Quorum-sensing contributes to virulence, twitching motility, seed attachment and biofilm formation in the wild type strain Aac-5 of *Acidovorax citrulli*

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Abstract

Acidovorax citrulli is a seed-borne pathogen causing bacterial fruit blotch of cucurbits including melon and watermelon. We investigated the roles of quorum sensing in the wild-type group II strain Aac-5 of *A. citrulli* by generating *aacR* and *aacI* knockout mutants and their complementation strains. We found that twitching motility and virulence were reduced, but biofilm formation and seed attachment were increased significantly in the two mutants as compared to the wild type strain. Deletion of *aacR* and *aacI*, however, had no effect on swimming motility and polar flagella formation of Aac-5. Furthermore, deletion of *aacR* resulted in reduced gene expression of *hrpE*, *hrcN* and *pilT*, while deletion of *aacI* affected only the expression of *hrpE* and *pilT*, not *hrcN*.

Keywords: Quorum sensing, *Acidovorax citrulli*, biofilm formation, twitching motility

Introduction

Bacteria communicate with each other using a process called “quorum sensing” (QS), which utilizes self-generated signaling molecules termed autoinducers (AIs). N-acyl-homoserine lactone (AHL) is the major class of QS molecules in Gram-negative bacteria, and they are produced by more than 50 different bacterial species [1, 2, 3, 4]. A common method of AHL regulation in Gram-negative bacteria is achieved by two proteins: LuxI, an AI synthase protein and LuxR, a regulatory protein. AI binds to the LuxR family regulators, which subsequently regulate the expression of a wide range of related genes [5]. Traits regulated by QS include the production of extracellular polysaccharides, degradative enzymes, antibiotics, siderophores, and pigments, as well as Hrp protein secretion, Ti plasmid transfer, motility, biofilm formation, and epiphytic fitness [6, 7, 8, 9].

Type III secretion system (T3SS) is a needle like device that bacteria use to deliver type III effector proteins into host cells. The T3SS is encoded by the hrp (HR and pathogenicity) cluster, which includes over 20 genes including hrc (HR and conserved) and hrp genes that encode a type III secretion tunnel [10]. In the hrp cluster, *hrcN* encodes HrcN, which is a translocase ATPase that is indispensable for T3SS [11]. The *hrpE* encodes a major component of a surface appendage named the Hrp pilus [12] that is involved in secretion of effector proteins. Type IV pili (TFP) are appendages of bacterial surface that are involved in many processes in bacteria including adherence and colonization, motility, and biofilm formation [13, 14]. The *pilT* gene encodes an ATPase responsible for TFP retraction that facilitates twitching motility [15]. The *pilT* deletion mutant strain of *Pseudomonas aeruginosa* completely lost twitching motility [16].

Acidovorax citrulli causes bacterial fruit blotch (BFB) of cucurbits, which results in substantial economic losses to cucurbit growers worldwide [17, 18, 19]. Strains of *A. citrulli* are divided into two groups: group I strains are pathogenic to most cucurbit hosts, and group II strains are comparatively more pathogenic to watermelon seedlings than group I strains [20, 21, 22, 23]. In addition, unlike the group I strains, group II strains are copper sensitive and unable to form biofilms [15]. The role of the QS system in *A. citrulli* was first studied in the group I strain XJL12 isolated in China [24]. Strain XJL12 was reduced in virulence, growth rate and swimming motility when its *aacI* (*luxI* homologue) gene was mutated, but the mutation had no effect on biofilm formation or exopolysaccharide production [25]. A recent study analyzing *aacI* and *aacR*, homologues of *luxI* and *luxR*, respectively, in the group II strain AAC00-1 isolated in the United States found that *aacI* is involved in seed-to-seedling transmission of BFB in watermelon [26], but biofilm formation was not studied in the study. Since there is no biofilm production in group II strains and it is unknown whether the QS system affects group I and group II strains of *A. citrulli* differently, we investigated the roles of QS in virulence, twitching and swimming motilities, flagella formation, biofilm formation and seed adhesion ability of a group II strain of *A. citrulli*.

isolated from watermelon in China [19]. Our results suggest there is a functional difference in the QS systems between the two groups of *A. citrulli* strains.

