed for S. enteric ybeD gene, only the ybeD_st1 site is functional (Fig. S2E), while the other one was not (Fig. S2F).

**Figure 6.** Binding of Shewanella lipBA to the cAMP-CRP functional complex. (A) Multiple sequence alignment of CRP-recognizable sites from Shewanella lipBA operon. Multiple sequence alignment was performed as described in Figure 2. Identical residues are indicated with white letters on a red background, similar residues are black letters on yellow, and varied residues are in black letters. Totally, the CRP-binding sites are sampled from 13 different species of Shewanella. (B) Sequence logo for the CRP palindromic consensus sequences. The palindromic sequences used here are identical to those listed in (A), and the sequence logo was generated using WebLogo (http://weblogo.berkeley.edu/logo.cgi). (C) Escherichia coli CRP binds to Shewanella lipBA promoter, but not E. coli lipA promoter. The CRP site of E. coli fadD (fadD_ec) is used as positive control, while the possible CRP site of E. coli lipA (lipA_ec) is referred to negative control (Table 2). The plus sign represents addition of the CRP protein and/or cAMP, whereas the minus sign denotes no addition of the CRP protein and/or cAMP. Designations: ec, E. coli; she, Shewanella. (D) Dose-dependent binding of E. coli CRP binds to Shewanella lipBA promoter. The level of CRP protein in (A) is 2 pmol, and the amount of cAMP is 20 pmol. The protein samples were incubated with 0.2 pmol of DIG-labeled lipBA_she probe (43 bp) in a total volume of 20 μL. A representative result from three independent gel shift assays (7% native PAGE) is given. CRP, cAMP-receptor protein.

A regulatory role for CRP in lipBA expression of S. oneidensis

Two approaches (lipBA_she-lacZ transcriptional fusion and the real-time qRT-PCR) were used to examine the
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in vivo regulatory role of cAMP-CRP complex in expression of \( S.\ oneidensis \) lipBA operon encoding lipoic acid synthesis machinery. First, \( S.\ oneidensis \) lipBA promoter was fused to a LacZ reporter gene to allow direct assaying of \( \beta \)-gal activity of lipBA\_she-lacZ transcriptional fusion integrated into \( E.\ coli \) chromosome (Table 1). In light of the functional equivalence of \textit{Shewanella} CRP to \textit{E. coli} CRP, we firstly compared the alteration of \( \beta \)-gal activity in the model organism \( E.\ coli \) (\( \Delta crp \) mutant and its parental strain of \( E.\ coli \)). As anticipated, MacConkey plates-based experiments visualized that the lipBA\_she promoter-driven \( \beta \)-gal activity is appreciably stronger (illustrated with purple) in the \( \Delta crp \) mutant than that of the wild type \( E.\ coli \) (low activity denoted by yellow) (Fig. 8A). Direct measurement of LacZ activity revealed that deletion of \textit{crp} gene gave three- to fourfold increment of lipBA\_she transcription level (Fig. 8B). A similar lacZ reporter construct was also integrated into the chromosome of \( S.\ oneidensis \) wild-type and its \( \Delta crp \) mutant strains (Fig. 8D and E) (Fu et al. 2014). Consequently, the significant alteration/improvement of lipBA\_she-lacZ expression level was detected upon the removal of the \textit{crp} gene from \( S.\ oneidensis \) (Fig. 8D and E). Second, the real-time qPCR-based analyses of transcriptional profile showed that no less threefold increment of lipA and/or lipB expression was observed in the \( \Delta crp \) mutant of \( S.\ oneidensis \) in relative to the wild type strain (Fig. 8C). Of particular note, repression of \( S.\ oneidensis \) lipBA expression by CRP depends on production of cyclic AMP (Fig. 8E and F). Given the above combined in vitro and in vivo data, we concluded that the cAMP-CRP complex is a repressor for lipBA expression in \( S.\ oneidensis \).

