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MOLECULAR PATHOGENESIS

CitAB Two-Component System-Regulated Citrate Utilization Contributes to *Vibrio cholerae* Competitiveness with the Gut Microbiota

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ABSTRACT Citrate is a ubiquitous compound and can be utilized by many bacterial species, including enteric pathogens, as a carbon and energy source. Genes involved in citrate utilization have been extensively studied in some enteric bacteria, such as *Klebsiella pneumoniae*; however, their role in pathogenesis is still not clear. In this study, we investigated citrate utilization and regulation in *Vibrio cholerae*, the causative agent of cholera. The putative anaerobic citrate fermentation genes in *V. cholerae*, consisting of *citCDEFXG*, *citS-oadGAB*, and the two-component system (TCS) genes *citAB*, are highly homologous to those in *K. pneumoniae*. Deletion analysis shows that these *cit* genes are essential for *V. cholerae* growth when citrate is the sole carbon source. The expression of *citC* and *citS* operons was dependent on citrate and CitAB, whose transcription was autorepressed and regulated by another TCS regulator, ArcA. In addition, citrate fermentation was under the control of catabolite repression. Mouse colonization experiments showed that *V. cholerae* can utilize citrate *in vivo* using the citrate fermentation pathway and that *V. cholerae* likely needs to compete with other members of the gut microbiota to access citrate in the gut.

KEYWORDS *Vibrio cholerae*, citrate, fermentation, intestinal colonization, two-component regulatory systems

Citrate is widely distributed in all living cells and natural environments. Particularly high concentrations are found in citrus fruits. It is an important chemical used in medicines and food. Citrate can be utilized by bacteria as a carbon and energy source. The molecular mechanisms to utilize citrate in various bacterial species are highly complicated and diversified (1–6). Under aerobic conditions this compound is transported into the cell by citrate transporters and produces ATP to support growth through the tricarboxylic acid cycle (TCA) pathway (3). Some facultative anaerobes, such as *Klebsiella pneumoniae* (1), lactic acid bacteria (7), and *Salmonella enterica* serovar Typhimurium (8), have developed several catabolic pathways to utilize citrate under fermentative conditions. Genes involved in citrate fermentation have been extensively studied in *K. pneumoniae* (9). These genes are located in two divergently transcribed operons, *citCDEFG* and *citS-oadGAB-citAB*, whose expression is modulated by the citrate-sensing CitA/CitB two-component system (TCS) (1, 10) (Fig. 1A). The sensor histidine kinase CitA senses environmental citrate, which leads to the autophosphorylation of a conserved histidine residue (11, 12). The phosphoryl group subsequently is transferred to a conserved aspartate residue on the cognate response regulator CitB (12). Phosphorylated CitB activates the transcription of two *cit* operons: the *citCDEFG* operon, encoding citrate lyase ligase, citrate lyase, and triphosphoribosyl-dephospho-coenzyme A synthase, and

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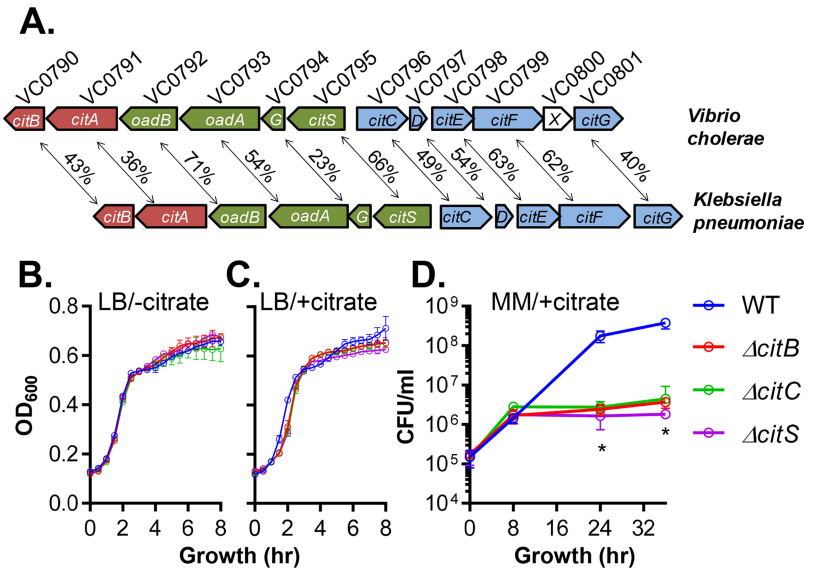

FIG 1 Citrate fermentation genes in *V. cholerae* and their effects on growth. (A) Comparisons of amino acid sequences of citrate-regulated fermentation genes between *V. cholerae* (GenBank accession no. CP028827) and *K. pneumoniae* (CP000647). The amino acid homologies between the conserved proteins are indicated. (B to D) *In vitro* growth. Overnight cultures of the wild type and different *cit* mutants were inoculated 1:100 into fresh LB medium in the absence (B) or presence (C) of 20 mM citrate, statically grown at 37°C. The OD_{600} was measured at the time points indicated. (D) Overnight cultures were spun down, washed twice with M9 minimal medium, and then inoculated at 1:100 into M9 minimal medium (MM) supplemented with 20 mM citrate as the sole carbon source. The cultures were incubated anaerobically, and cell numbers were enumerated by serial dilution and spread onto LB plates at time points indicated. The means of three independent assays are shown, and error bars represent the standard deviations. *, $P < 0.05$ (Student *t* test).

