Li Ganwu (Orcid ID: 0000-0002-6370-9573)

Citrate utilization under anaerobic environment in *Escherichia coli* is under direct control of Fnr and indirect control of ArcA and Fnr via CitA-CitB system

Running Head: ArcA and Fnr control Citrate utilization in *E. coli*

Fengwei Jiang[a,b], Xinxin Huang[c], Nicolle Barbieri[d], Catherine M. Logue[d], Lisa K. Nolan[e], Ganwu Li [a,b, #]

State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China[a]; Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa, USA[b]; Technical Center for Animal, Plant, and Food Inspection and Quarantine of Shanghai[c]; Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602[d]; Department of Infectious Disease, College of Veterinary Medicine, University of Georgia, Athens, GA 30602[e]

Address correspondence to: Ganwu Li, liganwu@iastate.edu

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Summary

Most *Escherichia coli* (*E. coli*) strains do not cause disease, naturally living in the lower intestine and is expelled into the environment within fecal matter. *E. coli* can utilize citrate under anaerobic conditions but not aerobic conditions. However, the underlying regulatory mechanisms are poorly understood. In this study, we explored regulatory mechanisms of citrate fermentation genes by global regulators ArcA and Fnr under anaerobic conditions. A gel mobility shift assay showed that the regulator proteins ArcA and Fnr bind to the promoter region localized between the *citAB* and *citCDEFXGT* operons. Subsequent assays confirmed that ArcA indirectly control the expression of citrate fermentation genes via regulating CitA-CitB system, while Fnr directly regulate but also indirect modulate citrate fermentation genes via control CitA-CitB system. Deletions of *arcA* and *fnr* significantly reduced the growth of *Escherichia coli* in M9 medium with a citrate carbon source. We conclude that both ArcA and Fnr can indirectly control the citrate utilization via CitA-CitB system, while, Fnr can also directly regulate the expression of citrate fermentation genes in *E. coli* under anaerobic conditions.

Importance

Escherichia coli generally can utilize citrate only in anaerobic conditions and in the presence of an oxidizable co-substrate. Several genes that are important for citrate metabolism are organized into the *citCDEFXGT* gene cluster and probably form one operon that share the same promoter. The coordinated expression of the *citCDEFXGT*

gene cluster is activated by a two-component system consisting of the sensor kinase CitA and the response regulator CitB. In this study, we show that the oxygen sensor regulators ArcA and Fnr bind to the promoter region localized between the *citAB* and *citCDEFXGT* operons and affect the expression of citrate fermentation genes indirectly via direct regulation of the CitA-CitB system, Fnr can also directly regulate the expression of citrate fermentation genes in *E. coli* under anaerobic conditions.

Keywords: Avian Pathogenic *E. coli* (APEC), ArcA, Fnr, CitA-CitB TCS, citrate fermentation genes

Introduction

Although some *Escherichia coli* strains that possess a plasmid-encoding citrate uptake systems can grow on citrate in aerobic environments (Ishiguro and Sato, 1985), a key characteristic of most *E. coli* strains is that they cannot use citrate under these conditions due to the lack of a functional citrate transporter (Pos *et al*., 1998). However, under anaerobic conditions, in the presence of an oxidizable co-substrate, such as glucose or glycerol, *E. coli* can utilize citrate (Lütgens and Gottschalk, 1980). The citrate uptake system and citrate lyase are specifically required for the fermentation of this tricarboxylic acid. The gene *citT*, located at 13.90 min on the *E. coli* chromosome, encodes a protein comprised of 487 amino acids. An analysis of CitT's primary sequence showed that it is highly hydrophobic with 12 putative transmembrane helices (Pos *et al*., 1998). Transport studies showed that CitT is a citrate carrier catalyzing the homologous exchange of citrate or a heterologous exchange against succinate, fumarate, or tartrate. The genes *citD*, *citE*, and *citF* encode three subunits of citrate lyase that cleaves citrate to acetate and oxaloacetate (Bott and Dimroth, 1994). The gene *citC* encodes a ligase required for the acetylation of the 2-(5′′-phosphoribosyl)-3′-dephosphocoenzyme-A prosthetic group, whereas *citG* and *citX* encode proteins presumably involved in the biosynthesis of the prosthetic group (Schneider *et al*., 2002). All these genes located as a gene cluster probably form one operon and share a single promoter.

The coordinated expression of the *citCDEFXGT* gene cluster is activated by a two-component system consisting of the sensor kinase CitA and the response

regulator CitB in *Klebsiella pneumoniae* and *E. coli* (Bott *et al*., 1995; Yamamoto *et al*., 2009). *E. coli* CitA is a typical histidine kinase consisting of an extracellular domain located in the periplasm and a cytoplasmic domain. The periplasmic domains of CitA histidine kinases sense the signal and bind citrate with high specificity and affinity, whereupon they become self-phosphorylated and activated (Kaspar *et al*., 1999; Kaspar and Bott, 2002; Sevvana *et al*., 2008). CitB, activated after trans-phosphorylation by CitA, binds to the intergenic region between *citA* and *citC* promoters and activates the transcription of genes involved in citrate transport and catabolism (Yamamoto *et al*., 2008). CitAB is known as a master regulatory system that activates the promoters of genes involved in citrate fermentation in the presence of citrate and under anaerobic conditions in *E. coli* (Yamamoto *et al*., 2008; Scheu *et al*., 2012). Nevertheless, because CitAB is not an oxygen sensor, the regulatory mechanisms by which the genes involved in citrate fermentation are only expressed under anaerobic, but not aerobic conditions, remain unknown.

