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Tn5 transposon mutagenesis in *Acidovorax citrulli* for identification of genes required for pathogenicity on cucumber

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An *Acidovorax citrulli*–cucumber pathosystem was established through which *A. citrulli* mutants with altered pathogenicity, generated by transposon mutagenesis, were identified on cucumber cotyledons. The *A. citrulli* group I strain FC440 was shown to grow faster in cucumber leaf tissues than a group II strain and was used for Tn5 transposon mutagenesis. A total of 2100 Tn5 insertional mutants were generated, and analysis of the mutant library showed that the transposon insertions were single, independent and stable. A conserved non-flagellar type III secretion system (NF-T3SS) ATPase gene *hrcN* was identified and confirmed to be essential for pathogenicity and functionality of NF-T3SS in *A. citrulli*. Comparative sequence analysis of the HrcN protein and its homologues in other representative bacterial plant pathogens revealed that the NF-T3SS of *A. citrulli* is close to that of *Ralstonia solanacearum* and *Xanthomonas campestris*, but distant from that of *Pseudomonas syringae* and *Erwinia amylovora*. The generated Tn5 insertional mutant collection is valuable for identification of genes required for *A. citrulli* pathogenesis, and the established *A. citrulli*–cucumber pathosystem will facilitate an improved understanding of *A. citrulli* biology and pathology.

Keywords: *Acidovorax citrulli*, bacterial fruit blotch, *Cucumis sativus*, pathogenicity, T3SS ATPase, transposon mutagenesis

Introduction

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB), a sporadic but devastating disease of cucurbits worldwide (Schaad *et al.*, 1978, 2008; Willems *et al.*, 1992). Since the first natural outbreaks in 1987 and 1989 in commercial watermelon (*Citrullus lanatus*) fields on the Mariana Islands in the South Pacific and in Florida, USA (Wall & Santos, 1988; Somodi *et al.*, 1991), BFB has spread worldwide and has been observed to cause diseases on other cucurbits, such as honeydew melon (*Cucumis melo*) (Isakeit *et al.*, 1997), citron melon (*Citrullus lanatus* var. *citroides*) (Isakeit *et al.*, 1998; Pallen *et al.*, 2005), pumpkin (*Cucurbita pepo*) (Langston *et al.*, 1999; Walcott *et al.*, 2004), squash (*Cucurbita maxima*) (Walcott *et al.*, 2004) and cucumber (*Cucumis sativus*) (Martin *et al.*, 1999; Burdman *et al.*, 2005; Liu *et al.*, 2009).

Acidovorax citrulli is a Gram-negative, rod-shaped bacterium that can be transmitted through seed. Under high relative humidity and high temperature, the bacteria spread rapidly throughout transplant greenhouses and in the field, leading to seedling lesions, blight or fruit rot (Schaad *et al.*, 2003). BFB has great potential to cause significant economic losses to cucurbit production, and has been responsible for losses of up to 50–90% of marketable fruits in some watermelon fields (Latin & Rane, 1990; Somodi *et al.*, 1991). Currently, strategies for managing BFB are limited and there are no available resistant commercial cultivars, making BFB a serious threat to the cucurbit industry worldwide (Bahar & Burdman, 2010). Understanding the complex interactions between the host plant and the pathogen is critical for the development of effective disease control measures. Unfortunately, the genetic and biochemical mechanisms employed by *A. citrulli* to ensure infection and colonization in the host plant are largely unknown.

Current knowledge on the biology and pathology of *A. citrulli* is limited. One of the important findings is the identification of pathogen differentiation in population structure and host range, which is useful for screening for BFB resistance and understanding pathogenicity mechanisms of *A. citrulli*, and for providing the framework for further investigation of the evolutionary, ecological and epidemiological significance of the pathogen (Walcott

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et al., 2000). Since the end of the 1980s, BFB has been a serious threat, mainly for watermelon. In 1991, it was reported that *A. citrulli* isolated from the 1989 BFB outbreak in Florida produced a hypersensitive response on tobacco and tomato, whereas the subspecies type strain did not (Somodi *et al.*, 1991). Subsequent discoveries have aroused serious concern worldwide: trends of devastating BFB outbreaks in other non-watermelon cucurbits (Isakeit *et al.*, 1997, 1998; Langston *et al.*, 1999; Martin *et al.*, 1999), and the discovery of two subgroups (group I and group II) within *A. citrulli* with significant differences in host range and pathogenicity (O'Brien & Martin, 1999; Walcott *et al.*, 2000, 2004; Burdman *et al.*, 2005). It was found that group I strains could moderately infect all cucurbit hosts, and group II strains were more aggressive on watermelon than on other hosts. These discoveries indicated that the population structure of the pathogen has been changing and the host range of *A. citrulli* has been expanding.

Another important finding is the identification of genes required for *A. citrulli* pathogenicity. The complete genome sequence of the group II strain AAC00-1 of *A. citrulli* was determined in 2007 (released by the Joint Genome Institute; GenBank accession number NC_008752) which facilitated the study of the genetic basis of BFB pathogenesis. The type III secretion system (T3SS) has been discovered in *A. citrulli* through genome sequencing (GenBank accession number AY898625) and was confirmed to be essential for pathogenicity in *A. citrulli* on the host plants watermelon and melon by gene disruption experiments of the T3SS structural genes *hrcR* and *hrcC* (Ren *et al.*, 2009; Johnson *et al.*, 2011). In addition, the type IV secretion system has been identified as required for virulence (Bahar *et al.*, 2009). However, successful establishment of *A. citrulli* in the host tissues requires the coordinated activity of many genes, for which the identities and modes of action are still largely unknown.

Several research groups have used *A. citrulli*–watermelon or *A. citrulli*–melon interaction models (Bahar *et al.*, 2009; Ren *et al.*, 2009; Johnson *et al.*, 2011) to explore the molecular basis of BFB pathogenesis. The reported findings have focused mainly on the pathogenesis on these two hosts. However, it should be noted that both group I and II strains resulted in higher percentages of symptomless seedlings in watermelon and melon than in cucumber in seed transmission assays; the percentages of symptomless seedlings with group I and II strains were 13% and 79%, respectively, for watermelon; 9% and 90%, respectively, for melon; and only 1·7% and 46·2%, respectively, for cucumber (Burdman *et al.*, 2005). These data suggest cucumber is probably a more suitable host for investigating BFB pathogenesis. With a relatively small and sequenced genome (Huang *et al.*, 2009) and a higher transformation efficiency (Selvaraj *et al.*, 2010) than other cucurbits, cucumber emerges as a model species in cucurbits. It is therefore very likely that the *A. citrulli*–cucumber interaction system will promote an increased understanding of the molecular basis of the pathogenicity of this pathogen.