the *citS-oadGAB* operon, which encodes citrate carrier and oxaloacetate decarboxylase (10). Citrate fermentation involves the uptake of citrate by a Na^+-dependent citrate carrier, cleavage of citrate into oxaloacetate and acetate by citrate lyase, and decarboxylation of oxaloacetate to pyruvate by oxaloacetate decarboxylase. Pyruvate can be further converted to end products: acetate, formate, and CO_2 (1, 13). Although it has been shown that mutations in citrate fermentation pathways result in growth defects when citrate is the sole carbon source, the role of citrate fermentation in pathogenic bacteria during infection is not clear. Of note, unlike *K. pneumoniae* and other members of the *Enterobacteriaceae*, *Escherichia coli* is unable to utilize citrate as a sole carbon source. Due to the lack of oxaloacetate decarboxylase, *E. coli* converts citrate to acetate and succinate during growth under anaerobic conditions (2, 14).

Little is known of how *Vibrio cholerae*, a Gram-negative bacterium that causes cholera, utilizes citrate. *V. cholerae* resides in aquatic ecosystems (in both coastal and estuarine environments) and can colonize the upper small intestine in humans. Outbreaks of cholera commonly occur in undeveloped countries due to the ingestion of *V. cholerae*-contaminated water or food (15, 16). After ingestion, *V. cholerae* colonizes the small intestine, where it responds to host signals to express a number of virulence factors, including cholera toxin (17–19), causing vomiting and watery diarrhea. During colonization, *V. cholerae* is confronted by a number of environmental challenges, such as limiting nutrients, reactive oxygen and nitrogen species, and colonization resistance from the gut microbiota (20–24). However, the metabolic pathways that allow *V. cholerae* to colonize the host and outcompete the gut microbiota are poorly understood. In this study, we identified the genes that are involved in citrate fermentation and their regulation in *V. cholerae*. We also demonstrated the role of citrate utilization in *V. cholerae* colonization.

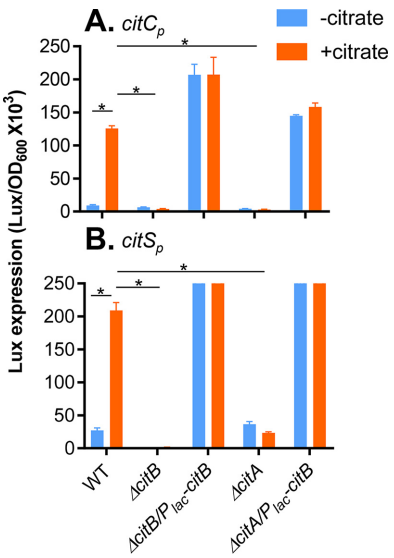

FIG 2 Role of the response regulator CitB in regulation of citrate fermentation. Overnight cultures of wild type (WT; pSRKKm), Δ*citA* (pSRKKm) mutant, and Δ*citB* (pSRK-*citB*^{WT}) mutant containing either P_{citC}-*luxCDABE* (A) or P_{citS}-*luxCDABE* (B) transcriptional fusion plasmids were inoculated 1:100 into M9 minimal medium supplemented with glycerol and 0.05 mM IPTG. When indicated, 20 mM citrate was included. The cultures were incubated statically at 37°C and luminescence was measured at the mid-log phase and normalized to the corresponding OD_{600}. The mean of three independent assays is shown and error bars represent the standard deviation. *, $P < 0.05$ (Student *t* test).

RESULTS AND DISCUSSION

Citrate fermentation pathway is required for *V. cholerae* to grow anaerobically with citrate as a sole carbon source. To define the citrate utilization pathway in *V. cholerae*, we analyzed the genome of *V. cholerae* El Tor strain and found that the amino acid sequences encoded by genes from VC0790 to VC0801 on the large chromosome are highly homologous to the anaerobic citrate fermentation gene products in *K. pneumoniae*, ranging from 23 to 71% identity (Fig. 1A). The genetic organization of this cluster is also similar to that of *K. pneumoniae*, except *citX* (VC0800). In *K. pneumoniae*, *citX* encodes a holo-citrate lyase synthase and is located in a second genomic region involved in citrate fermentation (25). Interestingly, *E. coli citX* is located in the citrate lyase gene cluster, *citCDEFXG* (13), which is the same as in *V. cholerae*.

To determine whether this gene cluster is involved in citrate utilization in *V. cholerae*, we constructed *citS* and *citC* in-frame deletion mutants and a response regulator *citB* knockout mutant. We compared the growth of these mutants with wild type under different conditions. In rich medium such as Luria-Bertani (LB) broth, all *cit* mutants had growth rates similar to that of the wild type in the absence or presence of citrate under aerobic (data not shown) or anaerobic (Fig. 1B and C) culture conditions. When citrate was used as the sole carbon source, however, the *citB*, *citC*, and *citS* mutants grew poorly under anaerobic conditions (Fig. 1D). These data suggest that similar to *K. pneumoniae*, these *cit* genes are required for citrate fermentation in *V. cholerae*.

