Ferric Uptake Regulator (FurA) is Required for *Acidovorax citrulli* Virulence on Watermelon

Jun Liu,¹ Yanli Tian,¹ Yuqiang Zhao,² Rong Zeng,¹ Baohui Chen,¹ Baishi Hu,^{1,†} and Ron R. Walcott^{3,†}

¹ College of Plant Protection and Key Laboratory of Integrated Management of Crop Diseases and Pests, Ministry of Education, Nanjing Agricultural University, Nanjing 210095, China

² Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (Nanjing Botanical Garden Mem. Sun Yat-Sen), Nanjing 210014, China

³ Department of Plant Pathology, 4315 Miller Plant Sciences, the University of Georgia, Athens, GA 30602, U.S.A.

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ABSTRACT

Acidovorax citrulli is the causal agent of bacterial fruit blotch, a serious threat to commercial watermelon and melon crop production worldwide. Ferric uptake regulator (Fur) is a global transcription factor that affects a number of virulence-related functions in phytopathogenic bacteria; however, the role of *furA* has not been determined for *A. citrulli*. Hence, we constructed an *furA* deletion mutant and a corresponding complement in the background of *A. citrulli* strain xlj12 to investigate the role of the gene in siderophore production, concentration of intracellular Fe²⁺, bacterial sensitivity to hydrogen peroxide, biofilm formation, swimming motility, hypersensitive response induction, and virulence on melon seedlings. The *A. citrulli furA* deletion mutant displayed increased siderophore production, intracellular Fe²⁺ concentration, and increased sensitivity to hydrogen peroxide. In contrast, biofilm formation, swimming motility, and virulence on melon seedlings were significantly reduced in the *furA* mutant. As

expected, complementation of the *furA* deletion mutant restored all phenotypes to wild-type levels. In accordance with the phenotypic results, the expression levels of *bfrA* and *bfrB* that encode bacterioferritin, *sodB* that encodes iron/manganese superoxide dismutase, *fliS* that encodes a flagellar protein, *hrcN* that encodes the type III secretion system (T3SS) ATPase, and *hrcC* that encodes the T3SS outer membrane ring protein were significantly downregulated in the *A. citrulli furA* deletion mutant. In addition, the expression of *feo*-related genes and *feoA* and *feoB* was significantly upregulated in the *furA* mutant. Overall, these results indicated that, in *A. citrulli*, FurA contributes to the regulation of the iron balance system, and affects a variety of virulence-related traits.

Keywords: *Acidovorax citrulli*, bacteriology, ferric uptake regulator, iron, virulence-related factor

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB) of plant species in the Cucurbitaceae family (Burdman and Walcott 2012; Schaad et al. 1978, 2008; Willems et al. 1992). BFB has been observed in many countries, including Australia (O'Brien and Martin 1999), Turkey (Demir 1996), Japan (Shirakawa et al. 2000), Thailand (Schaad et al. 2003), Israel (Burdman et al. 2005), Brazil (Assis et al. 1999), and the United States (Somodi et al. 1991). The bacterium is seedborne and seed transmitted (Hopkins and Thompson 2002), and infected or infested seed represent the most important inoculum sources for BFB outbreaks. Understanding molecular host–pathogen interactions are critical for effective BFB management. Previous research showed that the type III secretion system (T3SS) genes *hrcR* and *hrcC* that encode a type III export apparatus and type III pilus (TFP) proteins, respectively, are critical for pathogenicity (Johnson et al. 2011; Ren et al. 2009). *hrcV*, a gene that encodes another type III export apparatus protein, was also shown to be important for *A. citrulli* pathogenicity and ability to induce a hypersensitive response (HR) on tobacco leaves (Bahar and Burdman 2010). In addition, TFP, and the type VI secretion

system (T6SS) contribute to *A. citrulli* virulence and biofilm formation (Bahar et al. 2009; Tian et al. 2015). Therefore, T3SS, TFP, and T6SS are important for *A. citrulli* virulence.

Environmental signals also play a major role in the survival and virulence of bacterial pathogens (Mekalanos 1992). One important environmental signal is iron, which is essential for the survival and growth of many organisms (Bender et al. 2007; Hentze et al. 2010). However, excessive iron uptake from the environment can lead to iron toxicity (Cha et al. 2008; Quatrini et al. 2005). Therefore, many pathogens must maintain intracellular iron homeostasis, and do so via the ferric uptake regulator (Fur) protein that is encoded by the *fur* gene (Hantke 2001; Pohl et al. 2003). Interestingly, the *fur* gene also regulates key virulence factors involved in quorum sensing, biofilm formation, exopolysaccharide production, swimming motility, and the oxidative stress response in plant-pathogenic bacteria, including *Pectobacterium carotovorum* subsp. *brasiliense* (Tanui et al. 2017), *Xanthomonas vesicatoria* (Liu et al. 2016), and *Pseudomonas syringae* pv. *tabaci* 11528 (Cha et al. 2008). Fur is a central global regulator of iron homeostasis in cells (Hantke 2001; Pasqua et al. 2017). Under iron-poor conditions, Fur binds Fe²⁺ as a biologically active protein, and also binds a conserved DNA sequence called the “*fur* box”. These *fur* boxes are located near promoter regions and thereby inhibit or activate transcription of the target genes (Troxell and Hassan 2013). In addition to regulating the expression of iron-related genes, Fur also regulates the expression of genes related to metabolic pathways, acid resistance, chemotaxis, oxidative stress response, electron transport systems, the tricarboxylic acid cycle, and virulence (Cha et al. 2008; Kitphati et al. 2007; Quatrini et al. 2005; Zhang et al. 2005).

Currently, the role of *fur* in *A. citrulli* biology has not been elucidated. In order to functionally characterize Fur in *A. citrulli*, we identified *furA* (Aave_4555) and *furB* (Aave_2813) from the

[†]Corresponding authors: B. Hu; hbs@njau.edu.cn; and R. R. Walcott; rwalcott@uga.edu

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J. Liu and Y. Tian contributed equally to this research.

