INTRODUCTION

Transcriptional regulators of the LuxR family have been found in a wide range of bacteria, where they regulate various bacterial functions, including plasmid conjugation, antibiotic production, cell motility, and sporulation (Pappas and Winans, 2003; Kuwana et al., 2004; Qian et al., 2013; Singer et al., 2013). These regulators are approximately 250 amino acids in size with a helix-turn-helix (HTH) DNA-binding domain at the C-terminus, and a variable domain at the N-terminus that interacts with signalling substances, for example quorum-sensing, two-component signalling transduction, and other signals (Patankar and González, 2009; Santos et al., 2012). LuxR is best known for its role in quorum sensing (QS), which bacteria use to communicate with each other, and often pairs with LuxI, an autoinducer synthase protein. In addition to QS, LuxR-type proteins are also involved in other cellular signalling pathways. Several
luxR genes not associated with a luxI gene are responsible for modulating the expression of virulence factors, biofilm formation, and host immune responses (Fusco et al., 2001; Santos et al., 2012). In Salmonella enterica, a LuxR-type transcriptional regulator, RflM, harbours a conserved HTH domain at the C-terminal, whereas it does not contain any domain at the N-terminal. RflM negatively regulates the expression of flhDC, which encodes FlhD2C2, the flagellar master regulatory complex, which, in turn, affects the flagellar assembly (Singer et al., 2013).

Bacterial fruit blotch is a seedborne disease that causes significant yield losses in melon and watermelon worldwide. Its causal agent is Acidovorax citrulli, a gram-negative bacterium with a single polar flagellum (Bahar and Burdman, 2010). Based on DNA-fingerprinting profiles, whole-cell fatty-acid composition, carbon-source utilization, pathogenicity assays, pulsed-field gel electrophoresis, and multilocus sequence typing, strains of A. citrulli are divided into two groups: group I includes strains isolated mainly from nonwatermelon hosts and are pathogenic to most cucurbit hosts, and group II is composed of strains isolated mainly from watermelon and more aggressive to cucumber and melon, indicating the important role QS plays in the pathogenicity of A. citrulli.

In addition to LuxR and AacR, many other LuxR-type regulators in the genome of A. citrulli have been identified in our preliminary studies (data not shown). In this study, we explored the regulatory role of one of the LuxR-type regulators, AcrR, in the group II strain Aac-5 of A. citrulli by mutational analysis and RNA sequencing. Our results showed that deletion of acrR affected flagellar biosynthesis, cell motility, biofilm formation, and virulence of Aac-5 of A. citrulli. In addition, our RNA sequencing (RNA-Seq) results revealed that the expression of many genes involved in various functions of strain Aac-5 of A. citrulli, including flagellar assembly, were changed in acrR deletion mutant strain, further suggesting that AcrR functions as a global transcriptional regulator, especially for flagella-related functions in A. citrulli.

## RESULTS

### 2.1 Identification of AcrR, a putative LuxR-type regulator of A. citrulli, and generation of acrR mutant and complemented strains

An open reading frame (ORF) containing 209 amino acids was identified in the genome sequence of the A. citrulli group II strain AAC00-1 (GenBank accession number CP0000512.1), located from nucleotide 4874913 to 4875542, with the locus tag of Aave_4382. It has a typical domain structure of the LuxR-family response regulators: a receiver domain at the N-terminus and an HTH DNA-binding domain at the C-terminus. In addition, a BLASTp search showed that this ORF has 100% amino acid sequence identity with the protein ADX48163.1 in Acidovorax avenae subsp. avenae ATCC 19860, annotated as a two-component transcriptional regulator in the LuxR family. The AcrR has no similarities with the quorum-sensing signal receptor aacR (located from nucleotide 4232080 to 4232808, with the locus tag of Ave_3810 in genome of AAC00-1, Wang et al., 2016) at the nucleotide or protein level. This suggests that the ORF in A. citrulli strain AAC00-1 is also a LuxR-type transcriptional regulator and was designated AcrR because it is an A. citrulli regulator that is different from aacR in the group II strain Aac-5 of A. citrulli.

The successful construction of the acrR mutant strain ∆acrR was confirmed by PCR amplification of strain ∆acrR with the LacrR-F and RacrR-R primers (Table 1) and subsequent sequencing of the PCR product. The product is 1,868 bp in size, containing 472-bp upstream and 541-bp downstream fragments of the acrR gene, separated by an 855-bp gentamicin cassette (data not shown). This was different from the PCR product of 1,643 bp in size that was amplified from the wild-type strain Aac-5, which contained the 630-bp acrR gene as well as the same 472-bp upstream and 541-bp downstream sequences.