Citrate-dependent activation of *citC* and *citS* operons is activated by the CitA/CitB two-component system. In many *Enterobacteriaceae*, expression of citrate fermentation genes is regulated by the CitA/CitB TCS in response to external citrate under anaerobic conditions (1, 14). To investigate how citrate fermentation genes are regulated in *V. cholerae*, we constructed *citC*- and *citS-luxCDABE* transcriptional fusion reporter plasmids and introduced them into the wild type and the response regulator *citB* deletion mutant. The expression of *citC* and *citS* was low in the absence of citrate; however, with the addition of citrate, the expression of both *citC* and *citS* was strongly induced (Fig. 2). In the *citB* mutant, *citS* and *citC* expression was completely abolished, indicating that the response regulator CitB is the positive regulator for *cit* gene expression. Complementation of the *citB* mutant in *trans* restored *cit* expression, confirming the direct role

of CitB in the regulation of the *citS* and *citC* operons. However, expression of *citB* from a constitutive P_{lac} promoter on a plasmid rendered *cit* activation in the absence of citrate signals. This may be due to the overproduction of CitB, since it has been reported in other TCSs that artificially overexpressing response regulators sometimes can activate target genes independently of their cognate sensor kinases (1, 26). To confirm this, we also expressed P_{lac}-*citB* in the sensor kinase *citA* mutant and found that the expression of *citC* and *citS* was restored, but was independent of citrate as well (Fig. 2). Taken together, these data suggest that the CitA/CitB TCS activates the expression of the *citC* and *citS* operons and that this activation is dependent on citrate.

Expression of citrate fermentation genes in *V. cholerae* involves multiple transcriptional regulators. Since CitA/CitB play a key role in activation of *V. cholerae* citrate fermentation, we investigated how the *citAB* operon itself is regulated. We first tested whether it is autoregulated. Many TCSs possess positive-feedback loops where the phosphorylated response regulator activates transcription of its own gene and also the gene encoding its partner histidine kinase (27). In the case of PhoP/PhoQ in *Salmonella*, autoregulation can lead to a transient surge in response regulator phosphorylation, which jump-starts virulence (28). When we measured *citA* expression in wild-type *V. cholerae*, we found that the addition of citrate repressed *citA* expression (Fig. 3A). In the *citB* mutant, *citA* expression was significantly induced with or without citrate. These data suggest that CitB negatively regulates *citA* and possibly *citB* itself. Negative autoregulation in TCSs is less common. One report shows that in *Streptococcus pyogenes*, phosphorylated CovR represses transcription of the *covR-covS* operon, and this repression may induce oscillatory behavior (29, 30). The physiological significance of *V. cholerae* CitB repression of *citAB* is not clear, and further investigation is needed.

It has been reported that ArcA, a global transcriptional regulator that facilitates the transition from aerobic to microaerophilic growth in the *Enterobacteriaceae*, positively regulates citrate fermentation in *E. coli* (31). We thus examined whether ArcA regulates *citAB* in *V. cholerae*. Figure 3A shows that in the *arcA* deletion mutant, *citA* expression was significantly higher than in the wild type with or without citrate. These results suggest that under anaerobic conditions, ArcA represses *citAB*, whose expression may lead to activation of the citrate fermentation process. Why *citAB* expression is repressed by ArcA and under autorepression regulation in *V. cholerae* is not clear, but we speculate that multiple layers of fine tuning of CitAB levels may be important for *V. cholerae* growth and survival under certain environmental conditions.

Catabolite repression is commonly found in the process of aerobic and anaerobic metabolism of carbohydrates in the *Enterobacteriaceae*. It has been previously demonstrated that the CRP-cAMP complex regulates citrate fermentation in *K. pneumoniae* (32). To test whether *V. cholerae citAB* expression is under the control of catabolite repression, we compared *citA* expression in minimal medium supplemented with citrate with or without addition of glucose. We found that in the presence of glucose, *citA* expression was repressed (Fig. 3B), suggesting that *citAB* is regulated by catabolite repression. We then examined the expression of the *citC* and *citS* genes in the absence or presence of glucose. We found that expression of both *citC* and *citS* was repressed by glucose (Fig. 3C). We identified consensus CRP binding sites ($TGTGAN_6TCACA$) (33) in both *citA* and *citC/citS* promoter regions. To confirm that CRP-cAMP complex directly regulates *citA* and *citC/citS*, we performed electrophoretic mobility shift assays (EMSAs) using purified recombinant CRP proteins and PCR-amplified *citA* and *citC/citS* promoter DNA. We found that purified CRP-cAMP could retard both *citA* and *citC/citS* promoter DNA in a dose-dependent fashion (Fig. 3D). As controls, heat-inactivated CRP did not bind *citA* or *citC* promoter DNA, and active CRP did not bind to a nonspecific promoter ($recA_p$) (Fig. 3D). These data suggest that the CRP regulation of *citA* and *citC-citS* promoters is specific and direct. Taken together, our results show that genes involved in citrate fermentation are tightly regulated in *V. cholerae* (Fig. 3E): two-component system CitA/CitB senses environmental citrate and activates *citC-citS* operons. CRP-