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A. citrulli AAC00-1 genome (GenBank accession number NC_008752). Previous studies revealed that the *Mycobacterium tuberculosis* genome includes two members of the Fur family (FurA and FurB): (i) FurA binds Fe²⁺ as a biologically active protein to maintain iron uptake and (ii) FurB requires Zn²⁺ to bind its target sequence in regulated genes, also called the zinc uptake transcriptional repressor (Lucarelli et al. 2008). The *A. citrulli furB* gene has no homology with that of other pathogens. However, the *A. citrulli furA* (Aave_4555) amino acid sequence displayed high homology with similar sequences from important plant-pathogenic bacteria, including *Pectobacterium carotovorum* subsp. *brasiliense*, *Pseudomonas syringae* pv. *tabaci* 11528, *X. campestris* pv. *campestris*, and *P. syringae* pv. *tomato* DC3000, (55, 52, 52, and 52% identity, respectively). Hence, to functionally characterize the role of FurA in *A. citrulli*, the *furA* gene was deleted and the resulting mutant was assessed for siderophore production, oxidative stress response, swimming motility, biofilm formation, HR induction on tobacco leaves, and virulence on melon seedlings.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and vectors used in this study are listed in Table 1. *A. citrulli* was cultured in Luria-Bertani (LB) medium (Sambrook et al. 1989) with or without 1.5% (wt/vol) agar with appropriate antibiotics for 48 h at 28°C with shaking at 220 rpm (in the case of broth). All *Escherichia coli* strains were grown in LB broth or agar with appropriate antibiotics for 24 h at 37°C with shaking at 220 rpm (in the case of broth). Bacterial growth was monitored by measuring the optical density of cell suspensions at 600 nm. *A. citrulli* and *E. coli* strains were stored at -80°C for long-term storage. When used, antibiotics were incorporated into media at the following concentrations: rifamycin (Rif) at 100 µg/ml, kanamycin (Km) at 50 µg/ml, and gentamicin (Gm) at 100 µg/ml.

Construction and complementation of the *furA* deletion mutant of *A. citrulli*. According to the *A. citrulli* AAC00-1 genome (NC-008752), the length of the *furA* gene was 438 bp and it was located at position 5,073,921 to 5,074,358. Based on the AAC00-1 genome sequence, two *furA* flanking regions were generated by PCR using the primer pairs *furA*-F1/R1 and *furA*-F2/R2. The *furA*-1 amplicon (digested with *Xba*I and *Bam*HI) and the *furA*-2 amplicon (digested with *Bam*HI and *Kpn*I) were ligated into pEX18GM that was digested with *Xba*I and *Kpn*I. At the same time, to facilitate the screening of mutants, a Km resistance gene sequence was generated by PCR using the primer pair Km-F/R and the Km fragment (digested with *Bam*HI) was inserted into the center of the *furA*-1 and *furA*-2 fragment to generate the recombinant vector pEX18*furAKm*. This recombinant vector pEX18*furAKm* was transformed into *A. citrulli* xjl12. Transformed colonies were selected on LB plates with 10% (wt/vol) sucrose, Rif (100 mg/ml),

and Km (50 mg/ml). The resulting mutants were confirmed by PCR amplification using primers *furA*-F1 and *furA*-R2 (data not shown). One of the confirmed mutants, named *AcΔfurA*, was selected for further study.

To complement the *furA* deletion mutant of *A. citrulli*, we used the online promoter prediction website (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) to predict the *furA* gene promoter. Based on the *furA* sequence, a pair of specific primers, *furA* comp-F1/R1, was designed to amplify a 672-bp fragment containing the *furA* gene and the predicted promoter site. The amplicon was subcloned into PMD19-T and this fragment was sequenced to ensure that there were no base mutations. The amplicon was then digested with *Xba*I and *Kpn*I and cloned into the expression vector PBRR1MCS-5 (after digestion with *Xba*I and *Kpn*I) (Kovach et al. 1995). The recombinant vector PBRR-*furA* was transferred into *AcΔfurA* by homologous recombination. Transformants were selected on LB agar plates amended with Gm (100 mg/ml) and Km (50 mg/ml). Finally, one complemented strain, named *AcΔfurA*(PBRR-*furA*), was confirmed by PCR and used for further study. All primer sequences use in this study are presented in Supplementary Table S1.

Growth curve assay. *A. citrulli* strains, including the wild-type (WT) strain, the *furA* mutant, and the *furA* complemented strain, were cultured in LB broth at 28°C for 24 h with shaking at 220 rpm. All strains were reinoculated into 25 ml of fresh LB broth and allowed to grow until optical density at 600 nm (OD₆₀₀) = 1.0. The culture was then diluted 1:100 with 25 ml of fresh LB and incubated at 28°C with shaking at 220 rpm. For each strain, OD₆₀₀ was measured at 2-h intervals for 24 h. The experiments were performed in triplicate and repeated three times.

Siderophore production. Siderophore production was determined using the Chrome Azurol S (CAS) agar plate method, as described previously (Louden et al. 2011; Schwyn and Neilands 1987). Briefly, all bacterial cell suspensions were adjusted to OD₆₀₀ = 1.0, and 2 µl of each strain was added to the CAS plate and incubated at 28°C for 3 days. Siderophore production was indicated by the presence of a yellow halo surrounding the bacterial colony and the diameter of the halo was measured. The experiment was conducted in triplicate and repeated three times.

Detection of intracellular iron. Bacterial strains were grown in LB broth for 24 h at 28°C with shaking at 220 rpm. All strains were then diluted 1:100 with 50 ml of fresh LB broth and incubated until OD₆₀₀ = 1.0. Then, 2,2'-dipyridyl was added to the LB broth culture of WT, *AcΔfurA*, and *AcΔfurA*(PBRR-*furA*) strains to a final concentration at 200 µmol/liter and cultures were incubated at 28°C for 1 h. The cell culture was then centrifuged at 6,000 × g for 5 min and resuspended in phosphate-buffered saline buffer (pH 7.2). Finally, cells were centrifuged at 6,000 × g for 5 min. Colonies with cells containing Fe²⁺ appeared red due to the formation of the

TABLE 1. Bacteria and plasmids used in this study

Strains and plasmids	Relevant characteristics ^a	Source
Bacteria strains		
<i>Acidovorax citrulli</i>		
xjl12	Wild-type, Rif ^R	This lab
<i>AcΔfurA</i>	<i>furA</i> mutant strain, containing truncated <i>furA</i> gene and Km cassette, Rif ^R , Km ^R	This study
<i>AcΔfurA</i> (PBRR- <i>furA</i>)	<i>furA</i> complementation strain, containing PBRR- <i>furA</i> , Rif ^R , Km ^R , Gm ^R	This study
<i>Escherichia coli</i>		
DH5α	Φ80 <i>lacZ</i> M15,Δ(<i>lacZ</i> YA-argF)U169. <i>recA</i> I, <i>endA</i> I. <i>thi</i> -1	TaKaRa, Dalian, China
S17-1(λpir)	<i>Apir pro hsdR, recA</i>	Simon et al. (1983)
Plasmids		
pEX18GM	Suicide vector with <i>sacB</i> gene, Gm ^R	Hoang et al. (1998)
pEX18 <i>furAKm</i>	Containing wild-type <i>furA</i> gene replaced by Km cassette, Km ^R , Rif ^R	This study
PMD19-T Simple	'TA' cloning vector	TaKaRa
PBRR1-MCS-5	Broad host range vector, Gm ^R	Kovach et al. (1995)
PBRR- <i>furA</i>	Containing fragment of <i>furA</i> and its promoter region, Gm ^R	This study

^a Rif^R, Gm^R, and Km^R indicate resistance to rifamycin, gentamicin, and kanamycin, respectively.

bipyridyl-ferrous iron complex. The experiment was repeated three times and each treatment was replicated three times per experiment.