The aim of this study was to explore the *A. citrulli*–cucumber interaction as a model pathosystem for the study of the molecular mechanisms of BFB pathogenesis. The *A. citrulli*–cucumber system was used to determine pathogenicity and identify mutants of *A. citrulli* with altered pathogenicity. In addition, the HrcN protein of *A. citrulli* was compared with its homologues in other representative plant pathogenic bacteria.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Acidovorax citrulli* group I strain FC440 and group II strain AW0601 were originally isolated from diseased cantaloupe (*Cucumis melo* var. *cantalupensis*) and watermelon (*Citrullus lanatus*) in Xinjiang, China, respectively (Xu, 2007). *Acidovorax citrulli* strains were grown in nutrient broth (Difco) or on King's medium B (KMB) (King *et al.*, 1954) at 28°C. *Escherichia coli* cells were cultivated at 37°C in Luria–Bertani (LB) liquid medium (Sambrook & Russell, 2001). Plasmids were introduced into *E. coli* by electroporation (Sambrook & Russell, 2001) and into *A. citrulli* by diparental conjugation (De Bruijn & Rossbach, 1994). Antibiotics were added to the media at the following final concentrations, if not stated otherwise: ampicillin, 30 μg mL^{-1}; kanamycin, 50 μg mL^{-1}; tetracycline, 10 μg mL^{-1}.

Growth of plants and bacteria

Cucumber cv. Nongcheng No. 3 and *Nicotiana benthamiana* were grown in growth chambers in plastic pots under a 16-h photoperiod at 25°C with standard potting soil. Fruits of cucumber cv. Jingyanmini No. 2 were purchased from the market. Inoculated plant materials were kept under the same growth conditions supplemented with 95% humidity. Bacterial inoculum was prepared based on the linear regression equation between the bacterial suspension concentration of *A. citrulli* and OD_{600} value, which was $y = 4 \times 10^{-10} x + 0{\cdot}1128$, where x was the concentration of bacterial cells suspension (CFU mL^{-1}) and y was the OD_{600} value (Peng *et al.*, 2007). To prepare inoculum, a fresh colony of bacteria was inoculated into 2 mL nutrient broth and incubated at 28°C with shaking at 220 rpm. After 18 h of incubation, cells from 1 mL culture ($OD_{600} \approx 1$) were collected by centrifuging at 1000 ***g*** for 10 min, and resuspended in 2 mL ddH_2O. The optical density of the cell suspension was adjusted to an OD_{600} value of 0·5, which corresponds to a concentration of approximately $9{\cdot}68 \times 10^8$ CFU mL^{-1}.

Plant inoculations

Fully expanded leaves of 6-week-old *N. benthamiana* plants were used for hypersensitive response assays by infiltration of bacterial suspension ($9{\cdot}68 \times 10^8$ CFU

Table 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristics	Reference/source
Acidovorax citrulli strains		
FC440	Amp^R; wildtype, group I strain	N. W. Schaad
AW0601	Amp^R; wildtype, group II strain	B. S. Hu
FC440(Δ*hrcN*)	Amp^R; Kan^R; FC440 mutant defective in *hrcN*	This work
FC440(Δ*hrcN* +pHC60)	Amp^R; Kan^R; Tet^R; FC440 (Δ*hrcN*) strain containing expression vector pHC60	This work
FC440(Δ*hrcN* + *hrcN*)	Amp^R; Kan^R; Tet^R; FC440 (Δ*hrcN*) complemented with *hrcN* gene expressed by vector pHC60	This work
Escherichia coli strains		
DH10B	F^- *endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139* Δ(*ara, leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ^-	Invitrogen
$S_{17\text{-}1}$ λ pir	*E. coli* 294, *thi* RP4-2-Tc::Mu-Kan::Tn7 chromosomally integrated	Simon *et al.* (1983)
Plasmids		
pRLl063a	Broad-host-range plasmid Kan::Tn5 with *Vibrio fischeri luxAB* as reporter	Wolk *et al.* (1991)
pHC60	Tet^R; expression vector; low copy number	Cheng & Walker (1998)
pHC60 *hrcN*	Tet^R; pHC60 containing a 1341-bp fragment with the *hrcN* gene; used to complement FC440(Δ*hrcN*)	This work

Amp^R, Kan^R and Tet^R = resistant to ampicillin, kanamycin and tetracycline, respectively.

mL^{-1}) from the abaxial side using a blunt syringe. Visible hypersensitive reaction (HR) was observed 48 h post-infiltration. On cucumber fruits, 100 μL inoculum (containing approximately $9{\cdot}7 \times 10^7$ bacterial cells) were injected through the peel for investigation of BFB symptom development. Sterile distilled water was used for controls. Bacterial pathogenicity assays were performed on cotyledons of 6-day-old cucumber seedlings by scratching the adaxial surface with a flamed needle and then inoculating with 20 μL bacterial suspension (approximately $1{\cdot}9 \times 10^7$ bacterial cells). Five days after inoculation, BFB severity was evaluated based on a 0–4 scale: 0, no symptoms; 1, slight water-soaked lesion or chlorotic area on cotyledons; 2, extensive water-soaking on cotyledons; 3, collapse of the infected cotyledons or spread of the infection to true leaves; 4, collapse of the whole seedling. Each *A. citrulli* strain tested included at least six replicates (three plants) per experiment. Mutants with altered pathogenicity were screened using the wildtype FC440 strain as the control. Except for the screening of altered-pathogenicity mutants from the mutant library, each experiment mentioned above was performed at least three times for each tested strain. Two-sided *t*-tests were conducted using the statistical software R (version 2·13·0) (R Development Core Team, 2011).