Results

Confirmation of the *aacR* and *aacI* mutants and their complementation strains of *A. citrulli*

The *aacR* gene encodes the LuxR homologue protein, which is 243 amino acids in length based on the genome sequence of *A. citrulli* AAC00-1 (GenBank accession number CP000512.1). PCR amplification of the *aacR* mutant strain with the Rup-F and Rdn-R primers and the subsequent sequencing of the PCR product confirmed that strain *aacR*⁻ contained a band of 1,918 bp, consisting of 515-bp upstream and 548-bp downstream fragments of the *aacR* gene replaced by an 855-bp gentamicin (Gm) cassette (data not shown). PCR amplification of the *aacI* mutant strain *aacI* with the Iup-F and Idn-A primers and the subsequent sequencing of the PCR product confirmed that strain *aacI* contained a band of 1,897-bp containing 526-bp upstream and 516-bp downstream fragments of the *aacI* gene replaced by the 855-bp Gm cassette (data not shown). The presence of the Gm cassette in *aacI* and *aacR*⁻ was further confirmed by Southern blot and was absent from the wild-type strain Aac5 (data not shown). The *aacI* and *aacR*⁻ strains were stable after continuous culturing for 20 times in KB medium.

The fact that the complementation strains *aacR*Comp and *aacI*Comp were Sp^R suggested the successful transfer of the plasmid pHMaacR and pHMaacI into the *aacI* and *aacR*⁻ strains, respectively. Furthermore, the presence of pHMaacR in *aacR*Comp was confirmed by PCR using the primer pair aacR-F/R, as the subsequent sequencing of the PCR product showed that the *aacR*Comp contained a 1,351-bp fragment including the coding region of *aacR* and its promotor region. Similarly, the presence of pHMaacI in *aacI*Comp was confirmed by PCR using the primer pair aacI-F/R, as the subsequent sequencing of the PCR product showed that the *aacI*Comp contained a 1,207-bp fragment including the coding region of *aacI* and its promotor region.

The *aacR* and *aacI* mutants of *A. citrulli* were reduced in twitching motility

To determine if the QS system has a role in twitching motility in *A. citrulli*, we compared the wild-type strain Aac-5 to its mutant strains *aacR*⁻ and *aacI* for the formation of corrugated trajectories or halos around their colonies as the bacterium migrating via twitching motility. Strain Aac-5 produced typical haloes, while little or no halos were produced by the *aacI* and *aacR*⁻ strains (Fig. 1A). The complemented strains *aacR*Comp and *aacI*Comp, however, produced similar halos as the wild-type strain Aac-5 (Fig. 1A).

Since TFP are reported to be involved in twitching motility [13, 14, 27, 28], we measured the expression of the TFP gene *pilT* using quantitative reverse transcription-PCR. Compared to the

wild-type strain Aac-5, *pilT* gene expression was reduced 7.7-fold in *aacI* strain, while little expression was detected in the *aacR*⁻ strain ($P < 0.01$) (Fig. 1B).

The *aacR* and *aacI* mutants of *A. citrulli* were increased in biofilm formation

Under our qualitative biofilm assay conditions, the wild-type strain Aac-5 did not form any ring of biofilm on the surface of glass flasks, but when the *aacR* and *aacI* genes were mutated, the mutant strains *aacR*⁻ and *aacI* formed visible rings of biofilm (Fig. 2A). When the mutant strains were complemented, however, the complementation strains *aacR*⁻Comp and *aacI*Comp formed little or no visible rings of biofilm (Fig. 2A). This observation was confirmed by our quantitative biofilm assay, as the mean absorption values of the biofilm by *aacR*⁻ and *aacI* were significantly higher than the wild-type strain Aac-5, as well as the mutant complementation strains *aacR*⁻Comp and *aacI*Comp ($P < 0.01$) (Fig. 2B).