H₂O₂ sensitivity assay. To determine the role of *furA* in *A. citrulli* sensitivity to H₂O₂, we measured the diameters of the zones of inhibition for *A. citrulli* WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains in LB agar plates containing different concentrations of H₂O₂. Bacterial strains were cultured in LB broth on an incubator-shaker (220 rpm, 28°C) until OD₆₀₀ = 1.0. Each cell suspension (1 ml) was added to 25 ml of LB agar medium and poured into Petri dishes. After the LB agar medium solidified, a sterilized paper disk (approximately 0.4 cm in diameter) was placed in the center of each plate and 5 μl of different concentrations of H₂O₂ (1, 5, and 10%) was added to the paper disk. The diameters of H₂O₂ inhibition zones were measured after incubation at 28°C for 24 h. This experiment was repeated three times and each treatment was replicated three times per experiment.

Biofilm formation assay. Biofilm formation assays were conducted as described by Bahar et al. (2009), with the following modifications. Briefly, the bacterial strains were grown in LB broth for 24 h at 28°C with shaking at 220 rpm. The cell suspensions were then adjusted to an OD₆₀₀ = 1.0 with fresh LB broth. Then, 40 μl of each bacterial strain was transferred to 4 ml of LB broth and the OD₆₀₀ was measured after 48 h at 28°C. The medium was decanted and the cells were dried at 60°C for 20 min. Subsequently, biofilms were stained with 1% methyl violet for 30 min at room temperature. A ring of violet precipitate on the inner wall of the plate indicated biofilm formation. The biofilm was then dissolved with 5 ml of ethanol and the OD₅₉₀ was measured. The biofilm was quantified by calculating OD₅₉₀/OD₆₀₀ (Kaplan et al. 2004). This experiment was repeated three times and each treatment was replicated three times per experiment.

Swimming motility assay. Swimming motility was assayed as described previously (Herrera et al. 2008; Young et al. 1999). Bacterial strains were grown in LB broth for 24 h at 28°C with shaking at 220 rpm and OD₆₀₀ was adjusted to 0.3. Each bacterial cell suspension (3 μl) was added to the center of 0.3% LB agar plates. The LB agar plates were then incubated at 28°C and the diameters of the swimming halos were measured after 3 days. This experiment was repeated three times and each treatment was replicated three times per experiment.

HR assays: The ability to induce. HR was tested on tobacco (*Nicotiana tabacum*) leaves. Bacterial strains were grown overnight in LB broth, and the bacterial cell concentration was adjusted to OD₆₀₀ = 0.3 with sterilized double-distilled H₂O. Using a 1-ml plastic syringe (Kangdelai), approximately 10 μl of each bacterial suspension was infiltrated into leaves of 4-week-old tobacco plants. Plants were incubated at 28°C and observed visually for HR after 12 h. The experiment was repeated three times and each treatment was replicated three times per experiment.

***A. citrulli* colonization of melon seedling cotyledons and germinating seed.** The role of *furA* in *A. citrulli* seedling colonization was determined by infiltrating melon cotyledons and seed. *A. citrulli* xjl12, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) (approximately 1 × 10³ CFU/ml) were inoculated into 15 melon cotyledons (cultivar Huanghou) using a sterilized syringe (Shoude). Sterilized water was used as a negative control. The inoculated melon seedlings were incubated in a growth chamber with a relative humidity (RH) of 100% at 28°C for 0, 24, 48, 72, and 96 h. Inoculated melon cotyledons were then ground and transferred to a 2-ml centrifuge tube (Shoude) containing 900 μl of sterilized water. Homogenate (100 μl) was diluted 10-, 100- and 1,000-fold and aliquots were spread onto LB plates amended with appropriate antibiotics. Plates were incubated for 24 to 96 h at 28°C and colonies were counted. As described previously (Tian et al. 2015; Walcott et al. 2006), the surface of the seed were disinfected with 70% ethanol for 5 min before inoculation. The front end of the seed was gently opened and each bacterial cell suspension (approximately 1 × 10³ CFU/ml) was inoculated into 15 melon seeds (Huanghou)

with a sterile syringe (Shoude). In addition, 15 melon seeds inoculated with sterilized water served as a negative control. All melon seed were incubated in transparent plastic boxes (Wuhao) on moist blotter paper (Whatman) at 28°C with 100% RH for 24, 48, 72, and 96 h. All melon seed were milled and transferred to a 2-ml centrifuge tube containing 900 μl of sterilized water. Then, 100 μl of seed homogenate was diluted 10-, 100-, and 1,000-fold and spread onto LB agar plates with Rif (100 μg/ml). After incubation for 24 to 96 h at 28°C, colonies were counted. The experiment was repeated three times and bacterial population dynamics were plotted over time.

Role of *furA* in *A. citrulli* virulence. The role of *furA* in *A. citrulli* virulence on melon was evaluated using three inoculation methods, including injection of melon seedling cotyledons, spray inoculation of melon euphylla, and soaking of melon seed. For injection of melon seedling cotyledons, overnight cultures of *A. citrulli* xjl12, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) were harvested by centrifugation and adjusted to a concentration of approximately 1 × 10³ CFU/ml with sterilized double-distilled H₂O. Each strain was inoculated onto 10 melon (Huanghou) cotyledons (on 2-week-old seedlings), which were then incubated at 28°C and observed for disease symptoms at 5 days postinoculation. Second, each strain was cultured to a concentration of approximately 1 × 10⁶ CFU/ml and spray inoculated onto five 2-week-old melon seedlings (Huanghou). Seedlings were then incubated at 100% RH and 28°C. After 10 days, seedlings were visually observed for BFB symptoms. Seedlings spray inoculated with sterilized double-distilled H₂O served as a negative controls. To determine the effect of *furA* on seed-to-seedling transmission of BFB, 25 melon seeds (Huanghou) were soaked in cell suspensions (approximately 1 × 10⁶ CFU/ml) of each strain for 2 h followed by air drying at room temperature. Five seeds were planted per sterilized test cup (Wuhao) and incubated at 28°C with 100% RH. At 12 days after planting, the 25 seedlings were observed for BFB symptoms. Twenty-five seedlings inoculated in a similar manner with double-distilled H₂O were used as negative controls. This experiment was repeated three times.