Quantification of *in planta* bacterial growth

To determine levels of bacterial growth *in planta*, leaves of 3-week-old cucumber seedlings were vacuum-infiltrated with bacterial suspensions of 10^5 CFU mL^{-1} following the previous reported procedure (Katagiri *et al.*, 2002) with some modification. Detached leaves were placed in the bacterial suspension and a vacuum was applied to 95 kPa followed by a slow release to infiltrate the leaves uniformly. Leaves were then transferred to plastic trays and the petioles were covered with cotton balls soaked in sterile distilled water. The trays were incubated in growth chambers for 5 days. To measure bacterial multiplication within leaves, three leaf discs from cucumber leaves (surface-disinfected with 70% ethanol solution before use) were ground in sterile ddH_2O, and serial dilutions were spread onto KMB plates supplemented with ampicillin for genetic selection. Bacterial population size was examined up to 5 days (bacterial populations in leaves were sampled immediately after infiltration and on the 3rd and 5th day thereafter) post-inoculation. Relative growth rates were calculated based on the initial pathogen population infiltrated into the plant tissue (0 days after infiltration). Two-sided *t*-tests were performed using statistical software R (version 2·13·0). Three independent experiments were carried out for each strain.

Molecular manipulation

Routine molecular manipulations were carried out using standard procedures (Sambrook & Russell, 2001). Unless otherwise stated, all molecular biology reagents, including restriction enzymes, DNA polymerase and T4 DNA ligase, were from TaKaRa Biotech Inc. Kits for plasmid and PCR product purification were purchased from Axygen Scientific Inc. Kits for genomic DNA isolation from bacterial cells were purchased from Tiangen Biotech Inc. Oligonucleotide primers and DNA sequencing were synthesized or performed by the Beijing Genomics Institute.

Generation of *A. citrulli* Tn5 transposon mutants by diparental conjugation

A suicide vector carrying the transposon Tn5, pRLl063a Kan::Tn5 (Wolk *et al.*, 1991), was used for *A. citrulli* mutagenesis. A broad-host *E. coli* strain $S_{17\text{-}1}$ (Simon *et al.*, 1983) was used as the host of the suicide vector. Diparental conjugation was carried out following a standard procedure (De Bruijn & Rossbach, 1994) with

minor modifications. A 2-mL culture was started with a fresh colony of wildtype FC440 in nutrient broth supplemented with ampicillin and was grown at 28°C with shaking at 220 rpm until log-phase growth. The cultures were diluted 1:10 in fresh medium and incubated for another 5 h. The same procedure was used for the donor strain $S_{17\text{-}1}$ pRL1063a, except the culture was grown at 37°C in LB broth supplemented with kanamycin. The *A. citrulli* FC440 and $S_{17\text{-}1}$ pRL1063a cells were pelleted at 1000 ***g*** for 10 min, then resuspended in 0·01 M PBS to an OD_{600} value of 0·3 for *A. citrulli* FC440 ($4{\cdot}68 \times 10^8$ CFU mL^{-1}) and 0·6 for $S_{17\text{-}1}$ pRL1063a. To generate Tn5 insertion mutants, equal volumes of *A. citrulli* FC440 and $S_{17\text{-}1}$ pRL1063a cells were mixed and 100 μL mixture was pipetted onto sterile mating filter membranes (25 mm nitrocellulose filter membrane) placed on KMB plates without antibiotics and incubated for 48 h at 28°C. In each set of mating experiments, 100 μL of *A. citrulli* FC440 and $S_{17\text{-}1}$ pRL1063a cells were spotted separately onto the mating membrane as controls. Cells from the mating filters were selected by culturing in 1 mL nutrient broth supplemented with ampicillin and kanamycin at 28°C with shaking at 220 rpm for 24 h. Each culture mixture was further streaked individually onto solid KMB containing ampicillin and kanamycin and incubated for an additional 48 h. One colony from each mating was chosen for the third round of selection. Candidate mutants were stored at −80°C with 15% glycerol. To verify the exconjugants after three rounds of selections, colony PCR assays were used for detection of *luxAB* in the genome of the exconjugants resulting from introduction of suicide vector pRLl063a and for verification of *A. citrulli*, using primer pairs of LUXP1/LUXP2 (Li *et al.*, 2004) (5′-TCG GCTTGGTATCGC/CTTAGGTCCATTCTCA-3′) and SEQID4^m/SEQID5 (5′-GTCATTACTGAATTTCAACA/CCTCCACCAACCAATACGCT-3′) (Schaad *et al.*, 2000; Walcott *et al.*, 2003), respectively. Both plasmid DNA of pRL1063a and cells of wildtype strain FC440 were used as positive controls. For determination of the transposition frequency, cells washed from the mating filter in 1 mL nutrient broth supplemented with ampicillin and kanamycin were serially diluted in 2 mL selection broth followed by incubation at 28°C with shaking at 220 rpm for 6, 12 and 24 h. Finally, 100 μL of each cell culture were plated onto selection plates and the number of colonies resistant to both antibiotics was scored after 48 h of incubation at 28°C. Transposition frequency was defined as the number of Tn5-containing exconjugants divided by the total number of recipient cells of *A. citrulli* FC440.

Southern blot analyses of *A. citrulli* Tn5 mutants

There are five *Pst*I restriction sites in the pRL1063a vector, including three in the region introduced into the *A. citrulli* genome through transposition of Tn5. By digestion with *Pst*I, a 3·8-kb fragment from plasmid pRL1063a and a fragment of at least 3·6 kb from the Tn5 insertion region in the *A. citrulli* genome carrying the *luxAB* gene are expected to be released. For Southern blot analyses, 1 μg total *A. citrulli* genomic DNA and 0·1 μg pRL1063a plasmid DNA were restriction-digested with *Pst*I, resolved in 1·0% agarose gel in 1× TAE and transferred to Hybond N^+ nylon membrane (Amersham–Pharmacia) using 20× SSC as the transfer agent following a standard procedure (Sambrook & Russell, 2001). The membrane was baked at 80°C for 2 h to fix the DNA. A 1·6-kb *luxAB* fragment was PCR-amplified from plasmid pRL1063a using primers LUXP1 and LUXP2, as described above, and used as a probe for Southern hybridization. Approximately 100 ng purified PCR product was ^{32}P-labelled with random primers. Membrane hybridizations were done at 65°C for 16 h using 6× SSPE with 5× Denhardt's solution (1× Denhardt's solution contains 0·02% Ficoll 400, 0·02% PVP, 0·02% BSA) and 0·5% SDS as the hybridization buffer as described by Sambrook & Russell (2001). Following hybridization, the membranes were washed at 65°C twice in 2× SSC with 0·1% SDS for 15 min, and twice in 0·2× SSC with 0·1% SDS for 15 min. The signals were exposed and visualized on a FLA-7000 phosphor-imager (Fuji Photo Film Co. Ltd).