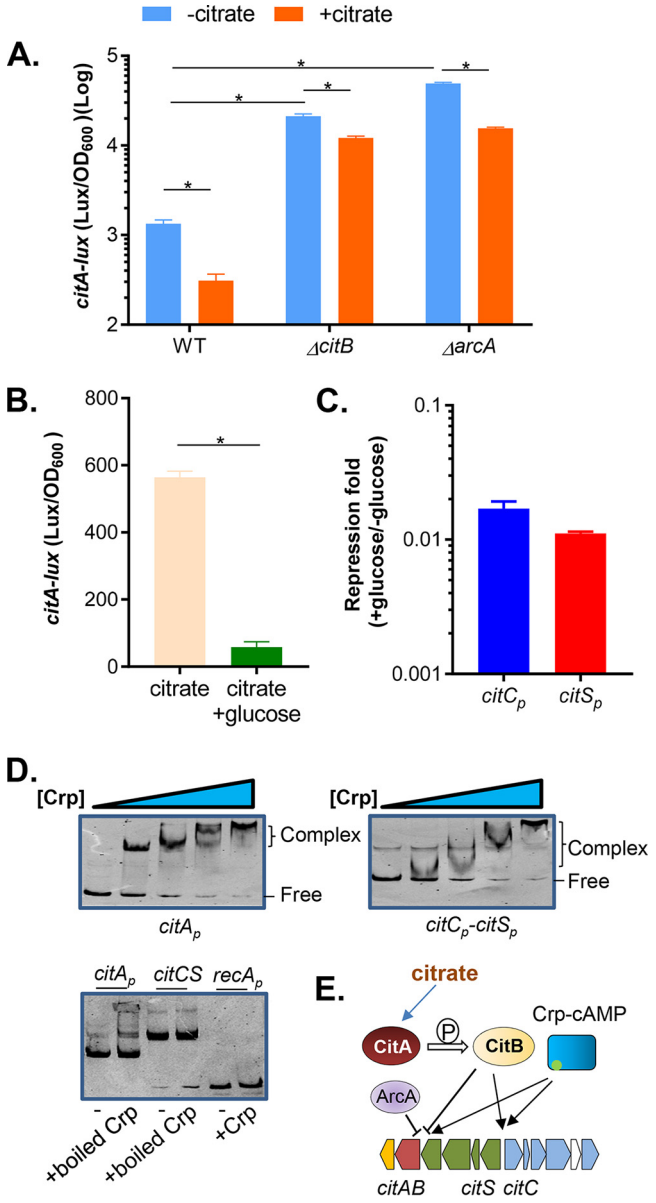

FIG 3 Regulatory components of citrate fermentation. (A) *citA* expression. Overnight cultures of wild-type, Δ*citB*, or Δ*arcA* strains containing the P_{citA}-*luxCDABE* transcriptional fusion plasmid were washed twice with M9 minimal medium and then inoculated at 1:100 into M9 medium containing glycerol in the absence or presence of 20 mM citrate. The cultures were incubated statically at 37°C, and the luminescence was measured at the mid-log phase and normalized to the corresponding OD_{600}. The means of three independent assays are shown, and error bars represent the standard deviations. (B and C) Wild-type strains containing P_{citA}-*luxCDABE* (B), P_{citC}-*luxCDABE*, or P_{citS}-*luxCDABE* (C) transcriptional fusion plasmids were grown in M9 minimal medium containing 20 mM citrate with or without the addition of 0.5% glucose. The cultures were incubated statically at 37°C, and the luminescence was measured at the mid-log phase and normalized to the corresponding OD_{600}. The fold changes were calculated as the ratio of value of glucose (+) over glucose (–). The means of three independent assays are shown, and error bars represent the standard deviations. *, $P < 0.05$ (Student *t* test). (D) EMSA results. Gel-purified DNA fragments containing the *citA* promoter (top left panel) or the *citC-citS* intergenic region (top right panel) were incubated with purified CRP-His_6 (0, 0.1, 0.3, 0.5, and 0.8 μg) for 20 min. The reaction mixes were then electrophoresed on nondenaturing 5% acrylamide gels. The gels were stained and imaged. The bottom gel shows results from control experiments. (E) Working model for the regulation of citrate fermentation in *V. cholerae*. →, activation; ⊣, repression.

cAMP is required to activate *citC-citS*, as well as *citAB*. Meanwhile, both autorepression and ArcA repression are involved in regulating *citAB* expression.

Citrate utilization contributes to *V. cholerae* competitiveness with the gut microbiota. Increasing lines of evidence in recent years suggest that many enteric