RNA isolation and quantitative real-time PCR analysis. Bacterial strains were cultured as described above. All cell suspensions were adjusted to OD₆₀₀ = 0.8 to 1.0, and cells were harvested by centrifugation at 1,000 × g for 1 min. Total RNA was extracted using the bacterial RNA kit (OMEGA) and DNase-treated RNA with reverse transcription use the primerScript RT reagent Kit with gDNA Eraser (TaKaRa). The cDNA was diluted 1:30 and used for quantitative real-time (qRT)-PCR with SYBR Premix Ex Taq (TaKaRa) in an ABI PRISM 7500 real-time PCR machine (Applied Biosystems). The *A. citrulli* 16S ribosomal RNA gene was used as an internal control for gene expression. qRT-PCR amplification was carried out according to the following thermal profile: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 59°C for 34 s, and a final dissociation curve analysis step from 60 to 95°C. Experiments were performed in triplicate and three biological replicates were included for each gene. The comparative 2^{-ΔΔCt} method was used to determine the fold change of gene expression.

Statistical analyses. SPSS 17.0 (SPSS Inc.) was used to perform one-way analysis of variance and least significant difference to determine *P* values. A significance level of *P* < 0.05 was used to determine differences in bacterial colonization, siderophore production, H₂O₂ sensitivity, swimming motility, biofilm formation, and RNA expression among WT and *furA* mutants of *A. citrulli*.

RESULTS

FurA is present in the *A. citrulli* AAC00-1 genome. Based on comparisons of amino acid sequences from other plant-pathogenic bacteria, the FurA protein was identified in the *A. citrulli* genome (Fig. 1). The *furA* open reading frame (ORF)

was 438 bp in length and located in the *A. citrulli* AAC00-1 genome at nucleotide position 5,073,921 to 5,074,358 (GenBank accession number NC_008752). BLASTP sequence homology analyses were performed using the NCBI BLAST. The *A. citrulli furA* amino acid sequence displayed 55, 52, 52, and 52% sequence identity with similar sequences from *Pectobacterium carotovorum* subsp. *brasiliense*, *Pseudomonas syringae* pv. *tabaci* 11528, *X. campestris* pv. *campestris*, and *P. syringae* pv. *tomato* DC3000, respectively (Fig. 1). Subsequently, we constructed *A. citrulli furA* mutant (*AcΔfurA*) and complemented *AcΔfurA*(PBBR-*furA*) strains by homologous recombination.

The role of *furA* in in vitro *A. citrulli* growth. *FurA* encodes the Fe²⁺-binding global iron repressor that maintains intracellular iron homeostasis. In the absence of *furA*, cell damage may occur due to increased intracellular iron concentration. Therefore, we compared the growth of *AcΔfurA* to WT and *AcΔfurA*(PBBR-*furA*) strains in low- and high-iron environments. In LB broth, *A. citrulli AcΔfurA* growth was significantly reduced (*P* < 0.05) to approximately 15% of the WT and *AcΔfurA*(PBBR-*furA*) strains (Fig. 2A). In LB broth amended with 100 μM 2'-bipyridine, *A. citrulli AcΔfurA* population growth returned to the WT levels observed in LB broth (Fig. 2B). However, when LB broth was amended with 50 μM FeCl₃, *A. citrulli AcΔfurA* growth was significantly reduced (*P* < 0.05) to approximately 30% of the WT and *AcΔfurA*(PBBR-*furA*) strains (Fig. 2C). These results indicated that iron is a limiting factor for *A. citrulli* growth in LB broth.

Role of *furA* in *A. citrulli* siderophore production. *AcΔfurA* displayed significantly increased siderophore production compared

with *A. citrulli* WT and *AcΔfurA*(PBBR-*furA*). The WT and *AcΔfurA*(PBBR-*furA*) strains produced smaller haloes than those of *AcΔfurA* (Fig. 3A). More specifically, the halo diameters of the WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains were 6.76 ± 0.65, 17.38 ± 1.11, 6.88 ± 0.85 mm, respectively (Fig. 3B). These data suggest that siderophore production is negatively regulated by *FurA*.

***furA* deletion increases intracellular iron in *A. citrulli*.** Iron is critical for growth and survival of most organisms because intracellular Fe²⁺ concentration must be maintained in balance. Cells containing high concentrations of Fe²⁺ will appear red due to the formation of the bipyridyl-ferrous iron complex (Worst et al. 1998). We observed that *AcΔfurA* colonies were redder than those of the WT and *AcΔfurA*(PBBR-*furA*) strains based on the 2'-bipyridine assay. This indicated an increase in iron uptake in the *AcΔfurA* cells (Fig. 4A). Therefore, we measured the expression levels of *feoA* and *feoB* (Fe²⁺ transport system) and *bfrA* and *bfrB* (which encode bacterioferritin) in the WT and *AcΔfurA* strains. Compared with the WT strain, *feoA* and *feoB* genes were upregulated (*P* < 0.05) 9.3- and 4.04-fold, respectively, in *AcΔfurA* (Fig. 4B). In contrast, *bfrA* and *bfrB* were downregulated (*P* < 0.05) 3.45- and 4.76-fold, respectively, in *AcΔfurA* (Fig. 4C). These results suggest that *furA* gene deletion led to increases in intracellular Fe²⁺ concentration.

Deletion of *furA* in *A. citrulli* increased bacterial sensitivity to H₂O₂. H₂O₂ sensitivity was assayed based on the diameter of the inhibition zone (Fig. 5A). The diameter of the inhibition zone for *AcΔfurA* was greater (*P* < 0.05) than that of the WT strain at all

Fig. 1. *Acidovorax citrulli* and other phytopathogenic bacteria *FurA* amino acid sequences. *Pst11528* = *Pseudomonas syringae* pv. *tabaci* 11528, *PstDC3000* = *P. syringae* pv. *tomato* DC3000, *Xcc1470* = *Xanthomonas campestris* pv. *campestris* 1470, and *Pcb1692* = *Pectobacterium carotovorum* subsp. *brasiliense* 1692. Conserved amino acids are shaded in black and homologous residues are shaded in gray.

concentrations of H_2O_2 tested (Fig. 5B). However, the sensitivity of *AcΔfurA*(PBBR-*furA*) to H_2O_2 was substantially restored to WT levels (Fig. 5B). Our results suggested that *furA* played a role in protecting *A. citrulli* from H_2O_2 stress on LB agar plates. Compared with the WT, the expression level of the superoxide dismutase-related gene *sodB* (iron/manganese superoxide dismutases) was significantly ($P < 0.05$) downregulated in *AcΔfurA* (Fig. 5C). These results suggest that the absence of *furA* reduced the oxidative stress tolerance of *A. citrulli*.