The *aacR* and *aacI* mutants of *A. citrulli* increased their ability to adhere to watermelon seeds

To determine if the QS system in *A. citrulli* plays a role in the adhesion ability of the bacterium to watermelon seeds, we measured adhesion of *A. citrulli* strains to watermelon seeds (Fig. 3). The wild type bacterial cells attached to watermelon seeds were estimated to be 8.67×10^6 cells, significantly lower than the two mutant strains (Fig. 3). The *aacI* was estimated to be 5.87×10^{13} cells, the highest among all tested strains (Fig. 3). The mutant complementation strain *aacI*Comp was significantly higher than the wild type strain Aac-5 but lower than the mutant strain *aacI*, while the mutant complementation strain *aacR*⁻Comp was similar to the mutant strain *aacR*⁻ (Fig. 3). To confirm that the difference in seed adhesion was not due to differences in growth ability among tested strains, growth rate was measured for each strain, and no significant differences were observed (data not shown).

The *aacR* and *aacI* mutants of *A. citrulli* were reduced in virulence

Ten days after inoculation, the disease index (DI) in watermelon seedlings caused by the QS mutants *aacR*⁻ and *aacI* were 22.47 and 25.37, respectively, which were significantly less than the DI of 39.30 caused by the wild-type strain ($P < 0.01$) (Fig. 4). DIs caused by the complementation strains *aacR*⁻Comp and *aacI*Comp were 37.16 and 35.77, respectively, not significantly different from the wild-type strain Aac-5 (Fig. 4).

The *aacR* and *aacI* mutants did not contribute to swimming motility and flagella biosynthesis of *A. citrulli*

The halo size as an indication of swimming ability was measured to be 3.07 cm, 3.05 cm and 3.04 cm in the wild-type Aac-5, and the mutants *aacR*⁻ and *aacI*, respectively, not significantly different from each other ($P > 0.05$) (Fig. 5A). In addition, using transmission electron microscopy,

polar flagella were observed in both of the mutant strains, similar to the ones in the wild-type strain (Fig. 5B).

The *aacR* mutant of *A. citrulli* were reduced in *hrpE* and *hrcN* gene expression, but the *aacI* mutant only affected *hrpE* gene expression

The expression level of the T3SS gene *hrpE* was decreased 5- and 1.4-fold in *aacR* and *aacI* mutant strains, respectively, as compared to the wild-type strain Aac-5 (Fig. 6). The expression level of another T3SS gene *hrcN* was reduced 8.4-fold in the *aacR* strain, but was at a similar level in the *aacI* strain, as compared to strain Aac-5, (Fig. 6).

Discussion

QS phenomenon is widespread in microbial communities. We investigated the functions of QS in the group II strain Aac-5 of *A. citrulli* by constructing *aacR* and *aacI* mutants and their complementation strains. Our results revealed that *aacR* and *aacI* positively regulate twitching motility and virulence, negatively regulate the biofilm formation and attachment of the bacterial cells to watermelon seeds, while have no significant effect on polar flagella formation and swimming motility of *A. citrulli* strain Aac-5.

Twitching is a form of surface motility mediated by TFP [14, 21] and is also involved in the initial stage of biofilm formation [32, 33, 34, 35]. The TFP of *P. aeruginosa* was shown to have multiple effects on biofilm formation by enabling microcolony formation under some conditions [28, 36]. Similarly, *pilT* mutants of *P. aeruginosa* have been shown to adhere to different surfaces at similar or higher extents than the wild type [32]. In *A. citrulli*, strains belonging to group II were previously reported to be unable to form biofilm [15], which was also confirmed in this study. The TFP in *A. citrulli* was known to be involved in twitching motility [15], and the *pilT* gene encodes an ATPase responsible for the TFP retraction that drives twitching motility [27]. When the *pilT* gene was deleted, the mutant exhibited declined twitching motility [15]. In our study, we constructed the QS mutants while keeping the TFP-related genes intact. The twitching motility was reduced significantly in the QS mutants, suggesting that QS regulates twitching motility in *A. citrulli* strain Aac-5, probably through the regulation of the TFP-related genes, including the *pilT* gene since the expression of this gene was significantly reduced (Fig. 1B). The reduced expression of *pilT* might also lead to the increased seed attachment by the bacterium to watermelon seeds as revealed by our results (Fig.3), probably due to restrained twitching motility. The *aacR* and *aacI* mutant complementation strains did not reduce or only partially reduced seed attachment to the wild type level, suggesting that the *aacR* and *aacI* genes may need to act in cis for wild type level of seed attachment.