Cloning of Tn5-tagged regions of *A. citrulli* mutants and sequence analysis

Because pRL1063a contains a replication origin (*oriT*) that functions in *E. coli*, though not in *A. citrulli*, DNA sequences surrounding the transposon insertion sites can be recovered from the genome of *A. citrulli* mutants using a plasmid rescue procedure (Wolk *et al.*, 1991). Briefly, the genomic DNA of the exconjugant was extracted and digested with *Eco*RI, which cuts the genome frequently but does not cut the transposon Tn5, keeping the Kan^R gene and the *oriT* fragment intact. Digestion products were then self-ligated and transformed into *E. coli* DH10B by electroporation (Sambrook & Russell, 2001). Colonies resistant to kanamycin were sequenced using primers MUP/MDP (5′-TATCAATGAGCTCGGTACCC/AGGAGGTCACATGGAATATCAGAT-3′) (Lü *et al.*, 2005). The obtained sequences were subjected to BLAST searches against the genome sequence of *A. citrulli* AAC00-1 (GenBank accession number NC_008752). To explore if GC content and gene density bias existed in the surveyed insertion sites, the background GC content of each kilobase was calculated without overlap, and gene density was computed every 50 kb with 1 kb moving each step. The calculation was performed by Perl 5 (Wall, 2011) and illustrated by R (version 2·13·0) based on the *A. citrulli* genome. To detect if position bias existed, chi-square tests were performed manually.

Construction of an *hrcN* expression vector and genetic complementation in *A. citrulli*

Expression vector pHC60 (Cheng & Walker, 1998) was used for genetic complementation assays of candidate

genes. *Escherichia coli* strain $S_{17\text{-}1}$ was used as the host of the expression vector. For the generation of an *hrcN* expression construct, *hrcN* was amplified by PCR from *A. citrulli* strain FC440 using primers ATPF/ATPR (5′-GCCGGTACCGGATGACGATGCATGACG/CTAG TCTAGAGGCATCAACCCGAGATGTCGG-3′) (*Kpn*I site and *Xba*I site underlined, respectively), and cloned into pHC60. The resulting construct, pHC60 *hrcN*, was transferred into *E. coli* strain $S_{17\text{-}1}$ and conjugated into strain FC440 (Δ*hrcN*), giving FC440 (Δ*hrcN*+*hrcN*). Plasmid pHC60 was transferred into FC440 (Δ*hrcN*) following the procedures used for strain FC440 (Δ*hrcN*+pHC60) (see above).

Protein phylogenetic analysis

The protein sequences of T3SS ATPase (HrcN), flagellum-specific ATP synthase (FliI), and F_0F_1 ATP synthase subunit beta (ATP_beta) used in this study are listed in Table 2. Amino acid sequences were aligned by ClustalW (Larkin *et al.*, 2007), and phylogenetic analyses were performed using the neighbour-joining method via MEGA 4 (Tamura *et al.*, 2007) with default parameters, bootstrapped 1000 times.

Results

Pathogenicity determination of *A. citrulli* on cotyledons of cucumber

As a model plant for the cucurbits, cucumber was selected as a host of *A. citrulli* for the study of its biology and pathogenicity. Two *A. citrulli* strains, FC440 and AW0601, representing groups I and II, respectively, were used for plant infection assays. Scratch-wound inoculation of cotyledons indicated that BFB severity was significantly different between the two group strains at the same bacterial concentration. Strain FC440 showed significantly more aggressiveness than AW0601 on cucumber (P = 3·851E-04) (Fig. 1a,b). Monitoring the multiplication of bacteria within leaf tissues further confirmed that strain FC440 grew significantly faster than strain AW0601 (P = 3·914E-05 and 1·148E-03 on the 3rd and 5th days, respectively; Fig. 1c). Group I strain FC440 was therefore chosen for use in subsequent experiments to establish an *A. citrulli*–cucumber pathosystem.

Strain FC440 was shown to successfully infect and colonize cotyledons, true leaves and fruits of cucumber by wound or needle injection inoculations (Fig. 1d–f). Symptoms on both cotyledons and true leaves were similar to those described previously (Martin *et al.*, 1999; Burdman *et al.*, 2005). Initial symptoms included chlorotic, water-soaked lesions that later dried out to form necrotic spots of light brown, dead tissue. Typically, small water-soaked spots developed around inoculation sites in the leaves, and large chlorotic areas formed on the surface of fruits. Water-soaked lesions were observed on cotyledons of 6-day-old seedlings 5 days post-inoculation, or on true leaves of 3-week-old seedlings 8 days post-inoculation. It took at least 8 days for visible lesions to develop on cucumber fruits. Therefore, the group I strain FC440 and cotyledons of 6-day-old cucumber seedlings were used for pathogenicity assays and mutant screening.

Generation of *A. citrulli* mutants by Tn5 transposon mutagenesis

It was difficult to achieve transposition in *A. citrulli*. Electroporation with the suicide vector pRL1063a failed to produce double antibiotic-resistant colonies (Amp^R and Kan^R). With diparental conjugation of wildtype FC440 and $S_{17\text{-}1}$ pRL1063a with 12 or 24 h conjugation followed by plating appropriate dilutions on selective agar media plates it was difficult to obtain positive colonies. When conjugation was extended to 48 h, the background on selection plates could be very low (no colonies) or very high, even diluted at 10^{-10} (too many colonies to select). To facilitate selection of exconjugants, the first round of selection was performed in liquid medium to enrich the exconjugants. Through a combination of 48 h conjugation and 24 h selection in double antibiotic liquid medium, an average of $1{\cdot}16 \times 10^4$ positive colonies were