Fig. 2. Population dynamics of *Acidovorax citrulli* xjl12 (wild-type [WT]), *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains in Luria-Bertani (LB) broth. OD₆₀₀ = optical density at 600 nm. **A**, Temporal population growth of *A. citrulli* WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains in LB broth. **B**, Temporal population growth of *A. citrulli* WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains in LB broth amended with 100 μ M 2,2'-bipyridine. **C**, Temporal population growth of *A. citrulli* WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains in LB broth amended with 50 μ M $FeCl_3$. WT = wild-type *A. citrulli* strain xjl12, *AcΔfurA* = *furA* gene deletion mutant of *A. citrulli*, and *AcΔfurA*(PBBR-*furA*) = complemented strain of *AcΔfurA*. Each treatment was replicated three times and the experiment was repeated three times with similar results. Dots represent the means of three replicates and error bars indicate standard deviations. Statistical tests were performed at 16, 20, and 24 h, respectively. Statistically significant differences were determined by one-way analysis of variance and P values less than 0.05 (an asterisk [*]) indicates $P < 0.05$, versus the WT *A. citrulli*.

The role of *furA* in *A. citrulli* biofilm formation. We investigated the role of *furA* in *A. citrulli* biofilm formation. *AcΔfurA* produced significantly less biofilm than the WT and *AcΔfurA*(PBBR-*furA*) *A. citrulli* strains (Fig. 6A). OD₅₉₀ and OD₆₀₀ values for stained biofilms from WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains were 0.336 ± 0.012 , 0.167 ± 0.005 , and 0.323 ± 0.013 , respectively (Fig. 6B). These results suggest that *furA* contributes to *A. citrulli* biofilm formation.

***furA* contributes to *A. citrulli* swimming motility.** The diameters of the swimming colonies of *A. citrulli* WT and *AcΔfurA*(PBBR-*furA*) strains were larger than those of *AcΔfurA* (Fig. 7A). The diameters of the WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains were 1.55 ± 0.13 , 0.95 ± 0.09 , and 1.48 ± 0.18 cm, respectively (Fig. 7B). The qRT-PCR assay showed that *fliS* (flagellar protein) expression was significantly downregulated ($P < 0.05$) in the *AcΔfurA* strain relative to the WT and *AcΔfurA*(PBBR-*furA*) strains (Fig. 7C). These results suggest that *furA* contributes to *A. citrulli* swimming motility.

***furA* does not affect *A. citrulli* HR induction but regulates T3SS gene expression.** HR induction on tobacco leaves by *AcΔfurA* was no different than that induced by the WT and *AcΔfurA*(PBBR-*furA*) strains at 10 h postinoculation (hpi). (Fig. 8A).

Fig. 3. Role of the ferric uptake regulator (*furA*) gene in *Acidovorax citrulli* siderophore production. The Chrome Azurol S (CAS) plate assay was used to compare siderophore production of *A. citrulli* *AcΔfurA* (*furA* gene deletion mutant) *AcΔfurA*(PBBR-*furA*) (complement of *AcΔfurA*), and xjl12 (wild-type) strains. **A**, Areas of haloes around the colonies represent the amount of siderophore production. WT = wild-type *A. citrulli* xjl12, *AcΔfurA* = *A. citrulli furA* mutant, and *AcΔfurA*(PBBR-*furA*) = *A. citrulli furA* deletion complement strain. **B**, Comparison of halo diameters produced by each strain on CAS plates. Experiments were repeated three times (with three replicates per treatment) and yielded similar results. Data shown represent the means $\pm$ standard deviations (error bars). Different lowercase letters indicate significant differences in halo size produced by the different strains. Statistically significant differences were determined by the one-way analysis of variance and $P < 0.05$.

However, qRT-PCR analysis revealed that the expression of *hrcN* (type III ATPase) and *hrcC* (TFP protein) genes were down-regulated ($P < 0.05$) in *AcΔfurA* relative to the WT strain (Fig. 8B). These results indicate that FurA affects the expression of some T3SS-related genes.

***furA* is required for *A. citrulli* virulence on melon seedlings.** To study the effect of *furA* on *A. citrulli* virulence on melon seedlings, we use three virulence assays, including injection into melon seedling cotyledons, spray inoculation on melon euphylla, and seed-to-seedling transmission. In all virulence assays, *AcΔfurA* did not induce symptoms on melon cotyledons, whereas *AcΔfurA*(PBBR-*furA*) developed symptoms similar to those induced by the WT strain but to a lesser degree (Fig. 9). These studies indicate that *furA* contributes to *A. citrulli* virulence on melon seedlings.

The role of *furA* in *A. citrulli* colonization of melon seedling cotyledons and germinating seed. To determine the role of *furA* in *A. citrulli* colonization of melon cotyledons and

germinating seed, WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains were syringe inoculated into the cotyledons of melon seedlings. Bacterial populations in seedling tissues were assessed at 0, 24, 48, 72, and 96 hpi. The average cell populations of *A. citrulli* WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains were approximately 1.71×10^5 , 1.52×10^4 , and 1.35×10^5 CFU/g, respectively, by 48 hpi (Fig. 10A). By 96 hpi, the average cell populations of WT, *AcΔfurA*,

Fig. 4. Iron uptake analysis of *Acidovorax citrulli* xjl12 (wild-type [WT]), *AcΔfurA* (*furA* gene deletion mutant), and *AcΔfurA*(PBBR-*furA*) (complement of *AcΔfurA*) strains. Colonies containing more Fe^{2+} appeared darker in the presence of 2'-bipyridine due to the formation of the bipyridyl-ferrous iron complex. **A**, Iron uptake analysis of the WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains. **B**, Expression levels of *feoA* and *feoB* in *AcΔfurA* and WT strains. **C**, Expression levels of *bfrA* and *bfrB* that encode Bacterioferritin in *AcΔfurA* and WT strains. Experiments were performed in triplicate and were repeated three times with similar results. Data represent the means of three replicates $\pm$ standard deviations (error bars). Different lowercase letters indicate a significant difference between the strains. Statistically significant differences were determined by the one-way analysis of variance and $P < 0.05$.