Glucose improves the expression of \( S.\ oneidensis \) lipBA in the alternative model microorganism \textit{E. coli}

It is well known that an addition of glucose into media can lower the level of cytosolic cAMP in \( E.\ coli \), which might in turn impair at least partially CRP-mediated regulation. Somewhat it is unusual that not all the species of \textit{Shewanella}-
la genus can utilize/metabolize glucose in that the S. oneidensis glucose transporter-encoding gene glcP is a pseudogene with a frame-shift (Romine et al. 2008; Rodionov et al. 2010). Given the above technical problem, we therefore attempted to examine the so-called “glucose effect” with the engineered E. coli strain FYJ457 carrying the lip-
BA-sher-lacZ transcriptional fusion (Fig. S3). As expected, we observed that the level of lipBA expression was induced by the addition of glucose (5 mmol/L) to about threefold higher than that grown in the M9 minimal media with acetate (5 mmol/L) as the sole carbon source (Fig. S3).

Together, we proposed for the first time that the global regulator, the cAMP-CRP complex represses bacterial lipoic acid synthesis in *Shewanella*, posing the relevance of the cAMP signaling to the production of the sulfur-containing C8 enzyme cofactor, lipoic acid (Fig. 1A). This regulatory network can respond to the status of glucose/cAMP level, i.e., the low glucose/high cAMP level shuts down lipBA expression (Fig. 1B), whereas the high glucose/low cAMP level de-represses lipBA transcription (Fig. 1C).

**Discussion**

Biotin and lipoic acid both are sulfur-containing fatty acid derivatives and act as enzyme cofactors required for central metabolism in the three domains of life. Unlike the fact that the regulation of bacterial biotin metabolism has been extensively investigated, the knowledge about genetic control of lipoic acid synthesis remains missing or lagged. Although a recent bioinformatics-based proposal was raised, that is, the PDH repressor involved in the production of this enzyme cofactor, lipoic acid (Fig. 1A). This regulatory network can respond to the status of glucose/cAMP level, i.e., the low glucose/high cAMP level shuts down lipBA expression (Fig. 1B), whereas the high glucose/low cAMP level de-represses lipBA transcription (Fig. 1C).

As an important second messenger, the pool of cAMP molecule is at least determined by the following three factors: First, The activity of cyclic adenylate cyclase (*cyaA*) is responsible for the formation of cAMP molecules (Of note, 90% of cAMP that is made by intracellular adenylyl cyclases) (Pastan and Perlman 1970; Hantke et al. 2011); Second, the cAMP phosphodiesterase (*CpdA*) has the opposite enzymatic activity to break a phosphodiester bond of cAMP (Imamura et al. 1996). Not only is the production of CyaA regulated by the CRP regulator at the transcriptional level (Aiba 1983; Schultz et al. 1991; Chandler 1992). However, the protein in *S. oneidensis* was initially characterized as a principal regulator controlling anaerobic respiration of many electron acceptors (Saffarini et al. 2003). In recent years, it has been repeatedly shown that the regulator in fact plays a more comprehensive role in the physiology, covering both aerobic and anaerobic respiration (Gao et al. 2010; Dong et al. 2012; Fu et al. 2013, 2014; Zhou et al. 2013). In contrast to the pathway-specific regulators BirA (Beckett 2007) and BioR (Feng et al. 2013a,b), both of which negotiate production of the other enzyme cofactor biotin, we believed that *Shewanella* genus have evolved an unknown strategy to share the cAMP-dependent CRP regulatory architecture with other biological processes to efficiently control lipoic acid synthesis. Given the fact that glucose can induce lipBA expressions (Fig. 1B), together
with the above information, we concluded that the logic for this kind of regulation does make sense. The reasons are described as follows: (1) the anaerobic growth environment preferred by Shewanella determines an entry of glucose into the glycolytic pathway, giving two pyruvate molecules each glucose; (2) in the Krebs cycle, the resulting pyruvate is catalyzed by PDH to give acetyl-CoA; (3) the full activity of PDH requires the lipoylation, a post-translational modification of protein (which is validated by the scenario seen in the Anti-LA Western blot, i.e., PDH is the prevalent protein form relative to OGDH, Figure 5); (4) the protein lipoylation depends on the availability of lipoic acids; (5) de novo LipB-LipA synthesis pathway is necessary to be turned on in addition to the LpLA-mediated scavenging route of lipoic acid; (6) de-repression of lipBA expression might facilitate meeting the physiological requirement for lipoic acid production in such situation (vice versa, Fig. 1C).