The complementation strain ∆acrRcomp showed resistance to kanamycin, suggesting the successful transfer of the plasmid pBBR-acrR into ∆acrR (Table 2). The presence of pBBR-acrR in ∆acrRcomp was further confirmed by PCR amplification of the strain ∆acrRcomp with the primers acrR-F and acrR-R (Table 1) and sequencing of the PCR product, which showed the PCR amplified the 630-bp acrR gene.

| Primers | Sequence (5’–3’, restriction enzyme sites are underlined) | Restriction enzyme | Product of PCR (bp) |
|---------|----------------------------------------------------------|-------------------|--------------------|
| LacrR-F | GAATTCGCCGACGCCCATCAT | EcoRI             | 472                |
| LacrR-R | GGATCCTCAGCCTCTCCGTGGTC | BamHI            | 541                |
| RacrR-F | GTCGAGCTCCGCCCGGCTCAT | Sall             | 630                |
| RacrR-R | AAGCTTCATGCGTCGCGCTCG | HindIII          |                    |
| acrR-F  | CCCAAGCTTTATGATCCCACGTCGTCG | HindIII          |                    |
| acrR-R  | CGGGATCCTCCACACACCGCTGTTG | BamHI         | 855                |
| GmF     | GGATCCGCCGACGCCCGGGGAAAC | BamHI          |                    |
| GmR     | GTCGAGCTCCGGCGGCTTAGGACAATTT | Sall           |                    |

### TABLE 1 Primers used for construction of mutant and complemented strains
The acrR mutant of *A. citrulli* was reduced in virulence

To investigate whether AcrR contributes to the virulence of *A. citrulli*, we compared the virulence of the wild-type strain Aac-5 in watermelon seedlings with the mutant strain ∆acrR and the complementation strain ∆acrRcomp. Ten days after inoculation, the disease index (DI) in the watermelon seedlings that was caused by the mutant strain ∆acrR was 31.71, which was significantly lower than the DI of 48.88 caused by the wild-type strain Aac-5 (*p* < .01). The DI caused by the complementation strain ∆acrRcomp was 47.29, similar to the wild-type strain Aac-5 (Figure 1).

To determine whether the mutant is defective in its ability to grow in planta, we injected watermelon cotyledons with bacterial cell suspensions of the wild-type strain Aac-5, the mutant strain ∆acrR, and the complementation strain ∆acrRcomp, as well as sterile water as a negative control. No symptoms were observed in inoculated cotyledons 1 and 24 hr after inoculation (hai) in all treatments, whereas water-soaking necrosis appeared 48 hai, and lesions started to develop 72 and 96 hai (Figure 2a) in cotyledons inoculated with the wild-type and complementation strains, but not in those inoculated with water and the mutant strain. The results from our quantitative bacterial in planta assay revealed that the populations of the Aac-5, the ∆acrR, and the ∆acrRcomp strains in cotyledons were not significantly different until 72 hai, when the populations of the ∆acrR and the ∆acrRcomp strain were significantly lower than that of the Aac-5 strain (Figure 2b). At 96 hai, the population of the ∆acrR strain remained similar to its population level at 72 hai but was significantly lower than that of the Aac-5 and the ∆acrRcomp strains (Figure 2b).

2.3 | Biofilm formation and growth rate of the acrR mutant of *A. citrulli* were increased

The wild-type strain Aac-5 did not form any biofilm when measured both qualitatively and quantitatively in our study (Figure 3). When the acrR gene was mutated, however, the mutant strain ∆acrR formed a visible ring of biofilm on the inner wall of a flask, while no such ring was observed for the complementation strain ∆acrRcomp (Figure 3a). This observation was confirmed by our quantitative biofilm assay, since the mean absorption value of the biofilm by ∆acrR was 2.23, while the absorption was below the detection levels for the wild-type strain Aac-5 and the complementation strain ∆acrRcomp (Figure 3b). Our results showed that the mutation of the acrR gene enhanced the biofilm formation of *A. citrulli*.

The growth ability of Aac-5, ∆acrR, and its complementation strain ∆acrRcomp was determined by measuring the optical density of cell suspensions incubated in King’s B broth at 28 °C. The mutant strain ∆acrR was increased in growth ability, with the OD<sub>600</sub> value reaching 1.14 at 12 hr of incubation, and in the meantime the OD<sub>600</sub> value of the complementation strain ∆acrRcomp reached 0.53, whereas the wild-type strain Aac-5 had an OD<sub>600</sub> value of 0.11 at
When incubated for 36 hr, the OD₆₀₀ value of ∆acrR was 1.87, while those of the Aac-5 and ∆acrRcomp were 1.38 and 1.52, respectively (Figure 4).