The Arc two-component signal transduction system, comprised of the kinase sensor ArcB and its cognate response regulator ArcA, is one of the mechanisms that enable *E. coli* adaptation to changing oxygen availability (Lynch and Lin, 1996). Previous studies showed that under anaerobic conditions, ArcA inhibits the expression of genes required for aerobic metabolism, energy generation, amino acid transport, carbon metabolism, and fatty acid transport, and also activates genes contributing to anaerobic respiratory and metabolism pathways (Salmon *et al*., 2005; Park *et al*., 2013). Fnr, another oxygen sensor regulator, controls the expression of a large number

of enzymes in response to the extracellular oxygen status. It has been shown to modulate directly or indirectly the expression of 712 genes involved in the regulatory networks that facilitate the transition of *Escherichia coli* cells from an aerobic to an anaerobic growth state (Salmon *et al*., 2003; Kang *et al*., 2005; Constantinidou *et al*., 2006).

Here, we provide solid evidence that the oxygen response regulators ArcA and Fnr directly regulate the expression of CitAB and indirectly affect the expression of citrate fermentation genes, while Fnr can directly regulate citrate fermentation genes as well, permitting their transcription only under microaerobic and anaerobic conditions.

Results

Mapping of *citC* and *citA* transcription start sites.

Bioinformatics analysis predicted that the gene cluster *citCDEFXGT*, which is involved in citrate fermentation under anaerobic conditions, forms one operon and shares one promoter. To map the transcription start sites (TSS) of the *citC* operon and *citA* gene, 5'-rapid amplification of cDNA ends (RACE) PCR was performed. Total RNA was isolated from *E. coli* strain XM, cultivated at 37 °C under anaerobic conditions in M9 medium with citrate as carbon source. Random primer was used for the first-strand cDNA synthesis. Universal Primer A Mix and gene specific primers were used for 5'ends amplification. The transcription start site of *citC* was mapped to the position 129 bp upstream of the *citC* coding region and the TSS of *citA* was mapped to the position 74 bp upstream of the *citA* coding region (Fig. 1). For both of the transcription start sites, -10 boxes could be identified in the sequence, and

corresponding -35 boxes were present at the proper distance to the transcription start sites that were mapped to the upstream of *citC* and *citA* coding region (Fig. 1).

Deletion of *arcA* reduces the expression of the *citCDEFXGT* and *citAB.*

The transcriptional regulation of the *citCDEFXGT* operon by ArcA was determined using chromosomal *citC-lacZ* reporter gene fusion. Under anaerobic conditions, the expression of *citC-lacZ* was significantly reduced in Δ*arcA* mutant strain and it was 26-fold lower than that in WT strain. Furthermore, the re-introduction of the complementation plasmid carrying *arcA* restored *citC-lacZ* expression (Fig. 2A), confirming that ArcA regulates the expression of the gene cluster *citCDEFXGT.* We then examined whether ArcA affects the expression of the CitA/CitB signaling system. Bioinformatics analysis predicted that *citA* and *citB* form one operon and share one promoter. Since chromosomal *citA-lacZ* reporter gene fusion might affect the expression of regulator CitB, qPCR was used to compare the expression levels of *citA* in Δ*arcA* mutant and WT strains in M9 cultures with citrate as carbon source under anaerobic conditions. The *citA* gene expression level was low in both strains, but it was eightfold higher in WT strain than in Δ*arcA* mutant strain (Fig. 2B), which indicated that the *citA* gene is under the control of ArcA.

ArcA directly regulates the expression of CitA/CitB through binding to its promoter region.

Two potential binding sites of ArcA (BS_{ArcA1} and BS_{ArcA2}) (at positions of −277~−268 and −86~−77 of *citA*) were predicted in the intergenic region of the *citAB* and

citCDEFXGT operons (http://www.prodoric.de/vfp/vfp_promoter.php) (Fig. 1). For both predicted binding sites, a 240bp DNA fragment containing 60 bp upstream, 120bp ArcA1 or ArcA2 binding site, and 60 bp downstream sequence were amplified using PCR, and a control DNA fragment only containing 60 bp upstream and 60 bp downstream, but without the 120bp binding site were constructed. As shown in Fig. 3A, phosphorylated ArcA could shift the DNA fragments containing either ArcA1 or ArcA2, but not the control fragments. Moreover, 30-bp DNA fragments with the original ArcA binding site ArcA1 and ArcA2 or with mutated binding site sequences were synthesized (information about the primers is provided in supplemental Table S2) and EMSAs were performed. As shown in Fig. S1, ArcA shifted both DNA fragments with the original binding sites ArcA1 and ArcA2 but not those with mutated binding site sequences. Collectively, these results validated both binding sites ArcA1 and ArcA2.

Expression studies were further performed to determine if ArcA directly regulates the expression of *citAB* through binding to sites BS_{ArcA1} and/or BS_{ArcA2}. Three *lacZ* fusions with *citA* promoter regions P_{citA1} (*citA* promoter region includes both $ArcA_1$ and ArcA2 binding sites from positions −409 to +73), P_{citA2} (*citA* promoter region only including ArcA2 binding site from positions −184 to +73), and P_{citA3} (*citA* promoter region including neither ArcA1 nor ArcA2 binding sites) were constructed using plasmid pCJ112. The expression of *citA* in the *lacZ* fusion with P_{citA1} showed no significant difference with that of P_{citA2}. However, the *citA* expression of P_{citA3} was extremely lower ($P< 0.01$) (Fig. 3B). Furthermore, A 6-nucleotide substitution of the

binding site ArcA1 or ArcA2 was made in the *citA* promoter P_{citA1} and expression studies were performed in WT and Δ*arcA* mutant strains. As shown in Fig. 3B, mutation of binding site ArcA1 significantly down-regulated the *citA* expression in the WT ($P< 0.01$), while mutation of binding site ArcA2 did not significantly change the *citA* expression. The *citA* expression in all constructs in the Δ*arcA* mutant was significantly lower than that in the WT except the P_{citA3}. These results indicated that ArcA directly regulate the expression of *citAB* through binding to ArcA1 site, but not ArcA2.