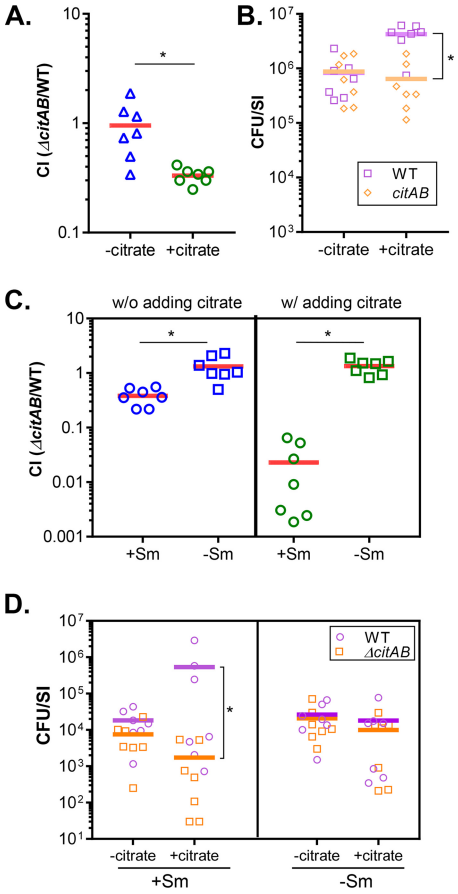

FIG 4 Effects of citrate utilization on *V. cholerae* pathogenesis and colonization. (A and B) Infant mouse colonization. Five-day-old CD-1 infant mice were intragastrically administered a 1:1 mixture of wild type (*lacZ*$^+$) and Δ*citAB* (*lacZ* mutant) strains. At 24 h postinoculation, the small intestines were harvested, and colonizing bacteria were enumerated. (A) The competitive index (CI) was calculated as the ratio of mutant to wild-type colonies normalized to the input ratio of the mutant to the wild type. (B) CFU/small intestine. Horizontal bars represent means from seven mice. *, $P < 0.05$ (Student *t* test). (C and D) Adult mouse colonization assays. Five-week-old CD-1 adult mice were divided into four groups as described in Materials and Methods. +Sm, mice were continuously treated with streptomycin; –Sm, streptomycin was removed from drinking water at 12 h postinoculation. When indicated, 50 mM citrate was included in the drinking water. Approximately 10^8 CFU of each of the wild-type (*lacZ*$^+$) and Δ*citAB* mutant (*lacZ* mutant) in a 1:1 mixture was intragastrically inoculated into mice after overnight streptomycin treatment. At 5 days postinoculation, the small intestines were collected and homogenized. (C) The CI was calculated as the ratio of mutant to wild-type colonies normalized to the input ratio of mutant to wild type. (D) CFU/small intestine. Horizontal bars represent means from seven mice. *, $P < 0.05$ (two-way ANOVA). Differences in the values between the –citrate/–Sm and +citrate/–Sm treatment groups or between the –citrate/+Sm and +citrate/+Sm treatment groups were not significant.

pathogens exploit signals and nutrients derived from both the microbiota and the host to regulate their genetic programs to promote their colonization of the intestinal tract (20, 34). Citrate fermentation has been extensively studied in enteric bacteria, and it has been shown that mutants with mutations in citrate fermentation pathways have growth defects when citrate is the sole carbon source. However, the role of citrate utilization in enteric bacteria during infection is still not clear. To investigate whether the citrate fermentation pathway is involved in *V. cholerae* pathogenesis, we first used an infant mouse competition model (35) to test whether mutation of citrate fermentation affects colonization. We coinoculated *citAB* mutants with wild-type cells and we found that in the 24-h postinoculation period, the *citAB* mutant colonized infant mice as well as the wild type did (Fig. 4A, triangles). When citrate was added intragastrically to infant mice, colonization of the *citAB* mutant was slightly reduced (Fig. 4A, circles). As shown in Fig. 4B, in the presence of citrate, the number of colonized wild-type cells

was higher than that of the *citAB* mutant. This colonization reduction of the *citAB* mutant may not be due to changes in virulence gene expression as when the expression of *tcpA*, the major virulence determinant (36), was measured using *in vitro* virulence-inducing conditions (37), neither citrate nor deletion of *citAB* affected *tcpA* expression (data not shown). These data implied that the suckling mouse gut contains little citrate and that *V. cholerae* may utilize citrate *in vivo* when it is available.

To prolong the colonization time and include possible gut microbiota effects, we next used a modified adult mouse model (38) to examine the effects of citrate utilization on colonization. Adult mice treated with streptomycin prior to and during *V. cholerae* infection have been used to model *in vivo* pathogenesis, such as resistance to reactive oxygen/nitrogen species (18, 21, 22). In order to assess whether the gut microbiota affect citrate availability, we compared colonization of citrate fermentation mutants with the wild-type strain in mice that continued to have streptomycin in their drinking water after inoculation (+Sm mice) and in mice that had streptomycin removed from their drinking water 12 h after *V. cholerae* inoculation (–Sm mice). The gut microbiota are partially restored after streptomycin removal (38). In addition, a recent report (39) demonstrated that in mice, spontaneous postantibiotic recovery can lead to partial reconstitution of the gut mucosal microbiota. Figure 4C shows that the *citAB* mutant displayed colonization defect in +Sm mice (left panel, circles), whereas in –Sm mice, the *citAB* mutant colonized as well as the wild type did (left panel, squares). When citrate was included in the drinking water, the *citAB* mutant was severely attenuated in colonizing the +Sm mice but not the –Sm mice (Fig. 4C, right panel), indicating that citrate may be limited in the regular mouse gut. Of note, the number of *V. cholerae* bacteria that colonized the small intestinal tracts of adult mice was relatively low (Fig. 4D) compared to that in the large intestinal tracts (approximately 10^7CFU/g stool pellets [data not shown]). Whether the colonization of adult mouse small intestine is physiologically relevant requires further investigation. Nevertheless, our data suggest that the ability of *V. cholerae* to utilize citrate provides a colonization advantage when the gut microbiota are lacking, whereas in the presence of the normal gut microbiota, Cit^+ *V. cholerae* no longer has a growth advantage *in vivo*.