The T3SS is critical to the pathogenicity of gram-negative plant-pathogenic bacteria including *A.*

citrulli. The *hrpE* gene in T3SS is essential for the hrp pilus that plays a role in secretion of virulence factors [12]. The *hrcN* gene encodes the ATPase HrcN, which hydrolyzes ATP in vitro and is essential for T3SS and bacterial pathogenicity [11]. It is therefore not surprising that the deletion of these genes resulted in reduced or even loss of virulence of Aac-5 in both melon and watermelon seedlings [40]. When the *aacR* and *aacI* genes were mutated in our study, the virulence of the mutants was significantly reduced, suggesting that the QS contributes to virulence of *A. citrulli*. When the expression of the *hrpE* gene was measured, its level was significantly reduced 5- and 1.4-fold in the *aacR* and *aacI* mutants respectively (Fig. 6), which may result in the reduction in virulence of the mutant strains to watermelon and melon seedlings. When the expression of *hrcN* was measured, however, its level was significantly decreased in *aacR* but not in *aacI* mutant, suggesting a more significant effect of the *aacR* gene on the regulation of the T3SS-related gene *hrcN* than the *aacI* gene.

In summary, the QS system regulates the twitching motility, virulence, seed adhesion ability and biofilm formation but not flagella formation or swimming motility of the group II strain Aac-5 of *A. citrulli*. Our result differs from the previous study regarding the group I strain XJL12 of *A. citrulli* in that the biofilm formation of the *aacI* mutant in XJL12 was similar to the wild-type strain but the growth rate of the mutant was decreased [25], suggesting different effects of *aacI* on biofilm formation of different groups of *A. citrulli* strains. Our result is also different from the previous study using the group II strain AAC00-1 isolated in the United States, since no significant difference in seed-to-seedling transmission between the *aacR* mutant and the wild-type strain AAC00-1 was observed [26], although seed attachment, not seed to seedling transmission was studied in our study. It is possible that the QS system in different groups of *A. citrulli* strains or strains isolated from different geographical locations may control a particular trait differently, which warrant further studies in the future.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *A. citrulli* wild-type and mutant strains were grown in King's B (KB) broth or KA plate (KB containing agar at 15 g/liter) with appropriate antibiotics at 28°C. *Escherichia coli* strains were grown in Luria-Bertani medium (LB) medium at 37°C [41]. The antibiotics used were ampicillin (Ap), kanamycin (Km), spectinomycin (Sp) and gentamicin (Gm) at concentrations of 100 µg mL⁻¹ for Ap and 50 µg mL⁻¹ for the other antibiotics.

Construction of the *aacI* and *aacR* mutants and their complemented strains

The *aacI* and *aacR* genes in the wild-type strain Aac-5 were inactivated by homologous