ArcA indirectly affects the expression of operon *citCDEFXGT* through the regulation of CitA/CitB

To determine whether ArcA directly regulates *citCDEFXGT* and/or indirectly through CitAB, we compared the expression level of *citC* in the WT, Δ*arcA* mutant strain, Δ*citB* mutant strain, and the mutant strain with double deletions of *arcA* and *citB* (Δ*arcA*Δ*citB* mutant strain). Similar to the results of previous study (Yamamoto *et al*., 2008; Scheu *et al*., 2012) , *citB* was essential for the expression of *citC* and deletion of *citB* completely abolished *citC* expression. Further deletion of *citB* in the *arcA* mutant strain further decreased the expression of *citC* (Fig. 4A). These results indicated that ArcA might affect expression of *citCDEFXGT* indirectly through two-component system CitAB**.** To further test this hypothesis, a plasmid with multiple copy number that constitutively expressed *citAB* (pGEN-Pcm-*citAB*) with P(cat) promoter of BBa_I14033 (http://parts.igem.org/Promoters/Catalog/Constitutive) was constructed and transformed into Δ*arcA* mutant. The *citAB* was also constitutively expressed

under the control of promoter BBa_J23116 (with low constitutive expression) in a single copy plasmid pBeloBAC11. As shown in Fig. 4B, constitutive expression of CitAB partially rescued the expression of *citC* in the Δ*arcA* mutant strain regardless its expression levels, while constitutive expression of ArcA could not increase the expression of *citC* in the Δ*citB* mutant strain (data not shown). Moreover, the double complementation by *arcA* and *citAB* was achieved by transforming both the pGEN-MCS-*arcA* and pBeloBAC11-*citAB* into the Δ*arcA* strains and the *citC* expression was not significantly different compared with the single complementation of *citAB* (Fig. 4C). All together, these results suggested that ArcA indirectly regulate *citC* expression through the regulation of *citAB.*

Inactivation of *fnr* decreases the expression *citCDEFXGT* and *citAB.*

Fnr is a well-known global regulator mediating bacterial adaptation to the anaerobic environment. To determine if Fnr affects the citrate utilization under anaerobic conditions, the transcriptional expression of *citCDEFXGT* was monitored by using *citC-lacZ* gene fusion measuring *β*-galactosidase activity in WT, Δ*fnr* mutant, and complemented Δ*fnr* mutant strains. As shown in Fig. 5A, *citC* expression was significantly down-regulated in Δ*fnr* mutant compared to that in WT strain, whereas *citC* expression was partly rescued in the complemented strain. These results demonstrated that Fnr plays an important role in the regulation of citrate fermentation genes under anaerobic conditions. To examine if Fnr regulates CitA/CitB two-component system, qPCR was performed to compare the expression level of *citA* in Δ*fnr* mutant and WT strains in M9 cultures with citrate as sole carbon source under

anaerobic conditions. The *citA* gene expression level in WT was 12-fold higher than that in Δ*fnr* mutant strain. Therefore, the results presented in Fig. 5B demonstrate that *citAB* is under the control of Fnr.

Fnr directly regulates CitA/CitB two-component system.

Bioinformatics analysis using Patser software (version 3d) predicted that there is a potential Fnr binding site localized in the intergenic region between the *citAB* and *citCDEFXGT* operons (Fig. 1). Electrophoretic mobility shift assays (EMSA) were performed to verify whether Fnr directly binds to the Fnr binding site. A DNA fragment containing the potential binding site of Fnr or excluding the Fnr binding site was amplified for use as a probe, and another DNA fragment, amplified from the *citC* coding region, was used as negative control. As shown in Fig. 6A, the Fnr fusion protein shifted the fragment of the intergenic region containing the Fnr binding site, but did not affect the fragment without the Fnr binding site and the control fragment. These results demonstrated that Fnr directly binds to the intergenic region between *citCDEFXGT* and *citAB* operons.

To examine if Fnr regulates the CitA/CitB two-component system, DNA fragments encompassing the *citA* promoters including or excluding the predicated Fnr binding sites were cloned into pCJ112 to generate two *lacZ* fusions. The *lacZ* fusion of the smallest *citA* fragment, which encompasses sequences from -14 to +73, directed an average of 11 units of *β*-galactosidase activity in the wild type strain. When the promoter fragment was extended to include Fnr binding sequences from positions

-409 to +73, the expression of *citA* in the WT (P_{citA1}) was 236 units, 21 times higher than the short fragment excluding the Fnr binding site (P_{citA2}) ($P< 0.01$) (Fig. 6B). Therefore, the results presented in Fig. 6B demonstrate that *citAB* is under the control of Fnr. To determine if Fnr directly regulates *citA*, the *citA* promoter regions with or without the Fnr binding site were cloned into pCJ112 to obtain pP_{citA}-*lacZ* and pP_{citA}-*lacZ* with an Fnr binding site deletion, respectively. The deleted Fnr binding site significantly down-regulated the expression of *citA* in the WT strain ($P< 0.05$), while the expression levels of *citA* with or without the Fnr binding site in the Δ*fnr* mutant strain were extremely low (Fig. 6B).

Fnr indirectly affects the expression of operon *citCDEFXGT* through the regulation of CitA/CitB

To determine whether Fnr directly regulates *citCDEFXGT* and/or indirectly through CitAB, the expression level of *citC* in the WT, Δ*fnr* mutant strain, Δ*citB* mutant strain, and the mutant strain with double deletions of *fnr* and *citB* (Δ*arcA*Δ*citB* mutant strain) was compared. As shown in Fig. 7A, *citB* was essential for the expression of *citC* and deletion of *citB* showed no detectable *citC* expression, further deletion of *citB* in the *fnr* mutant strain further decreased the expression of *citC*. A plasmid that constitutively expressed *citAB* (pGEN-Pcm-*citAB*) was further transformed into the *citC*-*lacZ*/Δ*fnr* mutant strain. As shown in Fig. 7B, the constitutive expression of *citC* could restore the effect of *fnr* deletion only partially. While, low constitutive expression of *citAB* from a single copy plasmid pBeloBAC11 could not increase the expression of *citC* in the Δ*fnr* mutant strain. In addition, a double complementation by

fnr in addition to *citAB* into the Δ*fnr* strains significantly increased *citC* expression compared with the single complementation of *citAB* (Fig. 7C). Moreover, a mutant with double deletions of both *arcA* and *fnr* genes was further constructed and CitAB was constitutively expressed in the mutant strain with double deletions. The expression of *citC* in the constitutive expression of CitAB strain could not restore any effect of double deletion of *arcA* and *fnr* (Fig. 7D). These results suggested that Fnr might also directly regulate the *citC* gene.