Table 2 Bacterial FliI, ATP_beta and HrcN protein sequences

Designation[a]	Description	Organism	No. of residues	Accession no.[b]
Ac FliI	Flagella-specific ATP synthase	*Acidovorax citrulli*	474	ABM34932
Ps FliI	Flagella-specific ATP synthase	*Pseudomonas syringae* pv. *tomato* DC3000	452	AAO55479
Xc FliI	Flagella-specific ATP synthase	*Xanthomonas campestris* pv. *campestris*	458	YP_001903624
Ac ATP_beta	F_0F_1 ATP synthase subunit beta	*Acidovorax citrulli*	476	YP_968753
Ps ATP_beta	F_0F_1 ATP synthase subunit beta	*Pseudomonas syringae* pv. *tomato* DC3000	459	NP_795317
Xc ATP_beta	F_0F_1 ATP synthase subunit beta	*Xanthomonas campestris* pv. *campestris*	468	YP_001905199
Ac HrcN	Type III secretion system ATPase	*Acidovorax citrulli*	442	ABM31070
Ps HrcN	Type III secretion system ATPase	*Pseudomonas syringae* pv. *tomato* DC3000	419	AAG33879
Xc HrcN	Type III secretion system ATPase	*Xanthomonas campestris* pv. *campestris*	438	YP_001904474
Rs HrcN	Type III secretion system ATPase	*Ralstonia solanacearum*	439	NP_522431
Ea HrcN	Type III secretion system ATPase	*Erwinia amylovora*	454	CBX79348

[a]The two-letter abbreviations of the source organism (e.g. Ac, *A. citrulli*) are followed by protein designations.
[b]Accession numbers in this table are retrieved from the GenBank database.

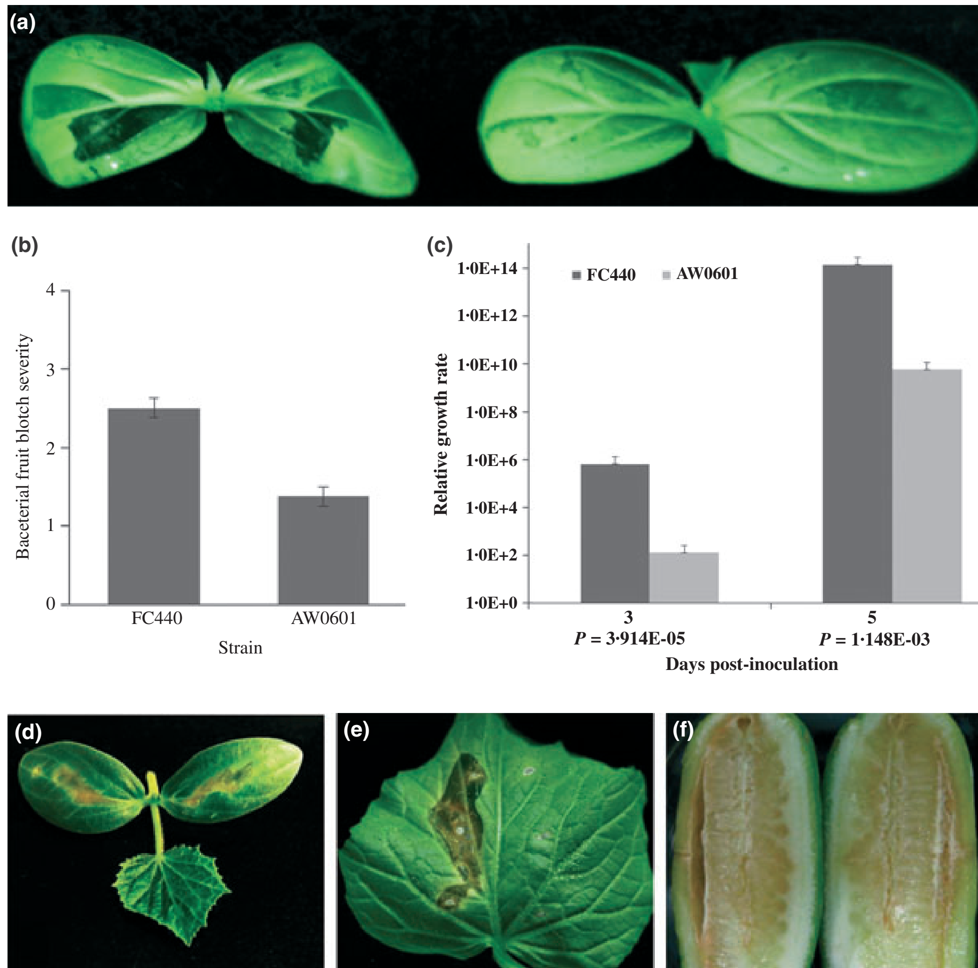

Figure 1 Pathogenicity determination of *Acidovorax citrulli* on cucumber (*Cucumis sativus* cv. Nongcheng No. 3). (a) Water-soaked lesions developed 5 days post-inoculation (dpi) on cotyledons of 6-day-old cucumber seedlings by scratch-wound inoculation with 20 μL bacterial cell suspension at $9{\cdot}68 \times 10^8$ CFU mL^{-1}. Left panel: cotyledons treated with strain FC440; right panel: cotyledons inoculated with strain AW0601. (b) Comparison of strains FC440 and AW0601 in causing bacterial fruit blotch severity. Symptom severity was scored 5 dpi according to the following scale: 0, no symptoms; 1, slight water-soaked lesion or chlorotic area on cotyledons; 2, extensive water-soaking on cotyledons; 3, collapse of the infected cotyledons or spread of the infection to true leaf; 4, collapse of the whole seedling. Error bars indicate standard errors from three independent experiments. (c) Multiplication of wildtype strains FC440 and AW0601 within cucumber leaves. Detached leaves were infiltrated with bacterial suspensions ($\sim 10^5$ CFU mL^{-1}), and relative growth rate (RGR) was determined at the indicated time points. Student's *t*-test showed the differences between treatments. Error bars indicate standard deviation. Three independent experiments were performed with similar results. Bacterial fruit blotch (BFB) symptoms formed on cotyledons 5 dpi (d), on true leaf 8 dpi (e) and on flesh of fruit 8 dpi (f). A droplet of 20 μL bacterial suspension ($9{\cdot}68 \times 10^8$ CFU mL^{-1}) of strain FC440 were wound-inoculated on cotyledons of 6-day-old seedlings or leaves of 3-week-old seedlings of cucumber cv. Nongcheng No. 3, respectively. To the right of the main vein on the true leaf: dH_2O negative control. Cucumber cv. Jingyanmini No. 2 fruits were injected with 100 μL bacterial suspension ($9{\cdot}68 \times 10^8$ CFU mL^{-1}).