2.4 | The acrR mutant of A. citrulli lost the ability to twitch and swim, as a result of the loss of flagella formation

We compared the wild-type strain Aac-5 to its mutant strain ∆acrR and the complementation strain ∆acrRcomp for the formation of corrugated trajectories or halos around their colonies as each bacterium migrated via twitching motility. Strain Aac-5 produced typical corrugated haloes, while smooth haloes were produced by the ∆acrR strain and the ∆acrRcomp strain (Figure 5a). These results show that the A. citrulli strain lost the twitching ability when its acrR gene was mutated.

Our assay for swimming motility revealed that the mutant strain ∆acrR did not spread 36 hr after inoculation of 10 µl of the bacterial suspension into the centre of a soft agar plate (0.3% agar), whereas the wild-type strain Aac-5 and the complementation strain ∆acrRcomp spread to approximately one quarter of the
When the bacterial suspension was placed on the basal medium plate, the average diameter of the drop was 1.01 cm. After 36 hr incubation at 28 °C, the average diameter of the bacterial lawn was 2.97 cm for the wild-type strain Aac-5, significantly larger than 1.16 cm for the mutant strain ∆acrR, but was similar to 2.83 cm for the complementation strain ∆acrRcomp (p < .01) (Figure 5c). The fact that the size of the bacterial lawn for the mutant strain ∆acrR remained similar 36 hr after incubation indicated the loss of swimming motility.

To determine what might cause the loss of swimming motility, we compared the flagellar formation of A. citrulli strains. Under transmission electron microscopy, polar flagella were observed on the plates (Figure 5b). When the bacterial suspension was placed on the basal medium plate, the average diameter of the drop was 1.01 cm. After 36 hr incubation at 28 °C, the average diameter of the bacterial lawn was 2.97 cm for the wild-type strain Aac-5, significantly larger than 1.16 cm for the mutant strain ∆acrR, but was similar to 2.83 cm for the complementation strain ∆acrRcomp (p < .01) (Figure 5c). The fact that the size of the bacterial lawn for the mutant strain ∆acrR remained similar 36 hr after incubation indicated the loss of swimming motility.

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in the wild-type and complemented strains but not in the mutant strain (Figure 6).

2.5 | RNA-Seq revealed that the acrR gene plays a role in the flagellar formation of A. citrulli

Because AcrR is identical to an annotated LuxR-type regulator in A. avenae subsp. avenae, we explored the possible regulatory role played by AcrR by comparing the acrR mutant strain ∆acrR of A. citrulli with its wild-type strain Aac-5 through transcriptome and gene ontology analyses. A total of 394 genes were differentially expressed in the ∆acrR mutant compared to its wild-type strain, including 219 highly and 175 lowly expressed genes (Table S1). The RNA-Seq results were validated in quantitative reverse transcription PCR (RT-qPCR) experiments with a set of 10 selected genes (Figure S1). Gene ontology analysis divided the differentially expressed genes (DEGs) into biological process, cellular component, and molecular function (Figure 7). Under the category of biological process, the DEGs related to processes of bacterial flagella and cell motility (subcategories cell motility, cellular component movement, ciliary or bacterial-type flagellar motility and locomotion)
were significantly lowly expressed, whereas the DEGs involved in cellular metabolic process were significantly highly expressed, indicating the regulatory role that acrR plays in the motility and metabolism of the A. citrulli strain Aac-5 (Figure 7). Under the categories of cellular component and molecular function, the numbers of highly expressed genes were more than that of lowly expressed genes, especially under the category of cellular component, which includes highly expressed genes hitting 13 out of 16 subcategories (Figure 7). Taken together, the results of the gene ontology analysis were consistent with the abolished motility and increased in vitro growth ability of acrR mutant of A. citrulli Aac-5.

The RNA-Seq data also showed that deletion of the acrR gene affected many genes involved in flagellar biosynthesis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that 33 DEGs were involved in flagellar biosynthesis, including 3 highly and 30 lowly expressed genes (Table 3). The lowly expressed genes encode critical components for flagellar assembly, including flagellin, flagella biosynthesis proteins, flagella L-g and P-ring proteins.

### Table 3: Differentially expressed genes involved in flagellar biosynthesis in Acidovorax citrulli ΔacrR strain compared to its wild-type strain Aac-5