To explore the possible mechanisms behind the phenomenon that the *citAB* mutant poorly colonizes the gut lacking commensal bacteria, we examined the impact of the gut microbiota on citrate availability. We first examined citrate concentration in the mouse chow and found that it contained 0.2 to 0.3 mmol/g of citrate (ca. 4% wt/wt). To examine citrate availability in the intestinal tract, we retrieved small intestine tissues from mice continuously treated with streptomycin (+Sm) and from those for which streptomycin was removed 12 h postinoculation (–Sm). We examined citrate concentration in the small intestines. We found that in the gut lacking commensal bacteria, citrate concentration was higher than in the gut with microbiota (Fig. 5A). We then loaded *V. cholerae* cells on the luminal surfaces of the small intestines and examined *citC* expression by quantitative PCR (qPCR) after 1 h of anaerobic incubation. We found that *citC* expression in *V. cholerae* incubated with the +Sm intestines was 6-fold higher than that with the –Sm intestines (Fig. 5B), implying that sufficient citrate signals are present in the +Sm intestines to induce the expression of the *cit* fermentation operons. We thus speculate that the streptomycin-sensitive gut microbiota may be responsible for reducing the amount of citrate available in the small intestines.

As a proof of concept study, we used MacConkey medium to estimate the number of nonfastidious Gram-negative enteric bacteria (such as *E. coli*) from mice with or without citrate supplementation. We found that more enteric bacteria were present in the mouse small and large intestinal tracts when citrate was added in the drinking water (Fig. 5C and D). This implies that enteric bacteria may preferentially utilize citrate in the gut which could impact *V. cholerae* citrate utilization. We then performed *in vitro* experiments to test this hypothesis. We established a model to mimic the intestinal lumen using M9 minimal medium with mucin as a carbon source and used *E. coli* as a representative member of the resident flora. The bacteria were grown anaerobically in this system. We observed that in the absence

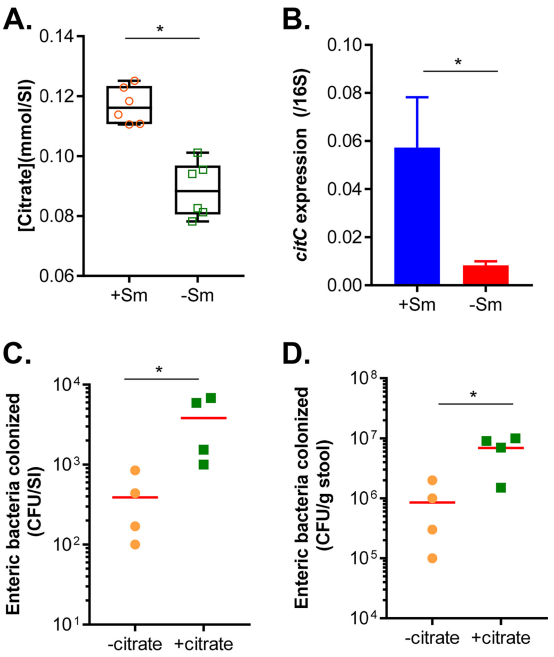

FIG 5 Enteric bacterial effects on citrate availability in small intestine. (A) Presence of citrate in the gut. Small intestines from mice treated with or without streptomycin were isolated, and the citrate concentration was assayed. *, $P < 0.05$ (Student *t* test). (B) *citC* induction. Small intestines from mice treated with or without streptomycin were harvested, and *V. cholerae* cells that were resuspended in M9 minimal medium containing 30 mM glycerol were loaded on the luminal surfaces of the opened small intestines. The samples were then incubated at 37°C for 2 h in an anaerobic jar. Bacterial cells were washed from the intestinal surface, and the total RNA was extracted for qPCR analysis of the *citC* expression (normalized against *V. cholerae*-specific 16S rRNA transcripts). The means of three independent assays are shown, and error bars represent the standard deviations. *, $P < 0.05$ (Student *t* test). (C and D) Citrate promotes enteric bacterial growth *in vivo*. Small intestine luminal contents and fecal pellets were collected from 5-week-old adult mice treated with or without 50 mM citrate in drinking water for 5 days. The enteric bacteria in the luminal contents and fecal pellets were enumerated and estimated on MacConkey plates. Horizontal bars represent means from four mice. *, $P < 0.05$ (Student *t* test).

of citrate, the *citAB* mutant grew as well as the wild type did with or without coincubation with *E. coli* MP1, a mouse isolate (40) (Fig. 6A). When citrate was included in the medium, in the absence of *E. coli*, the wild type grew significantly better than the *citAB* mutant (Fig. 6B, left panel). In contrast, coincubation with *E. coli* resulted in wild-type *V. cholerae* losing its growth advantage over the *citAB* mutant (Fig. 6B, middle panel). To test whether the *E. coli* effects on *V. cholerae* growth in the presence of citrate are due to citrate utilization by *E. coli*, we coincubated *V. cholerae* with the *E. coli dpiA* deletion mutant. *E. coli* DpiA is similar to CitB of *K. pneumoniae* in that it is required to activate citrate fermentation genes in *E. coli* (14). We found that when coincubated with the *E. coli dpiA* mutant in the presence of citrate, wild-type *V. cholerae* grew better than the *citAB* mutant (Fig. 6B, right panel). As for the growth of *E. coli*, we found that in the presence of citrate, the *dpiA* mutant grew more poorly than wild-type MP1, regardless of the presence or absence of *V. cholerae* (Fig. 6C). Taken together, these data suggest that, during colonization of the mucosal surface, *V. cholerae* may prefer citrate to mucin and that other commensal bacteria are likely to compete for citrate.