Fnr also directly regulates the expression of citrate fermentation genes.

To determine if Fnr directly regulates *citC*, we compared the expression levels of *citC* with or without the Fnr binding site in both WT and Δ*fnr* mutant strains. The DNA fragments encompassing the *citC* promoters including or excluding the predicated Fnr binding sites were cloned into pCJ112 to generate two *lacZ* fusions. The *lacZ* fusion of the smallest *citC* fragment, which encompasses sequences from +132 to +213, directed an average of 5 units of *β*-galactosidase activity in the wild type strain. When the promoter fragment was extended to include Fnr binding sequences from positions -227 to +213, the expression of *citC* in the WT (P_{citC1}) was 439 units, 88 times higher than the short fragment excluding the Fnr binding site (P_{citC2}) ($P< 0.01$) (Fig. 8).

The *citC-lacZ* fusion without the Fnr binding site was further amplified using overlap extension PCR to generate pP_{citC-} *lacZ* (Fig. 8) with a deletion of the FNR binding site. Deletion of the FNR binding site in the P_{citC1} significantly reduced the expression of *citC* expression in the WT ($P< 0.01$). These results suggest that Fnr directly regulates the expression of the *citCDEFXGT* operon. The expression of *citC* in the Δ*fnr* mutant

with pP_{citC}-*lacZ* was significantly lower than that in the WT strain with pP_{citC}-*lacZ* harboring a deletion of the Fnr binding sequence, suggesting that Fnr may also regulate the expression of *citC* indirectly.

ArcA and Fnr affect the utilization of citrate in the *E. coli* strain XM.

Growth properties of WT, Δ*arcA* mutant, Δ*fnr* mutant, Δ*arcA* and Δ*fnr* complemented strains were compared in M9 cultures with citrate as carbon source under anaerobic conditions. In addition, a Δ*citT* mutant was also included to determine if citrate metabolism contribute to the growth phenotype in the medium with citrate. As shown in Fig. 9A, deletion of *citT* significantly reduce the *E. coli*'s growth, while the Δ*arcA* mutant strain grew better than the Δ*citT* mutant, but significantly slower than the WT ($P< 0.01$). Reintroduction of the *arcA* gene back into the Δ*arcA* mutant restored its growth to the level observed in WT strain. Similarly, deletion of *fnr* also significantly reduced the growth of *E. coli* in M9 medium containing citrate as carbon source under anaerobic conditions. Furthermore, the growth was significantly improved in the complemented strain (Fig. 9B), suggesting that ArcA and Fnr indeed play important roles in citrate fermentation under anaerobic conditions.

Discussion

The global regulators ArcA and Fnr control expression of many genes under anaerobic conditions (Compan and Touati, 1994). In this study, we have demonstrated that the ArcA indirectly affects the expression of citrate fermentation genes, while Fnr not only indirectly modulates but also directly regulates these genes' expression. Both ArcA and Fnr directly regulate the expression of the two-component *citA/citB* system

thus affect the expression of citrate fermentation genes indirectly. The expression of citrate fermentation genes is induced by citrate and is dependent on CitAB under anaerobic conditions (Bott and Dimroth, 1994; Meyer *et al*., 2001; Scheu *et al*., 2012). Because CitAB is not an oxygen sensor, the regulators involved in the anaerobic induction of citrate fermentation genes have not been identified and their relationship to CitAB are largely unknown.

ArcA predominantly functions as a global repressor of pathways associated the oxidation of non-glycolytic carbon source, but also directly activated 11 operons in the previous study (Park *et al*., 2013). In our study, a new operon (*citAB*) was identified to be directly activated by the global regulator ArcA. Inactivation of ArcA retarded *E. coli*'s anaerobic growth in the medium with citrate as carbon source. Deletion of ArcA and/or mutation of binding site ArcA1 significantly reduced expression of the *citAB* operon, while constitutively, expression of CitAB in the ArcA mutant strain increased the expression of *citC* but could not recover the expression to WT levels. These results suggested that ArcA could directly regulate *citAB* expression. Two ArcA binding sites were identified in the intergenic region of the *citAB* and *citCDEFXGT* operons, but only one binding site, ArcA1, was confirmed to be important for positive regulation of both *citAB*. The positions of the identified ArcA binding sites relative to the TSS for each targeted operon were very variable with some binding sites located near to the mapped TSS, whereas others were as far as 200-400 bp upstream of the TSS (Park *et al*., 2013) . The binding site ArcA1 is localized at the position 86 bp upstream of TSS of *citA*, which was within the range in

previous findings. The other binding site, ArcA2, did not affected the expression of neither *citAB* in our study. For all ArcA-activated and impressed operons, the positions of the identified ArcA binding sites relative to the TSS ranged from + 80 to -500 bp (Park *et al*., 2013). ArcA2 is localized at position 269 upstream of the TSS of *citA* within the range; however, the underlying mechanism of why it is not important for *citA* expression remains unknown.