integration as described by Windgassen et al. [42], respectively, using the suicide vector pK18mobsacB [43]. Primers for PCR amplification of the two genes, *aacR*-F/R and *aacI*-F/R, were designed using the free online program Primer 3.0 (<http://www.simgene.com/Primer3>) (Table 2). Each reaction mixture contained 0.5 µl of DNA template, 6.25 µl of 2×PCR Mix (TaKaRa, Dalian, China) and 0.5 µl of each primer for a total reaction volume of 12.5 µl. The PCR conditions were 94°C for 3 min, 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 90 s, followed by 72°C for 5 min. The 1,918-bp fragment amplified from the wild-type strain Aac-5 by the Rup-F and Rdn-R primers (Table 2) contained a 729-bp coding region of the *aacR* gene, as well as 515- and 548-bp upstream and downstream sequences of the gene. The 1,897-bp fragment amplified from Aac-5 by the Iup-F and Rdn-R primers (Table 2) contained a 636-bp coding region of the *aacI* gene, as well as 526- and 516-bp upstream and downstream sequences of the gene. After confirmation by sequencing, the fragments were digested by *EcoRI* and *HindIII*, as well as by *HindIII*, respectively, and cloned into pK18mobsacB to create plasmids pK18-*aacR* and pK18-*aacI* (Table 1). The two plasmids were digested with *BamHI* and *NdeI*, and the *aacR* and *aacI* gene region were replaced with a *Gm* gene cassette (855 bp), respectively, to create plasmid pK18-*aacRGm* and pK18-*aacIGm* (Table 1). The pK18-*aacIGm* and pK18-*aacRGm* were introduced from *E. coli* DH5α into Aac-5, respectively, by triparental conjugation using pRK600 as a helper plasmid. Transconjugants were screened on KB supplemented with 10% sucrose and antibiotics (Ap and Gm) and confirmed by PCR using the Rup-F/Rdn-R and Iup-F/Rdn-R primers, respectively. To confirm the presence of the *Gm* cassette in the transconjugants, Southern blotting was performed with primers Gm-F/R using marker BM5000 (Biomed, 5,000 bp, 3,000 bp, 2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp) as the probe. The confirmed *aacR* and *aacI* mutant strains, *aacI*⁻ and *aacR*⁻ (Table 1), was used for subsequent studies.

To generate complementation strains, the *aacR* and *aacI* genes including their respective promoter region (1,351 bp and 1,207 bp, respectively) in Aac-5 were amplified using primers *aacI*-F/*aacI*-R and *aacR*-F/*aacR*-R respectively (Table 2). The gene fragments were cloned separately into pHM1 to generate pHM-*aacR* and pHM-*aacI* (Table 1), which were transferred into the mutant strains *aacI*⁻ and *aacR*⁻ by triparental conjugation, respectively. Transconjugants named *aacR*⁻Comp and *aacI*⁻Comp were identified through screening on KB (amended with Ap, Sp and Gm) (Table 1).

All obtained plasmids and *A. citrulli* strains were confirmed by PCR and DNA sequencing.

Assessment of twitching motility

Ten microliter suspension ($OD_{600}=1$) of *A. citrulli* strains were placed on KA plus Ap plates and incubated for 72 h, and their twitching motility was visualized using an inverted microscope (Olympus IX83, Japan) according to the protocol of Bahar et al. [15]. Colonies with twitching motility were characterized by the formation of corrugated trajectories or haloes around the colonies.

Virulence assays

Virulence of the *A. citrulli* strains were tested on 3-week-old watermelon seedlings (*Citrullus lanatus* cultivar Jingxin#6, provided by Beijing Academy of Agriculture and Forestry Sciences, Beijing, China). To prepare bacterial inoculum, *A. citrulli* strains were grown in KB liquid medium for 24 h, and their OD_{600} were adjusted with sterile distilled water to 0.6 (approximately 10^8 CFU/mL). Two hundred milliliters of each inoculum was spray inoculated into watermelon seedlings and the disease symptoms were evaluated each day for 10 days after inoculation using a disease index (DI) calculated from a modified 0 to 9 disease severity scale: 0, no symptoms; 1, 3, 5 and 7, necrotic lesions on approximately 25%, 50%, 75% and 100% of the cotyledons, respectively; and 9, total death of the seedling. The disease index (DI) was calculated each day based on the following formula:

$$DI = \sum (A \times B) \times 100 / \sum C \times 9$$

Where A is the disease scale (0, 1, 3, 5, 7 or 9), B is the number of seedlings in each disease scale, and C is the total number of seedlings in each treatment. The experiment was repeated three times.