In this study, we characterized the genes involved in citrate utilization and their regulatory pathways in *V. cholerae*. We found that *V. cholerae* citrate utilization loci are organized similar to *K. pneumoniae* (Fig. 1A). The *V. cholerae* CitA/CitB TCS senses exogenous citrate signals and activates genes for citrate transport and catabolism. *In vitro*, mutations in *cit* genes abolished the ability of *V. cholerae* to grow in minimal medium in which citrate is the sole carbon source (Fig. 1D). During *in vivo* colonization, *V. cholerae* can use citrate as energy source by expressing *cit*

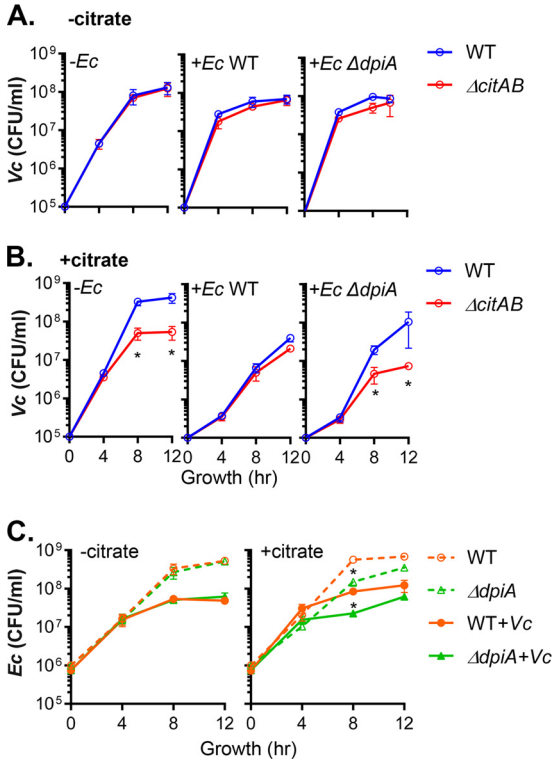

FIG 6 *E. coli* (*Ec*) effects on *V. cholerae* (*Vc*) citrate utilization *in vitro*. (A and B) Overnight cultures of *V. cholerae* wild-type and Δ*citAB* mutant strains were mixed at 1:1 without (left panels) or with wild-type MP1 (middle panels) or with Δ*dpiA* MP1 (right panels). *V. cholerae* and *E. coli* were inoculated at 1:10 into mucin broth supplemented without (A) or with (B) 10 mM citrate. The cultures were grown at 37°C statically. At the time points indicated, samples were withdrawn, and *V. cholerae* CFU were enumerated by serial dilutions and plating on selective LB agar plates. (C) *E. coli* CFU data from the same experiment. The means of three independent assays are shown, and error bars represent the standard deviations. *, $P < 0.05$ (Student *t* test).

genes (Fig. 4 and 5B). While the ability to use citrate was a marginal impediment to initial colonization, as shown in the infant mouse colonization model (Fig. 4A), the adult mouse model data indicate that citrate usage is important for long term carriage. Since citrate is present in food, *citAB* mutants displayed colonization defect in the microbiota-reduced guts of mice without additional citrate (Fig. 4C, left panel), and adding citrate in drinking water made the colonization defect of *citAB* mutants more severe (Fig. 4C, right panel). Given that the cholera toxin-induced secretory diarrhea clears much of the competing flora later in infection (23), it is possible that citrate metabolism becomes much more important later in infection as *cit* mutants are significantly counterselected in mice lacking the flora (Fig. 4C).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* El Tor C6706 (13) was used as the wild-type strain in this study. In-frame deletions were constructed by cloning the regions flanking target genes into the suicide vector pWM91 containing a *sacB* counterselectable marker (41). Double-crossover recombination mutants were screened using sucrose plates and confirmed via PCR. Transcriptional Lux reporters were constructed by cloning the promoter sequences of *citA* (VC0791), *citC* (VC0796), *citS* (VC0795), and *tcpA* (VC0828) (35) into the pBBR-lux vector, which contains a promoterless *luxCDABE* reporter (42). The plasmids overexpressing $citB^{WT}$ were obtained by cloning the complete *citB* sequence into the pSRKKm vector (43). For *in vitro* growth experiments, the bacteria were cultured in LB medium or M9 minimal medium containing indicated carbon sources, unless otherwise noted. For anaerobic growth, an anaerobic jar was used. Alternatively, bacterial cultures were filled up in screw-top vials and incubated statically at 37°C.