Fig. 5. Sensitivity of wild-type (WT), *AcΔfurA* (*furA* gene deletion mutant), and *AcΔfurA*(PBBR-*furA*) (complement of *AcΔfurA*) strains of *Acidovorax citrulli* xjl12 to H_2O_2 . **A**, Each strain (5 μl) was added to Luria-Bertani (LB) agar plates with 1, 5 and 10% H_2O_2 . The phenotype was observed after incubation at 28°C for 24 h. **B**, Inhibition zone diameters of each strain inoculated onto LB agar plates containing 1, 5, and 10% H_2O_2 . **C**, Expression level of *sodB* in WT and *AcΔfurA* strains of *A. citrulli*. The experiment was repeated three times. WT = WT strain of *A. citrulli*, xjl12, *AcΔfurA* = *furA* gene deletion mutant of *A. citrulli*, and *AcΔfurA*(PBBR-*furA*) = complemented strain of *AcΔfurA*. Experiments were performed in triplicate and were repeated three times with similar results. Data represent the means of three replicates $\pm$ standard deviations (error bars). Different lowercase letters indicate a significant difference between treatments. Statistically significant differences were determined by the one-way analysis of variance and $P < 0.05$.

and *AcΔfurA*(PBBR-*furA*) strains were approximately 9.75×10^9 , 9.42×10^4 , and 7.35×10^8 CFU/g, respectively (Fig. 10A). *AcΔfurA* population growth was significantly ($P < 0.05$) less than that of the WT strain in melon cotyledons. As expected, population growth of *AcΔfurA*(PBBR-*furA*) was not significantly different from that of the WT strain ($P > 0.05$).

A. citrulli WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains were inoculated into melon seed, and the seed were subsequently planted under conditions conducive for BFB development. Bacterial populations were quantified on the emerging melon seedling tissues at 0, 24, 48, 72, and 96 h after planting. The average cell populations of WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) were approximately 2.41×10^6 , 2.16×10^5 , and 2.16×10^6 CFU/g, respectively, at 48 h after planting (Fig. 10B). The average populations of WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) were approximately 1.21×10^9 , 6.15×10^5 , and 2.64×10^8 CFU/g, respectively, by 96 h after planting (Fig. 10B). The ability of *AcΔfurA* to colonize germinating melon seedlings was significantly less than that of the WT strain ($P < 0.05$). As expected, *AcΔfurA*(PBBR-*furA*) colonized germinating melon seed at similar levels as the WT strain ($P > 0.05$).

DISCUSSION

Fur plays an important role in many plant-pathogenic bacteria, including *X. vesicatoria* (Liu et al. 2016), *Pectobacterium carotovorum* subsp. *brasiliense* (Tanui et al. 2017), *Pseudomonas syringae* pv. *tabaci* 11528 (Cha et al. 2008), and *Edwardsiella tarda*

Fig. 6. Role of *furA* in *Acidovorax citrulli* biofilm formation in Luria-Bertani broth. **A**, Qualitative analysis of biofilm production of *A. citrulli* wild-type (WT), *AcΔfurA* (*furA* gene deletion mutant), and *AcΔfurA*(PBBR-*furA*) (complement of *AcΔfurA*) strains; biofilm production is indicated by a ring of dark precipitate on the inner wall of culture plate wells. **B**, Quantitative analysis of biofilm production by WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains of *A. citrulli*. Optical density at 600 nm (OD₆₀₀) was measured for 24-h liquid cultures of each strain in 12-plate wells and biofilm production was measured after staining with crystal violet using a spectrophotometer at OD₅₉₀/OD₆₀₀. WT = WT strain of *A. citrulli*, xjl12, *AcΔfurA* = *furA* gene deletion mutant of *A. citrulli*, *AcΔfurA*(PBBR-*furA*) = complemented strain of *AcΔfurA*, and NC = negative control. Experiments were performed in triplicate and were repeated three times with similar results. Data represent the means of three replicates $\pm$ standard deviations (error bars). Different lowercase letters indicate a significant difference between treatments. Statistically significant differences were determined by the one-way analysis of variance and $P < 0.05$.

Fig. 7. Role of *furA* in swimming motility of *Acidovorax citrulli*. **A**, Qualitative analysis of swimming motility of *A. citrulli* wild-type (WT), *AcΔfurA* (*furA* gene deletion mutant), and *AcΔfurA*(PBBR-*furA*) (complement of *AcΔfurA*) strains on 0.3% Luria-Bertani (LB) agar plates at 28°C for 3 days. **B**, Diameters of the haloes formed by each strain on LB agar measured at 3 days postinoculation. **C**, Expression level of *fliS* (that encodes flagellar protein) for *A. citrulli* WT and *AcΔfurA* (*furA* gene deletion mutant) strains. Each experiment was repeated three times. WT = wild-type strain of *A. citrulli*, xjl12, *AcΔfurA* = *furA* gene deletion mutant of *A. citrulli*, and *AcΔfurA*(PBBR-*furA*) = complemented strain of *AcΔfurA*. Different lowercase letters indicate a significant difference between the *furA* mutant, WT strain, and *furA* complemented strains. Experiments were performed in triplicate and repeated three times with similar results. Data shown represent the means of three replicates $\pm$ standard deviations (error bars). Different lowercase letters indicate a significant difference between strains. Statistically significant differences were determined by the one-way analysis of variance and $P < 0.05$.

(Wang et al. 2008). However, to date, there have been no reports regarding the role of *furA* in *A. citrulli*, an important threat to global cucurbit production. In this study, we functionally characterized the role of *furA* in *A. citrulli* strain xjl12 by constructing an *furA* mutant (*AcΔfurA*) and a corresponding *furA*-complemented strain (*AcΔfurA*(PBBR-*furA*)).

It is well established that iron plays a major role in the development of plant diseases (Cha et al. 2008; Jittawuttipoka et al. 2010). Specifically, iron is required for normal pathogen growth, and bacteria must regulate or maintain an intracellular iron balance (Jittawuttipoka et al. 2010). In many bacteria, iron uptake and storage affects a range of virulence factors (Cha et al. 2008; Kitphati et al. 2007). Because *fur* has been reported to control iron uptake and storage (Butcher et al. 2011; McHugh et al. 2003), it

likely plays an important role in host–pathogen interactions (Ratledge and Dover 2000).