recovered per mating, giving a transposition frequency of $5{\cdot}04 \times 10^{-4}$ per cell of *A. citrulli* FC440. However, this was most likely an overestimation because the initial recipient cells had multiplied for several generations and the numbers of exconjugants may also have increased during such a long conjugation and selection process. The results appeared to be in agreement with those for several other strains of *A. citrulli* for which transposon mutation also proved difficult (Tingchang Zhao, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, personal communication). The duration of conjugation was extended to 48 h and exconjugant growth in selective liquid medium to 24 h. To avoid inclusion of possible redundant colonies in the mutant collection and to minimize the effort of mutant screening in downstream experiments, particularly in screening for altered-pathogenicity mutants, one individual colony was selected from colonies produced by each diparental conjugation mating after the third round of selection. An average of 98·4% matings produced ampicillin- and

kanamycin-resistant colonies after three rounds of selection, and 98·3% of these colonies were verified to be *A. citrulli* by PCR amplification. A total of 2100 transconjugants were generated and stored at −80°C.

The quality of the library was assessed by Southern blotting analyses of five randomly selected candidate mutants. A single chromosome DNA fragment hybridized with the Tn5 probe, suggesting single Tn5 insertions in the chromosome (Fig. 2a). The transposon boundaries of 10 randomly selected mutants were sequenced in order to localize the insertion sites in the *A. citrulli* FC440 genome (Supplementary Table S1). Sequence analysis indicated that all 10 inserts generated 9-bp duplications at the boundaries, consistent with a Tn5 transposition. The majority of insertion sites were located inside recognized or hypothetical ORFs. The high percentage of intragenic insertion events implied a high probability of obtaining various kinds of functionally disabled mutants, including those with altered pathogenicity.

Library screening using the developed pathogenicity assays on cucumber cotyledons led to the identification of six altered-pathogenicity mutants from 1436 screened mutants, and three of them lost their abilities to cause BFB symptoms on susceptible hosts (cucumber, melon, watermelon, squash and pumpkin) and to induce HR in non-host tobacco. The remaining three showed reduced pathogenicity on host plants. Cloning and sequence analysis of the insertion sites revealed several interesting genes that probably contribute to pathogenicity. These included the glucose-inhibited division protein A gene, the flagellar hook-associated protein 3 gene, a predicted secretion-associated protein gene, and a predicted T3SS ATPase gene (genes Aave-0051, Aave-4431, Aave-0473 and Aave-0463, respectively, in the sequenced strain of AAC00-1; GenBank accession number NC_008752). The disruption of the former two individual genes led to reduced pathogenicity of *A. citrulli* on its hosts, whereas the disruption of the latter two caused pathogenicity deficiency in the pathogen. GC content and gene density bias were not found in the 10 randomly selected candidates (Fig. 2b). The insertion sites appeared to be clustered within the 4·1–5·2 Mb region of the *A. citrulli* genome,

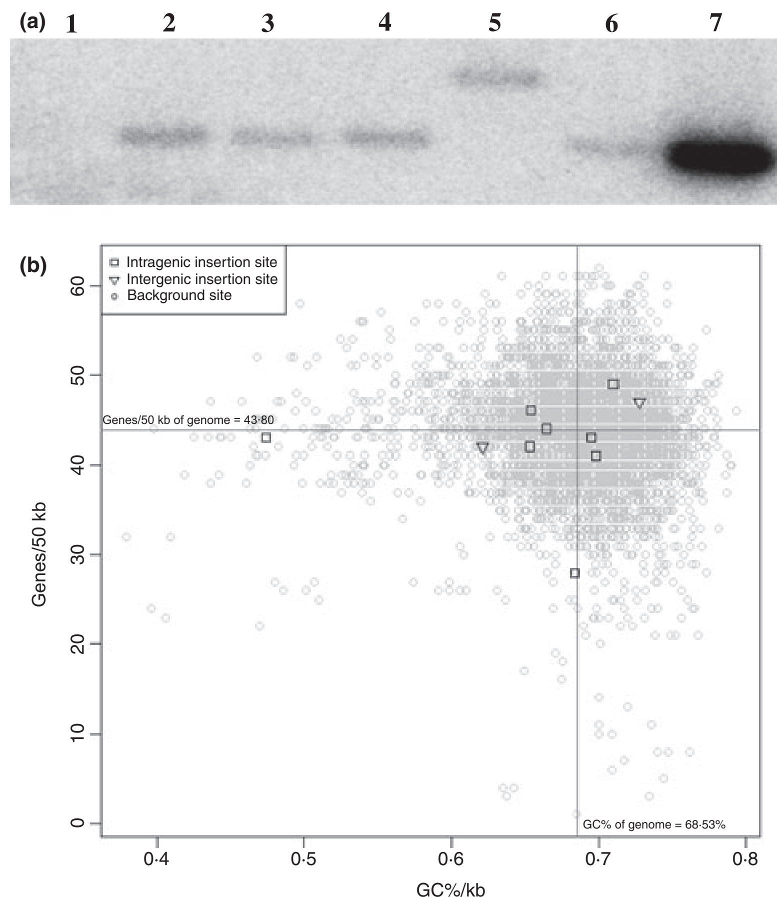

Figure 2 Analysis of transposon insertion mutants of *Acidovorax citrulli* FC440. (a) Southern blot analysis of five randomly selected individual transposition colonies. *Pst*I-digested genomic DNA (approximately 1 μg) isolated from wildtype *A. citrulli* (lane 1) and its Tn5 insertional mutants (lanes 2–6). Tn5 (3809 bp) was released from plasmid pRL1063a by *Pst*I digestion and used as positive control (100 ng) (lane 7). (b) GC content and gene density bias of the insertion sites in the *A. citrulli* genome were analysed with 10 randomly selected individual Tn5 mutants by Perl scripts and illustrated by R based on the *A. citrulli* genome.

but a chi-square test could not reject the hypothesis of their uniform distribution ($\chi^2 = 7 < \chi^2_{0{\cdot}10}(4) = 7{\cdot}779$).

To assess the stability of the *A. citrulli* Tn*5* mutants, transposon boundaries of the above 10 mutants, which remained pathogenic, were analysed after three rounds of inoculations of cucumber cotyledons using single colonies. The results showed that all tested mutants were pathologically and genetically stable.