Biofilm assays

The effect of *aacI/aacR* deletion on biofilm formation was determined qualitatively using glass flasks as described before [45] with modifications. Briefly, overnight cultures of the *A. citrulli* strains were adjusted to an OD_{600} of 1.0. One hundred microliters of each cell suspension were transferred into 10 milliliters of KB broth in glass flasks and incubated at 28°C for 7 d without agitation. The broth was then poured out slowly, and the flasks were rinsed three times with sterile distilled water. After fixating by heating at 80°C in an oven (Mettler, Schwabach, Germany) for 30 min, biofilms on the surface of the flasks were stained with 1% crystal violet for 2 h at room temperature, followed by three washes with sterile water. A ring of violet precipitation developing on the inner wall of the glass flasks indicated biofilm formation. Biofilm formation was also analyzed quantitatively by solubilizing the stained biofilms with 3 mL of anhydrous ethanol for 12 h and measuring the OD_{580} of the stained suspension with a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany). The experiment was repeated three times.

Growth rate assays

Growth rate of *A. citrulli* strains was determined as described before [46]. One hundred microliters of *A. citrulli* strains at OD₆₀₀ of 0.1 were diluted 1000-fold with 100 ml KB medium and incubated at 28°C with shaking at 180 rpm. OD₆₀₀ of the suspensions was measured every two hours for 36 hours.

Assay for seed adhesion by *A. citrulli* strains

The effect of *aacI/aacR* deletion on seed adhesion was determined using a modified method of Tian et al. [47]. Watermelon seeds were surface sterilized by soaking in 75% (vol/vol) ethanol twice, each time for 20 min, followed by vigorous rinsing with sterile water for three times. The seeds were air dried and stored at room temperature until use. The seeds were mixed with 10 ml of 10³ CFU suspension of each *A. citrulli* strain with gentle shaking. After incubation for 2 h, the seeds were transferred to a plastic culture tube containing 10 ml of sterile water. The tube was vortexed for 1 min to remove bacterial cells that were not strongly attached to the seed surface. One seed was then placed into each tube containing 1 mL of KB, and incubated at 28°C for 48 h. Dilution plating on KA plates was done for seed suspensions in each tube to predict relative initial cell adhesion. 10 seeds were used for each treatment and the experiment was repeated at least three times.

Assay for swimming motility and observation of flagella by transmission electron microscopy

A. citrulli strains were grown in KB, and their OD₆₀₀ was adjusted to 0.6. Ten microliters of each bacterial suspension were placed on the center of a soft nutrient agar plate (0.3% agar) (basal medium) [15] for 36 h at 28°C. The diameter of the colony on each plate was then measured. There were at least 9 plates inoculated by each strain in each experiment, and the experiment was repeated three times.

To observe flagella, each *A. citrulli* strain was grown on the basal medium. Ten microliters of sterile water were placed on 36 h-old colonies for 2 min, and a 200-mesh formvar-coated copper grid was placed on top of each water drop for 1 min. The copper grids were negatively stained with 1% uranyl acetate for three time, each time for 30s. Transmission electron microscopy was used to observe polar flagella in each *A. citrulli* strain.

RNA isolation and quantitative reverse transcription (qRT)-PCR analysis of gene expression

A. citrulli strains were grown in KB at 28°C overnight to an OD₆₀₀ of 0.8. The cells were washed twice with sterilized water and used for RNA extraction with the bacterial RNAout kit (Tiandz, Beijing, China) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (New England Biolabs, Beverly, MA, USA) to eliminate any potential DNA

contaminations. The RNA was then reverse transcribed by using FastQuant RT kit (TianGen, Beijing, China) according to the instructions. Quantitative PCR analysis was carried out with gene-specific primers (Table 3) designed in this study (Table 3) using the same online program as described above. cDNA was used as a template with SYBR Green added in the PCR reaction containing SYBR Supermix (Tiandz, Beijing, China). Relative levels of gene expression were determined using the $2^{-\Delta\Delta CT}$ method [48], with the *glyA* gene as the internal control [20]. There were three technical replicates in each experiment and the experiment was repeated three times.