| Gene ID   | Gene product description                        | logFC | p value     |
|-----------|------------------------------------------------|-------|-------------|
| Aave_2005 | Transcriptional activator FlhC                  | -2.77 | 6.64E-03    |
| Aave_2006 | Flagellar transcriptional regulator FlhD       | -2.83 | 5.87E-03    |
| Aave_2496 | Chemotaxis protein CheW                        | -1.92 | 2.84E-03    |
| Aave_4383 | Flagellar biosynthetic protein FlIR            | 4.68  | 4.69E-24    |
| Aave_4384 | Flagellar biosynthesis protein FlIQ             | 1.97  | 1.12E-04    |
| Aave_4385 | Flagellar biosynthetic protein FlIP             | 1.48  | 4.31E-03    |
| Aave_4387 | Flagellar motor switch protein FlIN             | -1.11 | 3.95E-02    |
| Aave_4390 | Flagellar hook-length control protein FlIK      | -1.91 | 1.18E-04    |
| Aave_4391 | Flagellar hook-length control protein FlJ       | -2.63 | 2.14E-03    |
| Aave_4392 | Flagellar protein export ATPase FII             | -1.68 | 1.98E-03    |
| Aave_4393 | Flagellar assembly protein FIIH                 | -1.91 | 2.83E-04    |
| Aave_4395 | Flagellar M-ring protein FlIF                   | -2.04 | 3.52E-05    |
| Aave_4397 | Flagellar protein FlIT                          | -2.89 | 1.40E-09    |
| Aave_4398 | Flagellar export chaperone FlIS                 | -3.13 | 1.88E-05    |
| Aave_4399 | Flagellar cap protein FlID                      | -1.34 | 6.45E-03    |
| Aave_4400 | Flagellin                                       | -4.35 | 9.36E-22    |
| Aave_4401 | Flagellin                                       | -2.28 | 4.33E-07    |
| Aave_4413 | Flagellar biosynthesis protein FlhA             | -3.97 | 1.34E-16    |
| Aave_4414 | Flagellar biosynthesis protein FlhF             | -3.29 | 3.55E-12    |
| Aave_4416 | RNA polymerase sigma factor FlIA                | -1.76 | 9.25E-06    |
| Aave_4418 | Flagellar biosynthesis anti-sigma factor FlgM   | -2.81 | 3.19E-07    |
| Aave_4419 | Flagella basal body P-ring formation protein FlgA | -2.2  | 1.22E-04    |
| Aave_4420 | Flagellar basal-body rod protein FlgB           | -3.74 | 2.00E-05    |
| Aave_4421 | Flagellar basal body rod protein FlgC           | -3.51 | 4.18E-06    |
| Aave_4422 | Flagellar basal body rod modification protein FlgD | -3.94  | 9.00E-14  |
| Aave_4423 | Flagellar hook protein FlgE                     | -4.35 | 6.65E-20    |
| Aave_4424 | Flagellar basal body rod protein FlgF           | -4.39 | 1.60E-14    |
| Aave_4426 | Flagellar L-ring protein FlgH                   | -3.55 | 3.19E-11    |
| Aave_4428 | Flagellar P-ring protein Flgl                   | -3.7  | 5.46E-13    |
| Aave_4429 | Flagellar rod assembly protein/muramidase FlgJ | -3.32 | 1.23E-10    |
| Aave_4430 | Flagellar hook-associated protein FlgK          | -2.92 | 2.69E-09    |
| Aave_4431 | Flagellar hook-associated protein 3 FlgL        | -2.86 | 8.98E-09    |
| Aave_4592 | Flagellar motor protein MotB                    | -1.95 | 1.07E-04    |

Note: Gene ID refers to the locus_tag of a differentially expressed gene in A. citrulli ΔacrR strain compared to strain Aac-5, identified by hits in a BLASTn search against the strain AAC00-1 genome (GenBank accession number CP000512.1). FC, fold-change
located in outer membrane and peptidoglycan layer, flagella cap, hook and basal-body rod related proteins, as well as transcriptional regulators FlhDC (Figure 8). The RNA-Seq results were consistent with the loss of flagella formation in the mutant strain when acrR was deleted (Figure 6). In addition, 46 DEGs were found involved in ribosomal proteins, all of which were significantly highly expressed (Table S1).

3 | DISCUSSION

In this study, we identified a LuxR-type transcriptional regulator AcrR in A. citrulli. We demonstrated that deletion of the acrR gene resulted in reduced virulence on watermelon seedlings, lost twitching and swimming motilities, and failure to form flagella, but increased biofilm formation and growth ability of the mutant strain compared to its wild-type strain of A. citrulli. Additionally, comparative RNA profiling analysis revealed that 394 genes were differentially expressed, including 33 involved in flagellar assembly, suggesting the regulatory role AcrR plays, especially in flagella-related functions of A. citrulli.

LuxR-type regulators are known to play a role not only in the QS system but also in other biological functions such as flagellar synthesis. For example, VisN and VisR are two regulators in Sinorhizobium meliloti that belong to the LuxR family and act as global regulators of chemotaxis, flagellar, and motility genes (Sourjik et al., 2000). As demonstrated in our study, the AcrR of A. citrulli either positively or negatively regulated multiple biological functions of A. citrulli, because the acrR mutant strain lost swimming motility and failed to twitch and form flagella but increased in biofilm production and growth ability.