We observed that *A. citrulli* *AcΔfurA* grew more slowly than the WT and *AcΔfurA*(PBBR-*furA*) strains in LB broth (Fig. 2A). This may be due to increased iron uptake leading to toxic levels of intracellular Fe²⁺, as described for other pathogens (Furano and Campagnari 2003; Jacobsen et al. 2005; Jittawuttipoka et al. 2010). We hypothesized that reduced iron levels in LB broth would recover the growth rate of *AcΔfurA* almost to WT levels. As expected, adding 100 μM 2′2-bipyridine to LB broth recovered the growth rate of *AcΔfurA* to WT levels (Fig. 2B). Meanwhile, adding iron (50 μM FeCl₃) to LB broth reduced *AcΔfurA* relative to the WT strain (Fig. 2C). These results suggest that the growth defect of *AcΔfurA* might be due to high intracellular iron levels.

Siderophore production is an important strategy by which bacteria acquire ferric iron in iron-poor environments (Touati 2000). As expected, the *A. citrulli* *furA* mutant showed increased siderophore production, resulting in the accumulation of intracellular iron (Jittawuttipoka et al. 2010; Tanui et al. 2017). In the current study, *AcΔfurA* increased siderophore production relative to the WT and *AcΔfurA*(PBBR-*furA*) strains. This finding demonstrated that the *furA* gene negatively regulates siderophore production.

Ferrous iron is considered to be the preferred form of iron for bacteria because of its high solubility relative to ferric iron (Kim et al. 2012). Approximately 80% of Gram-negative bacteria such as *Salmonella enterica*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* utilize a unique Feo uptake system for Fe²⁺ (Kim et al. 2013; Petermann et al. 2010; Seyedmohammad et al. 2014; Weaver et al. 2013). The *P. aeruginosa* PAO1 and *P. syringae* pv. *tomato* DC3000 *bfrA* and *bfrB* genes encode bacterioferritin that oxidizes the Fe²⁺ away from dioxygen, hydrogen peroxide, and superoxide to protect cells from toxic reactive oxygen species (ROS) (Butcher et al. 2011; Smith 2004). Therefore, when the intracellular Fe²⁺ concentration is high, ferritin is passed through the bacterioferritin ferrous iron redox center and oxidizes excess ferrous iron to nontoxic ferric iron (Arosio and Levi 2002; Carrondo 2003; Levi et al. 2001). In this study, we showed that the concentration of intracellular Fe²⁺ in *AcΔfurA* was significantly higher than that of the WT and *AcΔfurA*(PBBR-*furA*) strains (Fig. 4A). Using a qRT-PCR assay, we demonstrated that the Fe²⁺ transport system (*feoA* and *feoB*) was upregulated in *AcΔfurA* (Fig. 4B). In addition, we found *A. citrulli* *bfrA* and *bfrB* genes that encoded bacterioferritin homologs to BfrA and BfrB of *P. syringae* pv. *tomato* DC3000. These genes were significantly downregulated in *AcΔfurA* (Fig. 4C). Based on these results, we conclude that *AcΔfurA* displays increased levels of siderophore production and Fe²⁺ uptake and transport that led to the accumulation of intracellular iron. Meanwhile, because excess Fe²⁺ cannot be efficiently converted into nontoxic ferric iron due to a significant decrease in *bfrA* and *bfrB* expression in *AcΔfurA*, we concluded that the excess intracellular Fe²⁺ that could not be eliminated from the cell accumulated to toxic levels. This may also explain why the growth rate of the *AcΔfurA* was slower than that of *A. citrulli* xjl12 and *AcΔfurA*(PBBR-*furA*).

Excess Fe²⁺ can result in highly toxic hydroxyl radicals that damage cells through the Fenton reaction (Jeon et al. 2008; Rajasekaran et al. 2010). Hostile environmental conditions are encountered when bacteria infect leaf tissues. These conditions include ROS that limit bacterial colonization (Fones and Preston 2013). It was previously reported that the *A. tumefaciens* *rirA* (rhizobial iron regulator) mutant increased siderophore production but, due to excessive intracellular iron content, experienced increased ROS sensitivity and reduced expression of pathogenicity-related genes (Ngok-Ngam et al. 2009). In the current study, *AcΔfurA* displayed increased sensitivity to H₂O₂ (Fig. 5A) and significant downregulation of *sodB* (Fig. 5C). We hypothesized that accumulation of high concentrations of iron in

Fig. 8. Effect of *furA* on tobacco hypersensitive response (HR) induction by *Acidovorax citrulli* strains; 1 = wild-type (WT), 2 = double-distilled H₂O, 3 = *AcΔfurA* (*furA* gene deletion mutant), and 4 = *AcΔfurA*(PBBR-*furA*) (complemented strain of *AcΔfurA*). Approximately 10 μl of bacterial suspension (optical density at 600 nm = 0.3) was infiltrated at each inoculation site on a tobacco leaf and the plant was incubated at 28°C. The HR was observed at 10 h postinoculation (hpi). **A**, Tobacco HR induction 10 hpi and **B**, expression level of *hrcN* and *hrcC* genes in *A. citrulli* WT and *AcΔfurA* strains by the 2^{-ΔΔCt} method. Experiments were conducted in triplicate and repeated three times with similar results. Data shown represent the means of three replicates ± standard deviations. Error bars indicate standard deviations. Different lowercase letters indicate a significant difference between strains. Statistically significant differences were determined by the one-way analysis of variance and *P* < 0.05.

AcΔfurA cells resulted in the generation of ROS and reduced the oxidative stress response during plant infection. Therefore, the FurA protein is critical for maintaining the balance of intracellular iron in *A. citrulli*.

AcΔfurA displayed reductions in virulence and in planta growth. Although *AcΔfurA* is impaired in in vitro growth, the difference in growth between *AcΔfurA* and the WT in planta was more pronounced. Therefore, there might be two reasons for decreased virulence and in vivo growth in *AcΔfurA*. On the one hand, ferrous iron accumulates in *AcΔfurA* cells and reduces the ability to resist

oxidative stress during plant infection. On the other hand, reductions in virulence and in planta growth in *AcΔfurA* may be subject to other virulence factors such as biofilm, swimming motility, and T3SS.

For many pathogenic bacteria, disease development is positively correlated with biofilm formation (Luo et al. 2015). Bacterial biofilm is important for attachment to biological and nonbiological surfaces (Bahar et al. 2009). Previous studies have shown that *fur* mutants of *X. vesicatoria* and *Pectobacterium carotovorum* subsp. *brasiliense* resulted in reduced biofilm formation. We observed similar results for *AcΔfurA*; the biofilm formation ability of *AcΔfurA* was significantly reduced, suggesting that *furA* might be involved in this process. It is currently not clear how FurA regulates biofilm formation-related genes and this needs to be studied further.