Statistical analysis

Statistical analysis was performed using the Student's t test in the Excel 2010 software (Microsoft Inc., Seattle, WA, USA). For seed adhesion assay, the number of bacterial cells was analyzed using Duncan's test by using the free tool R 3.1.2. Differences were considered statistically significant if $P < 0.01$.

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
Strains		
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169(Φ80lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	[41]
<i>Acidovorax citrulli</i>		
Aac-5	Wild-type watermelon strain; Ap ^r	[20]
<i>aacR</i> ⁻	<i>aacR</i> mutant, Ap ^r ; Gm ^r	This study
<i>aacI</i> ⁻	<i>aacI</i> mutant, Ap ^r ; Gm ^r	This study
<i>aacR</i> ⁻ Comp	<i>aacR</i> complementation strain, <i>aacR</i> ⁻ containing pHMaacR; Ap ^r , Gm ^r , Sp ^r	This study
<i>aacI</i> ⁻ Comp	<i>aacI</i> ⁻ complementation strain, <i>aacI</i> ⁻ containing pHMaacI; Ap ^r , Gm ^r , Sp ^r	This study
Plasmids		
pK18mobsacB	Cloning and suicide vector with a <i>sacB</i> gene for mutagenesis; Km ^r	[43]
pK18- <i>aacR</i>	pK18mobsacB carrying 729-bp coding region of the <i>aacR</i> gene, as well as 515- and 548-bp upstream and downstream sequences of the gene; Km ^r	This study
pK18- <i>aacI</i>	pK18mobsacB carrying 636-bp coding region of the <i>aacI</i> gene, as well as 526- and 516-bp upstream and downstream sequences of the gene; Km ^r	This study
pK18- <i>aacR</i> Gm	pK18- <i>aacR</i> carrying 729-bp coding region of the <i>aacR</i> gene replaced by 855-bp Gm cassette; Gm ^r , Km ^r	This study
pK18- <i>aacI</i> Gm	pK18- <i>aacI</i> carrying 636-bp coding region of the <i>aacI</i> gene replaced by 855-bp Gm cassette; Gm ^r , Km ^r	This study
pHM1	Broad-host range expression vector; Sp ^r	[49]
pHMaacR	pHM1 carrying a 1351-bp fragment containing <i>aacR</i> and its promoter; Sp ^r	This study
pHMaacI	pHM1 carrying a 1207-bp fragment containing <i>aacI</i> and its promoter; Sp ^r	This study

^aKm^r, Ap^r, Gm^r, and Sp^r indicate resistance to kanamycin, ampicillin, gentamycin, and spectinomycin, respectively.

Table 2. Primers used for construction of mutant and complemented strains

Primers	Sequence (5'-3', restriction enzyme sites are underlined)	Restriction Enzyme	Product of PCR
Iup-F	<u>AAGCTT</u> ACCAGTTCGCCACGGTCTGTCCAC	<i>Hind</i> III	1,897 bp
Idn-R	<u>AAGCTT</u> CATTGGTGATGAAGCACTTG	<i>Hind</i> III	
Rup-F	<u>GAATTC</u> AGACGCTCTACGGCACGATA	<i>Eco</i> RI	1,918 bp
Rdn-R	<u>AAGCTT</u> ACCAGTTCGCCACGGTCTGTCCAC	<i>Hind</i> III	
GmF	<u>GGATCC</u> CGACGCACACCGTGGAAA	<i>Bam</i> HI	855 bp
GmR	<u>GTCGAC</u> GCGGCG GTGACAATTT	<i>Sal</i> I	
aacI-F	AAGCTTTTG GGAGGTCGGTACTGAG	<i>Sal</i> I	1,207 bp
aacI-R	<u>GTCGAC</u> GCAGGATTTTTCTGGCGACCGTGGC	<i>Hind</i> III	
aacR-F	<u>AAGCTT</u> GTAGGAAGGGCGGGGGGC	<i>Sal</i> I	1,351 bp
aacR-R	<u>GTCGAC</u> GCGGGATTGGCATTGGGGG	<i>Hind</i> III	