The bacterial flagellum is an organelle for cell propulsion (Macnab, 2004). In addition, the flagellum is involved in several

**Figure 8** Schematic diagram of the differentially expressed genes involved in flagellar assembly. Layers of inner and outer membrane, peptidoglycan layer, C-, MS-, P-, and L-rings, motor proteins (MotAB), rotor (FlgBCF), hollow rod, hook, and distal part of flagellum are shown. Components of flagellum transport apparatus, ATPase complex, chaperones of flagellar transport, and their substrates are presented schematically. Highly expressed genes are shaded grey and lowly expressed genes are highlighted in green.
functions associated with bacterial pathogenicity, including biofilm formation, protein export, and adhesion (Haiko and Westerlund-Wikström, 2013). The flagellum also serves as a virulence factor in many bacteria, including Salmonella typhimurium, Escherichia coli, Vibrio cholera, and Pseudomonas aeruginosa (Duan et al., 2013). Bahar et al. (2011) found that an intact flagellum was required to achieve full virulence of A. citrulli. They observed that the A. citrulli strain M6 was unable to form an intact flagellum when its flgC gene was mutated, and the mutant was reduced in virulence and twitching motility although not in biofilm formation (Bahar et al., 2011). In our study, the deletion of the acrR gene abolished the ability of A. citrulli strain Aac-5 to form a polar flagellum, which, in turn, may have resulted in reduced swimming motility, contributing to its reduced virulence on watermelon seedlings. Our RNA-Seq data support the role that AcrR plays in flagellar biosynthesis because 33 genes involved in flagellar assembly were differentially expressed in the acrR mutant strain in comparison with the wild-type strain (Table 3).

The lack of flagella could lead to enhanced growth ability in prokaryotes. Pyrococcus furiosus DSM363 exhibited an enhanced growth ability when its flagella were absent (Lewis et al., 2015). The transcription regulator SwrA stimulates the transcription of the genes for $\sigma^0$, which controls when Bacillus subtilis cells enter into the motile state with expression of flagellum biosynthesis genes from a state of growth as long and nonmotile chains (Kearns and Losick, 2005). For growth and maintenance on the host, bacteria may reduce or eliminate flagellar expression (Chaban et al., 2015). In our study, the deletion of the acrR gene not only eliminated the biosynthesis of flagellum of A. citrulli Aac-5 strain but also enhanced its growth ability, whereas the complementation strain showed similar growth ability to the Aac-5 strain, indicating that AcrR may regulate the Aac-5 switch between the growth and motility stages. The fact that 46 ribosomal genes were highly expressed in the mutant strain compared to the wild-type one (Table S1) suggests that these genes may contribute to the enhanced growth ability of the mutant strain.

Flagellar synthesis is a strictly hierarchical process in which more than 50 genes are involved (Wang et al., 2015). These genes are organized in multiple operons that can be divided into three classes based on the sequential order of the process: early (I), intermediate (II), and late (III) (Aldridge and Hughes, 2002; Osterman et al., 2015). Operon flhDC codes for transcription factor FlhDC, a master flagellar regulator. It is considered the sole class I transcription unit and is responsible for transcriptional activation of all structural and other regulatory components of the flagellar machinery (Fitzgerald et al., 2014). In A. citrulli, we found that among the 33 differentially expressed genes associated with flagellar assembly, flhC and flhD genes were lowly expressed with 2.77- and 2.83-log fold change, respectively, suggesting that the early stage of flagellar synthesis is regulated by AcrR. The class II sigma-factor flIA gene regulates the transition from early to late-stage flagellar gene expression (Fitzgerald et al., 2014; Osterman et al., 2015). Along with its cognate flgM gene, an anti-sigma factor, fliA was significantly lowly expressed in the acrR mutant, which in turn down-regulated its downstream class III flagellar synthesis genes flgKL, fliDST, motB, and cheW (Table 3).

Why the three genes, fliP, fliQ, and fliR, which code for FliP, FliQ, and FliR, respectively, in flagellar biosynthesis were highly expressed in the acrR mutant in comparison to the wild-type strain is unclear. FliP, FliQ, and FliR are components of the flagellar secretion apparatus that anchors at the cell membrane (Ward et al., 2018) and belongs to the flagellar type III secretion system (FT3SS). They reside within the MS-ring, a subdomain of the hook-basal-body, which is located within the cytoplasmic membrane (Figure 8) (Zhuang and Shapiro, 1995; Fan et al., 1997). Assembly of the flagellum begins with the MS-ring, comprising several transmembrane proteins, belonging to the FT3SS and inserted into the cellular membrane (Macnab, 2004). Because the acrR gene (nucleotides 487913 to 4875542) is located at the 3′ flanking region on the complementary strand of the operon fliRQP (nucleotides 4875558 to 4877416) in the genome of A. citrulli strain AAC00-1, the deletion of the acrR gene might not affect the promoter region of the operon fliRQP. The fact that all the differentially expressed flagellar assembly related genes except fliR, fliQ, and fliP were lowly expressed in the acrR mutant suggests that the fliR, fliQ, and fliP genes might be regulated by other factors when acrR is absent.