Motility is another important virulence factor in plant- and animal-pathogenic bacteria such as *Agrobacterium tumefaciens* (Chesnokova et al. 1997), *Pseudomonas syringae* pv. *tabaci* (Ichinose et al. 2003), *Helicobacter pylori* (Ottemann and Lowenthal 2002), and *V. anguillarum* (O'Toole et al. 1996). It enables bacterial cells to swim in a nutrient-rich environment or to avoid environmental stress (Fenchel 2002; Hossain et al. 2005). Previous studies have shown Fur-dependent positive regulation of several genes involved in swimming and chemotaxis in *H. pylori* G27, including *flgL*, *fliY*, *flgK*, *cheA*, and *flaB* (Danielli et al. 2006). Nevertheless, the influence of FurA on *A. citrulli* motility was not previously studied. We observed that swimming motility of *AcΔfurA* was significantly reduced relative to the WT strain. As further evidence, *fliS* was downregulated in *AcΔfurA*, suggesting that FurA positively regulates *A. citrulli* swimming motility. However, the regulatory relationship between *furA* and motility-related genes is still unclear. *A. citrulli furA* will be further studied indirectly or directly to understand its role in the regulation of motility-related genes. In addition, N-acyl homoserine lactone (AHL) is a cell-to-cell communication signal that is synthesized by specific enzymes and can be detected by specific sensors (Waters and Bassler 2005). Quorum sensing (QS) is involved in the regulation of many biological functions, including bioluminescence, swarming motility, and biofilm formation (Johnson and Walcott 2013). *Δfur* mutants have been reported to exhibit a decrease in the synthesis of AHL (Cha et al. 2008; Tanui et al. 2017). Therefore, we guessed that *furA* may positively regulate the synthesis of AHL in *A. citrulli*. It was previously reported that AHL-mediated QS comprised two genes, including *aacI* (*luxI* homolog) and *aacR* (*luxR* homolog), in *A. citrulli* (Johnson and Walcott 2013). However, the relationship between *furA* and QS has not been researched in the current study. The effects of *furA* on AHL synthesis and the regulation of AHL-related genes *aacI* and *aacR* will be further studied in the future.

Fig. 9. Role of *furA* in *Acidovorax citrulli* virulence on melon seedlings. **A**, Melon seedling cotyledons inoculated by injection with *A. citrulli* wild-type (WT), *AcΔfurA* (*furA* gene deletion mutant), and *AcΔfurA*(PBBR-*furA*) (complement of *AcΔfurA*) (approximately 1×10^3 CFU/ml) strains, with double-distilled H₂O (ddH₂O) as a negative control (NC). Seedlings were observed for bacterial fruit blotch symptoms at 5 days postinoculation. **B**, Melon seedlings euphylla spray inoculated with *A. citrulli* WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) (approximately 1×10^6 CFU/ml) strains and ddH₂O as NC and observed for bacterial fruit blotch symptoms 10 days postinoculation. **C**, Melon seedlings that developed 12 days after planting from seed that were soaked in inoculum (approximately 1×10^6 CFU/ml) of *A. citrulli* WT, *AcΔfurA*, and *AcΔfurA*(PBBR-*furA*) strains, and ddH₂O as NC. Experiments were repeated three times with similar results. WT = wild-type strain of *A. citrulli*, xjl12, NC = negative control (ddH₂O), *AcΔfurA* = *furA* gene deletion mutant of *A. citrulli*, and *AcΔfurA*(PBBR-*furA*) = complemented strain of *AcΔfurA*.

HilA is the central regulator of the *S. enterica* T3SS, and the *fur* gene of *S. enterica* affects T3SS by controlling *hilD* to regulate the expression of *hila* (Ellermeier and Schlauch 2008). The mechanism by which *fur* regulates the T3SS is different in *Shigella* spp. In *Shigella* spp., Fur regulates the small RNA, RyhB, which is involved in the regulation of the T3SS at the transcriptional level (Murphy and Payne 2007). Additionally, the cellular iron concentration of *P. syringae* was related to the expression of T3SS-related genes (Bronstein et al. 2008; Kim et al. 2009, 2010). Therefore, we hypothesize that Fur may affect the expression of T3SS-related genes in *A. citrulli*. The *hrcN* (type III ATPase) and *hrcC* (TFP protein) genes are T3SS genes that affect *A. citrulli* virulence. In this study, *hrcN* and *hrcC* were downregulated in the *AcΔfurA* relative to the WT strain, suggesting that *furA* positively regulates the expression of *hrcN* and *hrcC*. However, there was no difference in HR at 10 hpi with *AcΔfurA* and WT strains. We surmise that *AcΔfurA* does not fully inhibit the expression of T3SS-related genes but still induces an HR. Therefore, further investigation is needed to explore the relationship between *furA* and the T3SS.

Overall, we observed that FurA was involved in the regulation of a variety of *A. citrulli* virulence factors, including swimming motility, biofilm formation, T3SS-related genes, oxidative stress response, and siderophore production. Unsurprisingly, *AcΔfurA* displayed reduced virulence and reduced ability to colonize melon seedlings.

Fig. 10. Effect of *furA* on *Acidovorax citrulli* colonization of melon cotyledons and seed. **A**, Bacterial suspensions (approximately 1×10^3 CFU/ml) of each strain (WT = wild-type *A. citrulli* xj112, NC = negative control, double-distilled H₂O, *AcΔfurA* = *furA* gene deletion mutant of *A. citrulli*, and *AcΔfurA*(PBBR-*furA*) = complemented strain of *AcΔfurA*) were injected into cotyledons of melon seedlings and populations were quantified at 0, 24, 48, 72, and 96 h postinoculation. The experiment was repeated three times. **B**, Melon seed were inoculated with *A. citrulli* strains (approximately 1×10^3 CFU/ml) planted under conditions conducive for bacterial fruit blotch development and bacterial populations on emerging seedling tissues were quantified at 0, 24, 48, 72, and 96 h after planting. Experiments were repeated three times with similar results. Dots represent the means of three replicates. Error bars represent standard deviations. Statistically significant differences were determined by the one-way analysis of variance and $P < 0.05$.

In the future, we will investigate whether motility and antioxidative stress-related genes are directly regulated by FurA or indirectly regulated by increased levels of intracellular iron in *A. citrulli*.

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