Table 3. Primers designed in this study for quantitative reverse transcription PCR

Primer set	Sequences (5 - 3)	Size of PCR products (bp)	Target
pilT-F	ATGACGCTGTCCTTCGCCGC	142	<i>pilT</i>
pilT-R	GGGTGCCGAACACGAGGTGG		
hrcN-F	CGAACACCGAGGGCGGAAAG	184	<i>hrcN</i>
hrcN-R	TGCTGCTGATGATGGATTCTG		
hrpE-F	GTCAGGATGGACACGCAGGC	110	<i>hrpE</i>
hrpE-R	AACGCATGGTGCTGGCAGAG		
glyA-F	GCGGCAAGTGGTTCAACATC	139	<i>glyA</i>
glyA-R	GCGATGTCCACCCAGAAGAT		

Figure legend:

Fig. 1 Comparison of twitching motility in *A. citrulli* strains under inverted microscopy (A) and by *pilT* gene expression (B). The wild-type strain Aac-5 and its *aacR* and *aacI* mutant complementation strains *aacR*Comp and *aacI*Comp produced typical haloes around bacterial colonies, indicating bacterial motility via twitching. The *aacR* and *aacI* mutant strains *aacR*⁻ and *aacI*⁻, however, produced little or no haloes. The *pilT* gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method [47], and normalized using the expression level of the internal control gene *glyA*. Error bars represent standard deviations from three biological repeats. **: $P < 0.01$ by the Student's t test. The experiment was repeated three times with similar results.

Fig. 2 Effect of *aacR* and *aacI* on biofilm formation in *A. citrulli*, as visualized by the violet ring formed on the inner surface of the glass flasks (A) and measured under OD₅₈₀ using stained biofilm solubilized with ethanol (B). The error bar represents standard errors of the means from three experiments, and ** indicates $P < 0.01$ by the Student's t test. Aac-5, wild-type strain of *A. citrulli*; *aacR*⁻, *luxR* equivalent gene deletion mutant of Aac-5; *aacI*⁻, *luxI* equivalent gene deletion mutant of Aac-5; *aacR*Comp, *aacR* complemented strain of *aacR*⁻; *aacI*Comp, *aacI* complemented strain of *aacI*⁻.

Fig. 3 Effect of *aacR* and *aacI* on attachment of *A. citrulli* strains to watermelon seeds. The amount of bacterial cells attached to watermelon seeds were quantified by dilution plating. The bars represent standard errors of the means from three experiments for each strain. Different letters indicates significant differences ($P < 0.05$) among strains.

Fig. 4 Effect of *aacR* and *aacI* on *A. citrulli* virulence on watermelon leaves. Disease index was calculated based on a 0 to 9 disease severity scale of each seedling. The bars represent standard errors of the means from three experiments. ** indicates $P < 0.01$ by the Student's t test.

Fig. 5 Effect of *aacR* and *aacI* on formation of polar flagellum observed under transmission electron microscopy (A) and swimming motility of *A. citrulli* strains (B). *A. citrulli* swimming motility was indicated by the formation of haloes with its diameter measured 36 h after inoculation of the center of each of the soft basal medium plates with each strain of *A. citrulli*. The bars represent the standard errors of the means from five experiments with nine plates in each experiment for each strain. Polar flagella (indicated by arrows) were observed in all strains of *A. citrulli* including the wild-type strain Aac-5, and the mutant strains *aacR*⁻ and *aacI*⁻.

Fig. 6. Effect of *aacR* and *aacI* on the expression of the type III secretion system-related genes *hrpE* and *hrcN* in the wild-type strain Aac-5 (mathematically designated as 1) and the mutant strains *aacR*⁻ and *aacI*⁻ of *A. citrulli*. Each gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method [45], and normalized using the internal control gene *glyA*. Error bars represent standard deviations from three biological repeats. The experiment was repeated three times with similar results. ** indicates $P < 0.01$ by the Student's t test.

A

B