Biofilm formation is crucial for the virulence of some plant pathogenic bacteria (Dow et al., 2003; Fujishige et al., 2006). The phytopathogenic bacterium Xanthomonas citri showed reduced disease symptoms when it was unable to form a biofilm (Rigano et al., 2007). In contrast, our study revealed that biofilm formation of the acrR mutant of A. citrulli was not decreased but increased significantly compared to the wild-type strain Aac-5, while the virulence of the mutant was reduced. This result is in agreement with previous findings that some of the group II strains of A. citrulli are not able to form a biofilm, while the group I strain M6 is able to form a biofilm (Bahar et al., 2009; Chen et al., 2009), suggesting potential differences in the trajectory of biofilm formation processes between some of the strains that belong to the two groups of A. citrulli strains. Wiggling is a process that contributes to the adherence of bacterial cells to surfaces and colonization on bacterial hosts (Mattick, 2002; Craig et al., 2004). It is also required for the virulence and biofilm formation of A. citrulli (Bahar et al., 2009). When the acrR gene was mutated in Aac-5 of A. citrulli, no twitching motility was observed and the virulence was reduced, suggesting that acrR is important for twitching motility in A. citrulli, which, in turn, contributes to the virulence of the bacterium. An interesting finding of this study is that the acrR mutant lost the ability to twitch but increased its ability to form a biofilm compared to the wild-type strain Aac-5. This is different from previous findings that A. citrulli group I strain M6 and its mutants lacked the ability to twitch and were also decreased in biofilm formation (Bahar et al., 2009; Rosenberg et al., 2018). It is possible that the twitching motility and biofilm formation are regulated differently in group I and II strains, and the global transcriptional regulator AcrR may regulate many genes, including the ones involved in biofilm formation.

In summary, the acrR gene contributes to the virulence of A. citrulli strain Aac-5, either directly and/or indirectly though positive regulation of the twitching and swimming motilities and flagellar formation and negative regulation of the biofilm formation.
Additionally, our transcriptomic analysis revealed that the acrR gene also positively regulates flagellar assembly in *A. citrulli*, supporting the role that AcrR plays in flagellar biosynthesis. Because AcrR contains a receptor domain at the N-terminus, possibly the AcrR senses or interacts with certain signals that affect multiple biological functions, including virulence and flagellar formation of *A. citrulli*. Future research is needed to identify such signals and elucidate the molecular mechanisms behind the regulation of *A. citrulli*.

4 EXPERIMENTAL PROCEDURES

4.1 Bacterial strains, plasmids, growth conditions, and primer design

The bacterial strains and plasmids used in this study are listed in Table 1. *A. citrulli* strains were grown in King’s B (KB) broth or on a KBA plate (KB containing agar at 15 g/L) with appropriate antibiotics and at 28 °C. *E. coli* strains were grown in Luria Bertani medium at 37 °C. The antibiotics used were ampicillin (Ap), kanamycin (Km), and gentamicin (Gm) at concentrations of 100 µg/ml for Ap and 50 µg/ml for the other antibiotics. The primer pair acrR-F/R was designed based on *acrR* gene in AAC00-1 genome (GenBank accession number CP000512.1), while primer pairs LacR-F/R and RacrR-F/R were designed based on upstream and downstream sequences of the *acrR* gene (Table 1). Primers GmF and GmR were designed based on gentamicin cassette (Table 1). All primers used in this study were designed using the free online program Primer 3.0 (http://www.simgene.com/Primer3).

4.2 Construction of the *acrR* mutant and its complemented strain

The *acrR* gene was deleted by homologous double recombination as described previously (Wang et al., 2016). Briefly, the 472-bp upstream and 541-bp downstream sequences of the *acrR* gene were amplified from the wild-type strain Acac-5 using the LacR-F/LacrR-R and RacrR-F/RacrR-R primers (Table 3). After confirmation by sequencing, the PCR fragments were digested by appropriate restriction enzymes and ligated into pK18 mobsacB to create the plasmid pK18-acrR-Up&Down (Table 2). The plasmid was digested by *Bam*HI and *Sal*I, and a Gm gene cassette (855 bp) was inserted between the *Bam*HI and *Sal*I sites to create plasmid pK18-acrRGM (Table 3). The plasmid was then introduced into *E. coli DH5*α into the *A. citrulli* strain Acac-5 by triparental conjugation using pRK600 as a helper plasmid to create the *acrR* mutant strain ∆acrR (Table 2). Transconjugants were screened on KBA supplemented with 10% sucrose and antibiotics (Ap and Gm). The presence of the Gm cassette in the transconjugants was confirmed by PCR and sequencing of the amplified PCR product using the primer pair GmF/GmR (Table 1).