Fnr is another known global regulator that mediates the adaptation of bacteria to anaerobic conditions (Myers *et al*., 2013). Inactivation of Fnr and deletion of the Fnr binding site led to significant decreases in the expression of the *citC* gene cluster (Fig. 8), suggesting that Fnr directly regulates the expression of those genes. In addition, Fnr also directly regulates the expression of the two-component CitAB system (Fig. 6B). Because CitAB is a known regulatory system that is essential for *citC* gene cluster expression, this suggests that Fnr also indirectly modulates the expression of the *citC* gene cluster through control of CitAB. Accordingly, the introduction of constitutively expressed *citAB* into the Δ*fnr* mutant partly rescued *citC* expression but not to the WT level (Fig. 7B). The binding sites for promoters directly activated by Fnr are primarily centered at positions −41.5 and −61.5 with a few exceptions, e.g., at −74.5 and −132.5 in *dcuB* (Myers *et al*., 2013). Our results showed that binding Fnr sites was at −132 (*citA*). Binding at position -132 reflect a regulatory mechanism similar to that of *dcuB,* an anaerobic dicarboxylate transporter. Activation by Fnr occurs primarily through the interaction with the α subunit of RNA polymerase. Both ArcA and Fnr are known to mediate widespread changes in gene expression during

the transition from aerobic to anaerobic conditions. Two recent studies suggested that the functional roles of ArcA and Fnr are distinct: ArcA generally functions as a repressor of aerobic carbon oxidation pathways, whereas Fnr is more likely to function as an activator of anaerobic gene expression (Myers *et al*., 2013; Park *et al*., 2013). Although previous studies have suggested that there may be a large overlap between the genes regulated by ArcA and Fnr (Iuchi *et al*., 1994; Salmon *et al*., 2005), very little evidence of direct co-regulation actually exists. In this study, the *citAB* was added to the list of co-regulated operons. The operon *citAB* was not identified as a direct target of ArcA or Fnr in several previous studies (Myers *et al*., 2013; Park *et al*., 2013). This may be due to different culture conditions used in previous studies. No citrate was included in the culture medium of previous studies and the presence of glucose may even repress the expression of *citAB* (Zientz *et al*., 1998). In addition, the environmental stimuli probably change ArcA and/or Fnr binding site accessibility and affect transcription.

It should be noted that the expression of citrate fermentation genes is subject to multiple levels of regulation. CitAB positively regulates its own expression by binding to the promoter region of *citAB* (Yamamoto *et al*., 2008), and optimal *citC* expression requires DucSR, which directly regulates the expression of *citAB* (Scheu *et al*., 2012). On the other hand, TCS NarXL negatively influences DcuSR production (Goh *et al*., 2005); DcuS is up-regulated 2-fold by Fnr (Salmon *et al*., 2003); and in *Klebsiella pneumoniae*, the *citCDEX* cluster is also subject to catabolite repression, in which CRP is also involved (Bott *et al*., 1995).

In conclusion, we confirmed that regulator Fnr could directly and indirectly regulate the expression of citrate fermentation genes, while ArcA could only indirectly modulate citrate fermentation genes. The indirect regulation occurs through the two-component CitAB system, an essential and most direct regulator of citrate fermentation.

Materials and Methods

Bacterial strains, culture conditions and plasmids. The *E. coli* strain XM (O2:K1:H7), other bacterial strains, and plasmids used in this study are listed in Table S1. The DNA sequence of the intergenic region between *citC* and *citA* in the *E. coli* strain XM used in this study is 100% identical to that in *E. coli* K12 that the current knowledge of citrate fermentation and gene expression primarily comes from and additionally, the encoding regions of *citCDEFXGT* operon and *citAB* are 99% identical (GenBank: CP025328). Furthermore, the amino acid sequences of regulators Fnr, ArcA, and CitB are 100% identical to those in *E. coli* K12 (GenBank: CP025328). Aerobic growth was achieved by shaking in air at 160 rpm, whereas anaerobic growth was monitored during the incubation in a BugBox chamber (Ruskinn, ME, USA) filled with a mixture of N_2 (90%); CO_2 (5%), and H_2 (5%). For genetic manipulations, all *E. coli* strains were routinely grown in LB broth medium. For all growth tests and *β*-galactosidase assays，bacteria were grown under anaerobic conditions in M9 minimal salts base supplemented with 20mM citrate, 0.01% yeast extract, 50 mM glycerol and 20 mM dimethyl sulfoxide as substrates unless specified (Lara and Stokes, 1952; Kneuper *et al*., 2005; Krämer *et al*., 2007). Selective antibiotics and

isopropyl-*β*-D-thiogalactopyranoside (IPTG) were added when necessary at the following concentrations: ampicillin, 50 μg/mL; kanamycin, 50 μg/mL; chloramphenicol, 25 μg/mL; nalidixic acid, 30 μg/mL, and IPTG, 0.1 mM.

Recombinant DNA techniques. PCR, DNA ligation, electroporation, and DNA gel electrophoresis were performed as described by Sambrook and Russell (Joseph and David, 2001), unless otherwise indicated. All oligonucleotide primers were purchased from BGI Tech Solutions Co., Ltd. (BGI, Guangzhou, China), and are listed in Table S2 in the Supplemental Material. All restriction and DNA-modifying enzymes were purchased from TaKaRa Biotechnology Co., Ltd. (TaKaRa, Dalian, China), and used on the basis of the supplier's recommendations. Recombinant plasmids, PCR products, and restriction fragments were purified using TaKaRa MiniBEST plasmid purification kits or agarose gel extraction kits (TaKaRa, Shiga, Japan) as recommended by the supplier. DNA sequencing was performed at the DNA facility of Shanghai Sunny Biotechnology Co., Ltd.

Deletion mutants were constructed using the bacteriophage lambda red recombinase system described by Datsenko and Wanner (Datsenko and Wanner, 2000). The primers used for mutagenesis are listed in Table S2 in the Supplemental Material. The homologous recombination construction used PCR-purified products with selective antibiotic resistance genes and 60-nucleotide homology extensions. The mutants were confirmed by PCR and sequencing.

Plasmid construction. For complementation, gene coding sequences and their putative promoter regions were amplified using the primers listed in Table S2 of the

Supplemental Material from the WT strain and independently cloned into pGEN-MCS (Lane *et al*., 2007) using *Bam*HI and *Eco*RI (for *arcA*) or *Hin*dIII and *Bam*HI (for *fnr*).