Measuring transcription of the *cit* genes *in vitro* and *ex vivo*. Cells from overnight cultures grown in LB medium of the wild type, the *cit* mutants, and the *cit* complemented mutants bearing P_{citA}-*luxCDABE*, P_{citC}-*luxCDABE*, or P_{citS}-*luxCDABE* reporter plasmids were inoculated 1:100 into M9-glycerol medium supplemented with 0.3% hydrolyzed casein with or without 20 mM citrate. IPTG (isopropyl-β-

D-thiogalactopyranoside) was added to the cultures at a final concentration of 0.05 mM. Cultures were incubated statically at 37°C. At the time point indicated, samples were withdrawn. Luminescence was measured and normalized against the corresponding optical density at 600 nm (OD_{600}).

qRT-PCR. Wild-type cells from overnight cultures grown in LB medium were washed twice with M9 minimal medium. Approximately 10^5 cells were loaded onto the luminal surfaces of small intestines isolated from mice treated continuously with streptomycin or from mice for which streptomycin was removed 12 h postinoculation. The samples were then incubated anaerobically for 1 h. Bacterial cells were then collected and total RNA was extracted using TRIzol (Invitrogen). Single-stranded cDNA was synthesized from 0.5 μg of total RNA by using SuperScript III reverse transcriptase (Invitrogen) with random hexamers as a primer in a 20-μl reaction mixture. The resulting cDNA mixture was diluted to 10 μg/μl as a template for the subsequent assays. Reverse transcription-quantitative PCR (qRT-PCR) was carried out by using the CFX96 real-time PCR system (Bio-Rad) and a two-step RT-qPCR kit with SYBR green detection (TaKaRa). To standardize results, the relative abundance of *V. cholerae* 16S gene was used as the internal standard (16s-F, CGGTAATACGGAGGGTGCAA;16s-R, CACCTGCATGCGCTTTACG). The fold change in gene transcription was determined using the comparative threshold cycle (C_T) method (44).

Electrophoretic mobility shift assays. CRP-His_6 fusion proteins were 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 [DTT], 10% glycerol [pH 7.4]) at 4°C overnight with three buffer changes, as described previously (33). For EMSAs, the promoter region of *citA*, the *citC-citS* intergenic fragment, and *recA* were amplified and gel purified. EMSAs were performed by adding increasing amounts of purified CRP-His_6 fusion proteins to the DNA probe (50 ng) in 15 μl of binding buffer [50 mM Tris-HCl (pH 8.3), 0.25 M KCl, 2.5 mM DTT, 5 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, 0.05 mg/ml poly(dI-dC), 2.5 mM EDTA, 1% glycerol, 0.1 mM cyclic AMP]. The reaction mixtures were incubated for 20 min at room temperature. The reaction mixtures were then subjected to electrophoresis on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 180 V for 70 min. The gel was stained with GelRed (Biotium) for 15 min and then imaged using the molecular imager Gel Doc XR system (Bio Rad).

Virulence gene expression *in vitro*. Overnight cultures of wild-type and Δ*citAB* mutant strains containing P_{tcpA}-*luxCDABE* transcriptional fusion plasmids (35) were inoculated 1:10,000 into AKI medium (35) and statically incubated at 37°C. After 4 h, the luminescence was measured and normalized against OD_{600}.

Citrate content assays. Mouse food or intestinal contents were collected and resuspended in double-distilled H_2O. The citrate concentration was then determined by using a citric acid content assay kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

***V. cholerae* colonization in the mouse model.** Animal experiments were performed in accordance with the animal protocols that were approved by the Ethical Committee of Animal Experiments of Nanjing Agricultural University (permit SYXK [Su] 2017-0007).

The infant mouse model was used as previously described (35) with some modifications. Overnight cells of wild-type (*lacZ*$^+$) and Δ*citAB* mutant (*lacZ* mutant) were mixed in a 1:1 ratio, and approximately 10^5 *V. cholerae* cells were intragastrically inoculated into 5-day-old CD-1 suckling mice. Next, 0.1 ml of 20 mM citrate or phosphate-buffered saline was administered intragastrically every 6 h. After a 24-h inoculation, the small intestines were collected and homogenized, and colonizing bacteria were enumerated by serial dilution and plating on LB agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The competitive index (i.e., the output ratio of mutant to wild type over the input ratio of mutant to wild type) was then calculated.

The adult mouse model was used as described previously (38) with the following modifications. Five-week-old CD-1 mice were fed water containing streptomycin (0.5%) and aspartame (0.4%) for 12 h before inoculation with 10^8 cells of a 1:1 mixture of the wild type and the Δ*citAB* mutant. Mice were divided into four groups: (i) streptomycin was continuously administered in the drinking water (+Sm, −citrate); (ii) streptomycin was removed from drinking water 12 h after *V. cholerae* inoculation (−Sm, −citrate); (iii) streptomycin was continuously administered in the drinking water containing 50 mM citrate (+Sm, +citrate); and (iv) streptomycin was removed from drinking water containing citrate at 12 h after *V. cholerae* inoculation (−Sm, +citrate). The small intestines were collected and homogenized at day 5 postinoculation. The competitive index was then calculated.

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