The absence of the *acrR* gene in the transconjugants was confirmed by the lack of PCR product using primer pair acrR-F/acrR-R.

To generate a complementation strain of ∆acrR, the *acrR* gene in Acac-5 was amplified using primers acrR-F and acrR-R (Table 1). The PCR product was digested with *Hind*III and *Bam*HI and cloned into pBBR1MCS-2 to generate pBBR-acrR, which was transferred into the mutant strain ∆acrR by triparental conjugation (Table 2). The successful transconjugant, named ∆acrRcomp, was identified through screening on KBA (amended with Ap, Km, and Gm; Table 2). All obtained plasmids and *A. citrulli* strains were confirmed by PCR and DNA sequencing.

4.3 Virulence assays

The virulence of the *A. citrulli* strains was tested on 3-week-old watermelon seedlings (*Citrullus lanatus* ‘Jingxin#6’, provided by the Beijing Academy of Agriculture and Forestry Sciences, Beijing, China). The virulence assay was performed, and the DI was calculated as previously described (Wang et al., 2016). Briefly, *A. citrulli* strains were grown in KB broth and their OD 600 was adjusted to 0.6 (approximately 10⁸ cfu/ml). Two hundred milliliters of each bacterial suspension was sprayed onto watermelon seedlings grown at 28 °C in a growth chamber with 90% relative humidity. Disease symptoms were evaluated at the eighth day after inoculation using a disease severity scale: 0 for no symptoms; 1, 3, 5, and 7 for necrotic lesions; and 9 for complete death of the seedling. The disease index was calculated based on the formula

\[ DI = \frac{\sum (A \times B) \times 100}{\sum C \times 9} \]

where *A* is the disease scale, *B* is the number of seedlings in each disease scale, and *C* is the total number of seedlings in each treatment. For each *A. citrulli* strain, six watermelon seedlings in three pots were inoculated in each experiment, and the experiment was repeated three times.

4.4 In planta growth assays

The growth ability of *A. citrulli* strains in 2-week-old watermelon cotyledons (the same cultivar as the one used in virulence assay) was determined using the method of Johnson et al. (2011), with modifications. Briefly, *A. citrulli* was grown overnight in KB broth at 28 °C to an OD 600 of 0.8. The bacterial cells were then washed three times with sterile water and adjusted to 10⁶ cfu/ml with sterile water to make the bacterial inoculum. One millilitre of the inoculum was injected into 30 watermelon cotyledons using sterile syringes. Six injected cotyledons, each inoculated with the *A. citrulli* strain or water, were collected at 1, 24, 48, 72, and 96 hai, respectively. One 3-mm disc from each cotyledon and six discs in total were collected and homogenized in Lysing Matrix A tubes (MP Biomedicals Co., Ltd) containing 1 ml of sterile water using MP FastPrep-24™ 5G (MP Biomedicals Co., Ltd). The lysates were serially diluted with sterile water and plated on KBA plates. The plates were incubated at

\[ \text{DI} = \sum (A \times B) \times 100 / \sum C \times 9 \]
28 °C for 48 hr. The growth of colonies on the KBA plates was counted as a measurement of the population of A. citrulli strains growing in watermelon cotyledons. The experiment was repeated three times.

4.5 Assay for swimming and twitching motilities and observation of flagella by transmission electron microscopy

Swimming and twitching motilities of the A. citrulli strains were determined using the methods of Wang et al. (2016). For swimming motility, A. citrulli strains were incubated in KB broth and their OD600 was adjusted to 0.6. Five microlitres of each cell suspension was placed into the centre of a basal medium plate containing 0.3% agar and incubated for 36 hr at 28 °C. The diameter of the colony on each plate was then measured. For twitching motility, 10 μl of A. citrulli strains were placed on the KBA plates containing ampicillin and incubated for 72 hr. The twitching motility was visualized using a BX63 microscope (Olympus). Colonies with twitching motility were characterized by the formation of corrugated trajectories or haloes around the colonies. In both swimming and twitching motility assays, at least nine plates were inoculated by each strain in each experiment, and the experiment was repeated three times.

Flagella of each A. citrulli strain were observed using the method described by Wang et al. (2016). Briefly, each A. citrulli strain was grown on the basal medium that was used in the swimming motility assay for 36 hr. Ten microlitres of sterile water was placed on top of each colony for 2 min, and then covered by a 200-mesh copper grid for 1 min. The copper grids were negatively stained with 1% uranyl acetate three times, each time for 30 s, before they were dried on sterile filter paper. The presence or absence of polar flagella in each A. citrulli strain was examined under transmission electron microscopy.