Of particular note, we also detected functional CRP-binding sites ahead of ybeD with unknown function in limited species such as human pathogen S. enterica (Figs. 2 and S2). Although that lipB gene is adjacent to ybeD (of note, we lacked evidence proving if they are co-transcribed or not), it required further experimental evidence for CRP regulate lipB or not in this case. It is of interest to test this hypothesis. In fact, it has already been being our research direction in aiming to answer/pursue this question. To the best of our knowledge, our findings reveal, for the first time, a new molecular mechanism for genetic control of bacterial lipoic acid synthesis.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Characterization of Shewanella CRP protein. A. Sequence comparison of CRP proteins from three different organisms. As we described in Figures 2 and 4, the multiple alignments of CRP proteins were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Identical residues are in white letters with red background, similar residues are in black letters with yellow background, varied residues are in black letters, and dots represent gaps. The predicted secondary structure
was shown in top. \( \alpha \): \( \alpha \)-helix; \( \beta \): \( \beta \)-sheet; \( \gamma \): \( \beta \)-turns/coils.

The three organisms used here are *E. coli*, *V. cholerae*, and *S. oneidensis*, respectively. (B) SDS-PAGE profile of the purified Shewanella CRP protein. The protein sample was separated with 4–20% gradient Mini-PROTEAN@ TGXTM Gel (Bio-Rad). The monomeric CRP protein with the estimated molecular weight of \(~24\) kDa is indicated with an arrow. (C) Modeled structure of Shewanella CRP protein. Structure modeling was proceeded by the software of SPDBV_4.01 using *E. coli* CRP regulator with known structure (PDB: 2WC2) as structural template. N: N-terminus, C: C-terminus. (D) MS identification the recombinant Shewanella CRP protein. The peptide fragments that match Shewanella CRP protein are highlighted in bold and underlined type (70% coverage in total).

**Figure S2.** Diversity in binding of bacterial ybeD probes to CRP protein. (A) The CRP site of Shewanella lipBA gene (referred to lipBA_she) can interact with *E. coli* CRP protein. (B) The predicted CRP site in front of *E. coli* ybeD-lipB-lipA operon (ybeD_ec) has no ability to bind to the CRP protein. The putative CRP sites of the ybeD-lipB-lipA operon from Enterobacter sp. 638 (ybeD_es, C) and Klebsiella pneumonia (ybeD_kp, D) are functional. The predicted CRP site 1 of Salmonella enteric ybeD-lipB-ybeF-lipA operon is functional (E), whereas the site 2 is inactive (F). (G) No binding of the cAMP-CRP complex to the suspected CRP site in front of the ybeD-lipB-lipA operon of Yersinia pestis. All the EMSA experiments (7% native PAGE) were conducted as we described (Feng and Cronan 2012; Feng et al. 2013a) with a minor change. The level of cAMP added is 20 pmol. The *E. coli* CRP protein samples in various concentrations were incubated with 0.2 pmol of DIG-labeled probe in a total volume of 15 \( \mu \)L. A representative result is given. The sequences of all the DNA probes used here are listed in Tables 2 and 3. The minus sign denotes no addition of the CRP protein and/or cAMP molecule. Designations: she, Shewanella; ec, *E. coli*; es, Enterobacter sp. 638; kp, Klebsiella pneumonia; st, Salmonella typhimurium LT2, and yp, Yersinia pestis.

**Figure S3.** Induction of Shewanella lipBA expression by glucose in the alternative model *E. coli*. To test the effect of glucose on Shewanella lipBA expression, the *E. coli* strain carrying the lipBA_she-lacZ transcriptional fusion (FYJ457) was used here. Mid-log phase cultures in M9 media with acetate and/or glucose (5 mmol/L) as sole carbon source were sampled for assaying \( \beta \)-gal activity. The data from more than three independent experiments is expressed in average \( \pm \) standard deviation (SD), and error bars indicate SD.