Role of the HrcN protein in pathogenicity and NF-T3SS functionality in *A. citrulli*

One of the altered-pathogenicity mutants was shown to be disrupted with a predicted T3SS ATPase, as mentioned above, which is homologous to the NF-T3SS HrcN protein. The transposon insertion site was at bp 938 of the 1329-bp *hrcN* gene. This mutant, designated FC440 (Δ*hrcN*), lost the ability to cause BFB symptoms in cucumber (Fig. 3a, seedling 3) and failed to induce HR in tobacco (Fig. 3b, circle 3). The predicted HrcN protein in strain FC440 (GenBank accession number JF701985) shared 99·3% sequence identity with Aave-0463 in strain AAC00-1, indicating a very high level of conservation between the two subgroup strains in *A. citrulli*.

Typical and conserved characteristics of ATPase enzymes (Walker *et al.*, 1982) were found in the predicted ATPase HrcN sequence, including two Walker box motifs, Walker box A motif ^{173} PAGVGKS^{179} and Walker box B motif ^{258} LLMMD^{262}. Comparative sequence analyses of NF-T3SS ATPase (HrcN), F_0F_1 ATP synthase subunit beta and flagellum-specific ATP synthase (FliI) between *A. citrulli* and representative plant pathogenic bacteria, including *Pseudomonas syringae* and *Xanthomonas campestris*, showed that the predicted HrcN of *A. citrulli* fell in the NF-T3SS ATPase cluster, and was distant from the other two clusters (Fig. 4).

To verify that the transposon-disrupted *hrcN* gene plays a crucial role in *A. citrulli* pathogenicity and NF-T3SS functionality, and to exclude the possibility of a polar effect of the transposon insertion on genes adjacent to *hrcN*, a genetic complementation experiment was carried out by transforming the pHC60 plasmid carrying the wildtype *hrcN* gene into the mutant FC440 (Δ*hrcN*). The complemented strain FC440 (Δ*hrcN*+*hrcN*) showed restored ability to cause water-soaked lesions on cucumber cotyledons and to induce HR in tobacco leaves (Fig. 3a seedling 4 and Fig. 3b circle 4). Both the sequence comparison and genetic complementation assays

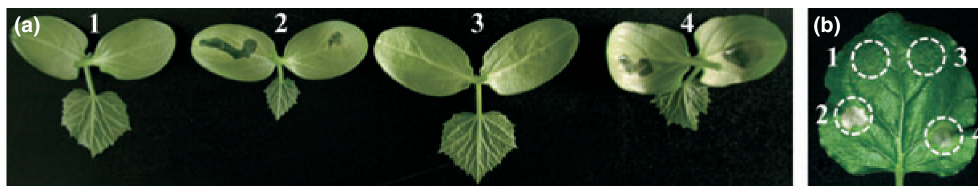

Figure 3 Analysis of *Acidovorax citrulli* mutant disrupted with NF-T3SS ATPase HrcN on host and non-host plants. Response of (a) 6-day-old cucumber (cv. Nongcheng No. 3) cotyledons (photo taken 5 days after scratch-wound inoculation) and (b) 6-week-old leaf of non-host plant *Nicotiana benthamiana* (photo taken 48 h after infiltration) to inoculation with *A. citrulli* wildtype FC440 (2); FC440 (Δ*hrcN*) carrying the empty vector (3); FC440 (Δ*hrcN*+*hrcN*) (i.e. FC440 [Δ*hrcN*] expressing *hrcN* gene) (4); ddH_2O was used as negative control (1).

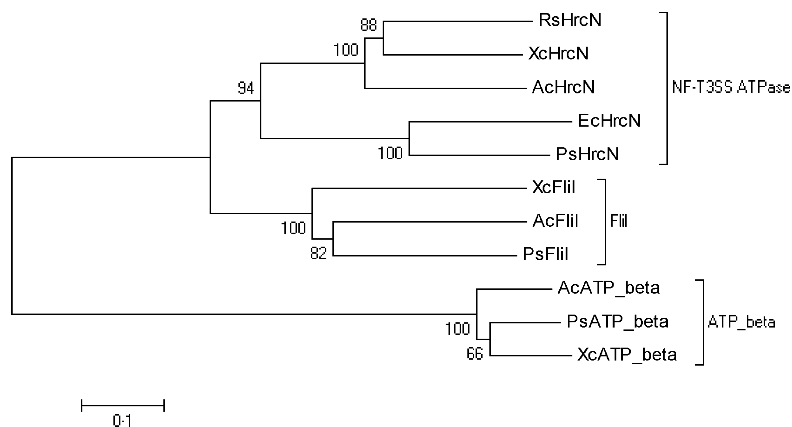

Figure 4 Phylogenetic analysis of HrcN-related ATPases. The abbreviations of NF-T3SS ATPase, FliI and ATP_beta represent non-flagellar type III secretion system ATPase, flagella-specific ATP synthase, and F_0F_1 ATP synthase subunit beta, respectively. Ac, Ea, Ps, Rs and Xc represent *Acidovorax citrulli*, *Erwinia amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas campestris*, respectively.

confirmed that the predicted T3SS ATPase was a NF-T3SS ATPase HrcN.

Comparison of the NF-T3SS of *A. citrulli* with those of *Ralstonia solanacearum* and *X. campestris*

The difference between two NF-T3SS groups of plant pathogenic bacteria (Alfano & Collmer, 1996) may be derived from the distinct T3SSs they possess. To evaluate the phylogenetic relationships between NF-T3SS in *A. citrulli* and other representative plant pathogenic bacteria, the NF-T3SS ATPase HrcN, a conserved and essential inner-membrane component in NF-T3SS, was chosen for comparative sequence analysis. The results showed that the NF-T3SS of *A. citrulli*, *R. solanacearum* and *X. campestris* are closely related to each other and are distinct from those of *P. syringae* and *E. amylovora* (Fig. 4), indicating that the NF-T3SS of *A. citrulli* belongs to group II with those of *R. solanacearum* and *X. campestris* (Alfano & Collmer, 1996).

Discussion

This study set up a model pathosystem for *A. citrulli* in which cucumber cotyledons were used for pathogenicity assays. The established *A. citrulli*–cucumber pathosystem provides a valuable model to dissect interactions between *A. citrulli* and cucurbits.