To construct the plasmid for constitutive expression of the *citAB* gene under the control of the constitutive promoter Pcat, the pKD3 promoter region was first cloned from the pKD3 plasmid and the *citAB* gene from WT strain, then these two fragments were combined by overlap PCR, and the assembled fragment inserted into pGEN-MCS (Lane *et al*., 2007) using *Eco*RI and *Not*I restriction sites. All constructs were confirmed by DNA sequencing.

The plasmid for constitutive expression of the *citAB* gene with single copy number under the control of low constitutive promoters BBa_J23116 (pBeloBAC11-*citAB*) was constructed by homologous recombination. The sequence of promoter sequence and RBS sites, followed by *citAB* gene were cloned and combined into pBeloBAC11 using the ClonExpress ® Ⅱ One Step Cloning Kitpurchased from Vazyme Biotech Co., Ltd (Vazyme, Nanjing, China).

Chromosomal transcriptional *lacZ* fusion was constructed by homologous recombination of the suicide plasmid pVIK112 carrying a fragment of the complete 5′-region of the target gene (Kalogeraki and Winans, 1997; Cai *et al*., 2013; Barbieri *et al*., 2014). Briefly, PCR fragments of target genes were cloned into pVIK112 using *Eco*RI and *Xba*I sites. The resulting pVIK112 derivatives were introduced into APEC XM Δ*lacZ* by conjugation. Conjugants were selected and confirmed by PCR. The plasmid pCJ112, was constructed by replacing the R6K origin in pVIK112

(Kalogeraki and Winans, 1997) with p15A origin from pBAD30 (Guzman *et al*., 1995) using *Eco*RI and *Bam*HI restriction sites, was created for making chromosomal transcriptional fusion of the *lacZ* reporter gene. The resulting plasmid was tested to show the functionality and an undetectable basal expression level without inserting any promoters upstream from the promoterless *lacZ*. The sequences of the whole *citA* promoter with intact or mutant ArcA potential binding sites were cloned by overlap PCR, inserted into pCJ112, and transformed into the Δ*arcA* mutant and WT strains. The same operation was applied to the *citC* promoter and Δ*fnr* mutants.

To construct the plasmid overproducing pET28a (+)-ArcA-His_6 fusion protein, a 717-bp fragment containing the coding region of *arcA* was obtained by PCR from genomic DNA using pET28a-*arcA*-F and pET28a-*arcA*-R (see Table S2 in the Supplemental Material), carrying codons for 6×His, and subsequently cloned into the pET28a (+) vectors (Novagen, Madison, WI, USA) using *Nco*I and *Hin*dIII sites. The resultant plasmid contained pET28a-ArcA-His_6 under the control of the T7 promoter.

Purification and phosphorylation of ArcA-His_6 protein. *E. coli* BL21 with pET28a-ArcA was grown in 200 mL of LB medium for 16 h at 28 °C, and protein expression was induced by adding 0.1 mM IPTG. The ArcA-His_6 fusion protein was purified to homogeneity using Ni-nitrilotriacetic acid spin columns and dialyzed in buffer (20 mM Tris, 50 mM NaCl, 40 mM EDTA, 4 mM dithiothreitol, 10% glycerol, pH 7.4) at 4 °C overnight with triple dialysis buffer changes. Protein concentration was estimated by using the BCA protein assay reagent (Thermo Fisher Scientific, Massachusetts, MA, USA), with bovine serum albumin as the standard. For the

preparation of His_6-ArcA-P for use in DNA binding assay, an *in vitro* phosphorylation of ArcA with lithium acetyl phosphate (Sigma-Aldrich, Shanghai, China) was performed. We used a standard phosphorylation reaction by incubating 20 μg of ArcA in a 10 μL buffer containing 50 mM Tris-HCl (PH 8.0), 0.5 mM EDTA, 10 mM glycerol, 10 mM $MgCl_2$ and lithium acetyl phosphate to a final concentration of 50 mM. The incubation was proceeded for 60 min at 25 ℃ as described (Drapal and Sawers, 1995; Lynch and Lin, 1996). The phosphorylated ArcA was used immediately for DNA studies.

Electrophoretic mobility shift assays (EMSA). To study the binding of ArcA to the DNA probe, electrophoretic mobility shift assays (EMSAs) were performed as described previously (Lynch and Lin, 1996; Gao *et al*., 2008). Briefly, DNA probes were amplified using specific primers and purified using a TaKaRa Mini-BEST gel extraction kit. To demonstrate the predicted binding sites by EMSAs, dsDNA containing ArcA mutant binding sites was obtained by mixing the pair of complementary bases with annealing buffer and incubated at 95 °C for 10 min and then allowed to stand at room temperature for 5-10 min to allow slow annealing to occur. A gel was run to qualify dsDNA and then, DNA fragments were diluted to appropriate concentrations. EMSAs were performed by adding increasing amounts of purified and phosphorylated ArcA-His_6 fusion protein (0 to 10.5 μM) to the DNA probe (0.1pmol) in binding buffer (10 mM Tris-HCl, 7 mM $MgCl_2$, 5% glycerol, 40 μg/mL bovine serum albumin, pH 8.0) for 30 min at 37 ℃. The reaction mixtures were then subjected to electrophoresis on a 6% polyacrylamide gel in 0.5×TBE buffer (44.5

mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 200 V for 45 min. The gel was stained in 0.5×TBE buffer containing 1×SYBR Gold nucleic acid staining solution (Life Technologies, Grand Island, NY, USA) for 30 min, and then the image was recorded.