4.6 Biofilm and growth ability assays

The effect of acrR deletion on biofilm formation was determined both qualitatively and quantitatively, and the growth rate of the A. citrulli strains was measured as described by Wang et al. (2016). Briefly, overnight cultures of A. citrulli strains were adjusted to an OD600 of 0.1. One hundred microlitres of each cell suspension was added to 10 ml KB broth in a glass flask and incubated at 28 °C for 7 days without agitation. Two millilitres of 0.1% (wt/vol) crystal violet was then added to the flask and incubated for 2 hr. After the liquid had slowly been poured off, the flasks were rinsed with distilled water and fixed by heating at 80 °C for 30 min. To quantify the biofilm formation, the stained biofilm was solubilized by 5 ml ethanol for 12 hr, and the OD580 of the stained suspension was measured with a spectrophotometer (Biophotometer, Eppendorf). Three biological replicates were performed for each A. citrulli strain in each experiment, and the experiment was repeated three times.

To measure bacterial growth in vitro, 2 μl of cell suspension for each A. citrulli strain at OD600 of 0.3 was diluted 100-fold with 198 μl KB broth in a well of a 96-well plate. The plate was incubated at 28 °C with shaking at 220 rpm. The OD600 of the suspension was measured every 2 hr for 72 hr by Bioscreen C (FP-1100-C, Oy Growth Curves Ab Ltd).

4.7 RNA isolation and quantitative reverse transcription PCR analysis of gene expression

RNA was isolated from the A. citrulli strains using the method of Wang et al. (2016). Briefly, A. citrulli was grown in KB broth at 28 °C overnight to an OD600 of 0.8. The bacterial cells were then washed twice with sterilized water and their RNA was extracted using a bacterial RNAout kit (Tiangz) based on the manufacturer’s instructions. cDNA was synthesized based on the method of Wang et al. (2016) using a FastQuant RT kit (TianGen). RT-qPCR analysis was carried out using primers designed for 10 DEGs (Table S2). cDNA was used as a template with SYBR Green added in the PCR, and relative levels of gene expression were determined as previously described (Wang et al., 2016). Three biological replicates were established in each experiment, and the experiment was repeated three times.

4.8 RNA-Seq library construction and sequencing

A total of 3 μg of RNA per A. citrulli strain was used as input material for subsequent RNA sample preparations. The RNA sequencing libraries were constructed and sequenced commercially by Novogene Co., Ltd using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB), and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v. 3-cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2500 platform, and 100-bp paired-end reads were generated.

4.9 RNA-Seq data analysis

The sequencing data were analysed commercially by Novogene Co., Ltd. Briefly, analysis for differential gene expression between the wild-type strain Aac-5 and the acrR mutant strain ΔacrR (three biological replicates per strain) was performed using the DESeq R package (v. 1.10.1). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting p values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted p value <0.05 found by DESeq were assigned as differentially expressed (Pan et al., 2018).
Gene ontology enrichment analysis of the DEGs was implemented by the goseq R package, in which the gene length bias was corrected. GO terms with a corrected p value less than .05 were considered significantly enriched by the DEGs (Pan et al., 2018).

4.10 Statistical analysis

Statistical analysis was performed using the Student’s t test in Excel 2010 software (Microsoft Inc.). Differences were considered statistically significant if p < .01.

ACKNOWLEDGEMENTS

This work was supported financially by National Natural Science Foundation of China (31701754), the National Key Research and Development Program of China (2016YFD0201004), and the China earmarked fund for Modern Agroindustry Technology Research System (CARS-26).

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

W.G., T.W., and T.Z. designed the research. W.G. wrote the paper. T.W., W.G., E.T., and B.L. executed the experiments. W.G., T.W., and Y.Y. performed the data analyses. Q.H. revised the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Wei Guan https://orcid.org/0000-0002-5533-6682

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

FIGURE S1 Relative expression of selected genes by RT-qPCR. The bacterial strains were incubated under the same condition as those for the RNA-Seq experiment (three biological replicates per strain).

The x axis represents log_{2} (fold-change) of each gene in acrR mutant compared to its wild-type strain Aac-5

TABLE S1 Differentially expressed genes between the Acidovorax citrulli acrR mutant strain and its wild-type strain Aac-5

TABLE S2 Primers used for RT-qPCR

How to cite this article: Guan W, Wang T, Huang Q, et al. A LuxR-type regulator, AcrR, regulates flagellar assembly and contributes to virulence, motility, biofilm formation, and growth ability of Acidovorax citrulli. Molecular Plant Pathology. 2020;21:489–501. https://doi.org/10.1111/mpp.12910