The established pathosystem was proved to be efficient by successful identification of pathogenicity-deficient *A. citrulli* insertional mutants generated by Tn5 transposon mutagenesis. A mutation in the predicted T3SS ATPase gene *hrcN* in *A. citrulli* led to loss of pathogenicity. Further phylogenetic analysis and genetic complementation experiments confirmed that NF-T3SS is essential for *A. citrulli* pathogenicity, consistent with the results from characterization of the interaction between *A. citrulli* and its original natural host melon or watermelon (Ren *et al.*, 2009). Comparative analysis of the conserved and essential NF-T3SS component HrcN protein and its homologues showed that the NF-T3SS of *A. citrulli* was in group II, which includes that of *R. solanacearum* and *X. campestris* (Alfano & Collmer, 1996).

It was shown that the group I strain of *A. citrulli* was more aggressive on cucumber than the group II strain, and the group I strain of the *A. citrulli*–cucumber model is probably more suitable for investigating *A. citrulli*–cucurbit interactions. Previous investigations showed that cucumber can be infected by *A. citrulli* under natural and experimental conditions, and typical BFB symptoms were characterized mainly on cucumber foliage; no symptoms on plant stems or fruits were reported (Martin *et al.*, 1999; Burdman *et al.*, 2005; Liu *et al.*, 2009). In the present study, typical symptoms were observed on cucumber fruits following bacterial inoculation by injection. Besides the capability to infect cucumber, *A. citrulli* strains in the two subgroups were reported to differ in pathogenicity on the host plant (O'Brien & Martin, 1999; Walcott *et al.*, 2000, 2004; Burdman *et al.*, 2005), with the group I strains being more aggressive than the group II strains on cucumber, as determined by both seedling pathogenicity and seed transmission assays (Burdman *et al.*, 2005). The present study also investigated differential BFB severity between strains of the two subgroups following wound inoculation on cucumber cotyledons. To exclude the possible influence of differences in motility, capability of surface attachment and adhesion between strains on pathogenicity determination, an infiltration method was employed for inoculation and to quantify the growth rates of the two subgroup strains within tissues. The data confirmed that the group I strain grew significantly faster than the group II strain within cucumber tissues, which could lead to more severe BFB development. Quantification of bacterial populations in inoculated leaf tissues further confirmed the difference in BFB severity between the strains on cotyledons, and verified that the pathogenicity assays on cucumber cotyledons were stable and repeatable. Large-scale screening for pathogenicity mutants requires an efficient procedure for pathogenicity determination. In view of the existence of certain percentages of symptomless mutants on seedlings, as revealed by seed transmission assays discussed above, and the tedious screening procedures reported, the simplified method for pathogenicity determination by cotyledon inoculation of cucumber seedlings will facilitate large-scale identification of pathogenicity genes in the BFB pathogen.

Identification of pathogenicity-related genes in the pathogen has been a major focus in the field of plant–pathogen interactions. To identify pathogenicity-associated genes in *A. citrulli*, transposon-mediated insertional mutagenesis has been applied to create mutants followed by *in planta* screening for altered-pathogenicity genes on original hosts of wildtype strains with seed-transmission assays (Bahar *et al.*, 2009; Ren *et al.*, 2009). The present study generated a high-quality, Tn5-mutated library of *A. citrulli*, which provided plenty of genetically stable single-insertion mutants of *A. citrulli*. The investigation of some mutants excluded the concern of potential genetic instability problems resulting from expression of transposase in the subsequent generations of *in vivo* transposition systems (Goryshin *et al.*, 2000). Thus, the newly developed protocol for *A. citrulli* mutagenesis will facilitate the use of genetic approaches to the study of *A. citrulli* biology and pathology. The valuable mutant resource generated can be applied in a wide range of research on biology and pathology of *A. citrulli*. The quality of the mutant collection and the more efficient library screening procedure was confirmed by successful identification of several genes required for pathogenicity, including the predicted NF-T3SS ATPase gene in *A. citrulli*.

The NF-T3SS ATPases shared substantial sequence similarities with FliI, the ATPase component of the flagellar assembly, and to a lesser degree with the catalytic beta subunit of the rotary F_0F_1 ATP synthase (Woestyn *et al.*, 1994; Akeda & Galan, 2004). Both the flagellar and non-flagellar T3SSs secrete macromolecules from the site of synthesis (cell cytoplasm) to their sites of action (Pallen

et al., 2005), but the process of secretion and the mode of gene action between the two types of mechanisms (flagellar apparatus and needle complex) can be very different. In animal pathogenic bacteria the flagellar T3SS ATPase (FliI) is not essential for flagellar T3SS, but is required for the initial entry of export substrate into the export gate (Minamino & Namba, 2008). However, in the plant pathogen *X. campestris* pv. *vesicatoria*, the secretion via NF-T3SS could not occur without the ATPase (Lorenz & Buttner, 2009). In the present study, sequence comparison and genetic complementation assays indicated that the predicted T3SS ATPase was a NF-T3SS ATPase HrcN and was essential for both *A. citrulli* pathogenicity on the host plant and the HR reaction on a non-host plant, suggesting that the HrcN protein was essential for functionality of NF-T3SS. The identification of HrcN not only verified the requirement of T3SS for pathogenicity in *A. citrulli* on watermelon and melon, as previously reported (Ren *et al.*, 2009; Johnson *et al.*, 2011), but also confirmed the *A. citrulli*–cucumber interaction system to be effective for studying the pathogenicity mechanism of BFB.

NF-T3SS is used by proteobacteria for pathogenic or symbiotic interaction with plant and animal hosts (Marie *et al.*, 2003). Without NF-T3SS, phytopathogenic bacteria are unable to defeat basal defences, grow in plants, produce disease symptoms in hosts, or evoke the hypersensitive response (HR) in non-hosts. Based on their possession of similar genes, operon structures and regulatory systems, the NF-T3SSs within phytopathogens are further divided into two groups (Alfano & Collmer, 1996). Being categorized in the same group, the inclusion of NF-T3SSs in the bacterial plant pathogens *R. solanacearum* and *X. campestris* will further aid understanding of the function of NF-T3SS in the pathogenesis of *A. citrulli*.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1. Sequence analysis and putative function of *Acidovorax citrulli* DNA flanking Tn5 transposon insertions in the FC440 strain.

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