We used the $(FnrD154A)_2$ protein variant because it displayed DNA-binding affinity and transcriptional regulatory activity with various Fnr-dependent promoters under aerobic conditions (Shan *et al*., 2012; Barbieri *et al*., 2014). Protein expression was performed as described previously (Lazazzera *et al*., 1993). To study the binding of Fnr to the DNA probe, EMSAs were performed as described elsewhere (Shan *et al*., 2012). Briefly, the $(FnrD154A)_2$-His_6 fusion protein was purified to homogeneity using Ni-nitrilotriacetic acid spin columns and dialyzed against binding buffer. DNA probes were amplified using specific primers and purified using a gel extraction kit (TaKaRa, Shiga, Japan). EMSAs were performed by adding increasing amounts of purified $(FnrD154A)_2$-His_6 fusion protein (0 to 0.26 μM) to the DNA probe (0.1 pmol) in binding buffer (20 mM Tris-HCl, 10 mM EDTA, 4 mM dithiothreitol, 50 mM NaCl, 5% glycerol, 0.5 mg/mL bovine serum albumin, pH 6.8) for 30 min at 37℃. The reaction mixtures were then subjected to electrophoresis on a 6% polyacrylamide gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 200V for 45 min. The gel was stained in 0.5× TBE buffer containing 1× SYBR Gold nucleic acid staining solution (Life Technologies, Grand Island, NY, USA) for 30 min, and then the image was recorded.

Mapping of transcription start sites by 5'-RACE. Total RNA was isolated from

ExPEC XM, cultivated at 37 °C under anaerobic conditions in M9 medium with citrate as carbon source. Genomic DNA was removed from the extracted RNA using the RNase-Free DNase set (Qiagen, California, CA, USA) and then was reverse transcribed to cDNA using reagents supplied in the SMARTer RACE 5'/3' Kit (TaKaRa) following the manufacturer's instructions. The obtained cDNA was then used for the 5'-RACE amplifying with the Universal Primer A Mix and gene specific primers (Table S2). The 5'-RACE products were cloned into the pRACE vector by in-fusion cloning. The fragments of several clones were sequenced for mapping of the primary transcription.

***β*-galactosidase activity.** Expression levels of the *citA-lacZ* and *citC-lacZ* reporter gene fusions was determined by measuring *β*-galactosidase activity of exponentially growing cultures at 37 °C under anaerobic conditions in M9 medium. For anaerobic growth, cultures were incubated in degassed medium in rubber-stoppered infusion bottles under N_2. Overnight LB cultures of *E. coli* containing the fusions of the gene of interest with *lacZ* were washed with phosphate-buffered saline, diluted 1:100 in M9 medium with citrate as carbon source, and grown at 37 °C to log phase or stationary phase. These cultures were diluted 1:1 in Z buffer and assayed for *β*-galactosidase activity using ortho-Nitrophenyl-*β*-galactoside as substrate as described previously (Miller, 1972). The values of *β*-galactosidase activity were measured at least in triplicate for each experiment.

Supplemental material

Supplementary Figure legends:

Fig. S1 Representative electrophoretic mobility shift assays of phosphorylated ArcA-His_6 binding to the ArcA1 and ArcA2 original and mutant binding sites.

Fig. S2 (A) Citrate effect for *citC* expression in *arcA* mutation strains when CitA-CitB are produced constitutively in single copy plasmid. (B) Citrate effect for *citC* expression in *fnr* mutation strains when CitA-CitB are produced constitutively in single copy plasmid. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments.

Table S1. Strains and plasmids. The genotypes of all strains of *E. coli* utilized or constructed in this study and information about the plasmids used in this study.

Table S2. Oligonucleotides. Oligonucleotide sequences used as PCR primers.

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Competing interests

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Fig. 1 The transcription start sites of *citC* and *citA*. The transcription start sites of *citC* and *citA* were determined by 5'-RACE PCR using total RNA isolated from ExPEC XM with citrate under anaerobic conditions. Italic and bold, start codon; bold and underlined, transcription start sites; shaded, -35 and -10 regions; underlined (solid), ArcA1 and ArcA2 binding sites; underlined (dashed), Fnr binding site; the endpoints of fragments used for the construction of the *citC* and *citA* promoter-*lacZ* fusions are marked with arrows with closed and open arrowheads, respectively. The fragments used in the gel shift assays of ArcA are marked by brackets and are labeled with "f" and the number of the fragment. The same predicted ArcA or Fnr sites were assayed once for DNA binding.

Fig. 2 Deletion of *arcA* reduces the expression of the *citCDEFXGT* and *citAB*. (A) Expression levels of *citC* were assessed by measuring *β*-galactosidase activity in *citC-lacZ* transcriptional fusion strains grown anaerobically at 37 °C in M9 medium with citrate overnight. (B) qPCR was performed to compare *citA* expression levels in Δ*arcA* mutant and wild type (WT) strains grown in M9 cultures with citrate as sole carbon source under anaerobic conditions. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments. Significant differences are indicated by asterisks (**, $P < 0.01$).

Fig. 3 ArcA directly regulates the expression of CitA/CitB through binding to its promoter region. (A) A representative nonradioactive electrophoretic mobility shift assay (EMSA) of ArcA-His_6 binding to the *citA* promotor region. For both BS_{ArcA1}

and BS_{ArcA2}, Probes with DNA coding regions of a 240bp DNA fragment containing 60 bp upstream, 120bp ArcA1 or ArcA2 binding site, and 60 bp downstream sequence were amplified using PCR, and a control DNA fragment only containing 60 bp upstream and 60 bp downstream, but without the 120bp binding site were constructed. Phosphorylated ArcA could shift the DNA fragments containing either ArcA1 or ArcA2, but not the control fragments. (B) Upper portion of Figure 3B: The expression directed by the *citC* promoter-*lacZ* fusions was determined in the wild-type strain and the Δ*arcA* mutant with plasmid pCJ112 for the expression of *citA*. The positions of the fragments are given relative to that of the transcription start of the *citA* promoter mapping to 129 bp upstream of the *citA* coding region. Binding of ArcA is schematically indicated as in Fig. 1. The bottom half of Figure 3B: Expression levels of *citA* were assessed by measuring *β*-galactosidase activity in the wild type (WT) and *arcA* mutant strains with different plasmids. The *citA* promoter regions containing the potential ArcA binding site (pP_{citA}-*lacZ*) or point mutated binding site ($pP_{citA\text{-}ArcA}$-*lacZ*) were cloned by overlap PCR and inserted into the new constructed plasmid pCJ112. BS_{ArcA1} or BS_{ArcA2}, ArcA1 or ArcA2 binding sites; ΔBS_{ArcA1} or ΔBS_{ArcA2}, substitution of ArcA1 or ArcA2 binding site. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments. Significant differences are indicated by asterisks (**, $P< 0.01$).

Fig. 4 ArcA indirectly affects the expression of operon *citCDEFXGT* through the regulation of CitA/CitB. (A) The expression level of *citC* in the WT, Δ*arcA* mutant strain, Δ*citB* mutant strain, and the mutant strain with double deletions of *arcA* and

citB. (B) Expression levels of *citC* in *arcA* mutation strains with multiple copy number that constitutively expressed *citAB* with P(cat) promoter of BBa_I14033 plasmid (pGEN-Pcm-*citAB*) and a single copy plasmid (pBeloBAC11-*citAB*) that constitutively expressed *citAB* with promoter of BBa_J23116. These *citC-lacZ* transcriptional fusion strains grown anaerobically at 37 °C in M9 medium with citrate overnight. (C) Expression levels of *citC* in *arcA* mutation strains with double complementation by *arcA* and *citAB*. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments. Significant differences are indicated by asterisks (**, P <0.01).

Fig. 5 Fnr regulates *citCDEFXGT* and *citAB*. (A) Expression levels of *citC* were assessed by measuring *β*-galactosidase activity in *citC-lacZ* transcriptional fusion strains grown anaerobically at 37 °C in M9 medium with citrate overnight. (B) qPCR was performed to compare *citA* expression levels in Δ*fnr* mutant and WT strains grown in M9 cultures with citrate as sole carbon source under anaerobic conditions. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments. Significant differences are indicated by asterisks (**, $P <$ 0.01).

Fig. 6 Fnr directly regulates CitA/CitB two-component system. (A) A representative nonradioactive electrophoretic mobility shift assay (EMSA) of $(FnrD154A)_2$-His_6 binding to the original but not the mutant Fnr binding sites of the *citA* promoter region. PCR products of *citA* promoter region with or without binding sites for the Fnr protein were used as probes at 50 ng per reaction mixture. Purified

$(FnrD154A)_2$-His_6 fusion protein was added to each reaction mixture at different concentrations, as indicated. Probes with *ydfZ* promoter region DNA with or without binding sites for the Fnr protein were used as positive controls. Probes with *fliC* DNA coding region were used as negative controls. DNA fragments were stained with SYBR Green. (A) Upper portion of Figure 6B: The expression directed by *citA* promoter-*lacZ* fusions was determined in the wild-type strain and the Δ*fnr* mutant with plasmid pCJ112 for the expression of *citA*. The positions of the fragments are given relative to that of the transcription start of the *citA* promoter mapping to 74 bp upstream of the *citA* coding region. Bottom portion of Figure 6B: Expression levels of *citA* were assessed by measuring *β*-galactosidase activity in WT and *fnr* mutant strains with different plasmids. The *citA* promoter regions with (pP_{citA}-*lacZ*) or without ($pP_{citA\text{-}FNR}$-*lacZ*) Fnr potential binding sites were cloned by overlap PCR and inserted into the newly constructed pCJ112 plasmid. (B) Constitutive expression of *citAB* partly rescued expression of *citC* in the Δ*fnr* mutant strain. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments. Significant differences are indicated by asterisks (**, $P< 0.01$, *, $P<0.05$).

Fig. 7 Fnr indirectly affects the expression of operon *citCDEFXGT* through the regulation of CitA/CitB. (A) The expression level of *citC* in the WT, Δ*fnr* mutant strain, Δ*citB* mutant strain, and the mutant strain with double deletions of *arcA* and *citB*. (B) Expression levels of *citC* in *fnr* mutation strains with multiple copy number that constitutively expressed *citAB* with P(cat) promoter of BBa_I14033 plasmid (pGEN-Pcm-*citAB*) and a single copy plasmid (pBeloBAC11-*citAB*) that

constitutively expressed *citAB* with promoter of BBa_J23116. These *citC-lacZ* transcriptional fusion strains grown anaerobically at 37 °C in M9 medium with citrate overnight. (C) Expression levels of *citC* in *fnr* mutation strains with double complementation by *fnr* and *citAB*. (D) Expression levels of *citC* with constitutive production of CitA-CitB in double deletion of *arcA* and *fnr* strains. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments. Significant differences are indicated by asterisks (**, P< 0.01).

Fig. 8 Upper portion of Figure 8: The expression directed by *citC* promoter-*lacZ* fusions was determined in the wild-type strain and the Δ*fnr* mutant with plasmid pCJ112 for the expression of *citC*. The positions of the fragments are given relative to that of the transcription start of the *citC* promoter mapping to 129 bp upstream of the *citC* coding region. Binding of Fnr is schematically indicated as in Fig. 1. Bottom portion of Figure 8: Expression levels of *citC* were assessed by measuring *β*-galactosidase activity in wild type and *fnr* mutant strains with different plasmids. The *citC* promoter regions with (pP_{citC}-*lacZ*) or without (p$P_{citC\text{-}FNR}$-*lacZ*) FNR potential binding sites were cloned by overlap PCR and inserted into pCJ112 plasmid. BS_{FNR}, Fnr binding site; ΔBS_{FNR}, mutation of Fnr binding site. Data are presented as the mean ± standard deviation of triplicate samples from three independent experiments. Significant differences are indicated by asterisks (**, P< 0.01).

Fig. 9 ArcA and Fnr regulate the utilization of citrate in the avian pathogenic *E. coli* strain XM (A) *In vitro* growth of WT, Δ*arcA* and Δ*citT* mutant strains in M9 medium containing citrate as carbon source under anaerobic conditions was assessed

by monitoring optical density of the cultures. Deletion of *citT* and *arcA* significantly down-regulated the utilization of citrate. (B) Deletion of *citT* and *fnr* significantly down-regulated the utilization of citrate. The experiments were performed at least three times.