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Pathogen profile

Acidovorax citrulli: generating basic and applied knowledge to tackle a global threat to the cucurbit industry

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SUMMARY

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB) of cucurbit plants. In recent years, the disease has spread to many parts of the world, mainly via the inadvertent distribution of contaminated commercial seeds. Because of the costly lawsuits filed by growers against seed companies and the lack of efficient management methods, BFB represents a serious threat to the cucurbit industry, and primarily to watermelons and melons. Despite the economic importance of the disease, little is known about the basic aspects of *A. citrulli* pathogenesis. Nevertheless, the release of the genome of one *A. citrulli* strain, as well as the optimization of molecular manipulation and inoculation methods, has prompted basic studies and allowed advances towards an understanding of *A. citrulli* pathogenicity. In this article, we summarize the current knowledge about this important pathogen, with emphasis on its epidemiology and the factors involved in its pathogenicity and virulence.

Taxonomy: Bacteria; Betaproteobacteria; order *Burkholderiales*; family *Comamonadaceae*; genus *Acidovorax*; species *citrulli*.

Microbiological properties: Gram-negative, strictly aerobic, rod-shaped; average dimensions of 0.5 μm × 1.7 μm; motile by means of an ~5.0-μm-long polar flagellum; colonies on King's medium B are round, smooth, transparent and nonpigmented; optimal temperatures for growth around 27–30 °C; induces a hypersensitive response on nonhost tobacco and tomato leaves.

Host range: *Acidovorax citrulli* strains are pathogenic to various species of the Cucurbitaceae family, including watermelon, melon, squash, pumpkin and cucumber. Significant economic losses have been reported in watermelon and melon.

Disease symptoms: Watermelon and melon seedlings and fruits are highly susceptible to *A. citrulli*. Typical seedling symptoms include water-soaked lesions on cotyledons that are often adjacent to the veins and later become necrotic, lesions on the hypocotyl, and seedling collapse and death. On watermelon fruits, symptoms begin as small, irregular, water-soaked lesions which later extend through the rind, turn brown and crack. On melon fruits, symptoms are characterized by small, often sunken rind lesions and internal fruit decay. Symptoms on the leaves of mature plants are difficult to diagnose because they are often inconspicuous or similar to those caused by other biotic or abiotic stresses. When they occur, leaf lesions can spread along the midrib and main veins. Lesions appear dark-brown to black on watermelon and light to reddish-brown on melon.

Useful websites: Bacterial fruit blotch of cucurbits at APSnet, http://www.apsnet.org/edcenter/intropp/lessons/prokaryotes/Pages/BacterialBlotch.aspx; bacterial fruit blotch guide from ASTA, http://www.amseed.com/pdfs/DiseaseGuide-BFB-English.pdf; *Acidovorax citrulli* AAC00-1 genome at JGI, http://genome.jgi-psf.org/aciav/aciav.info.html.

INTRODUCTION

Bacterial fruit blotch (BFB) of cucurbits is caused by the biotrophic Gram-negative bacterium *Acidovorax citrulli* (Schaad *et al*., 2008). *Acidovorax citrulli* (Fig. 1A,B) has rapidly emerged as an economically important seed-borne pathogen of watermelon (*Citrullus lanatus*) and melon (*Cucumis melo*) worldwide, with the ability to affect other cucurbits, such as cucumber, squash and pumpkin. Typical symptoms caused by *A. citrulli* on watermelon and melon are shown in Fig. 1C–F. Seed disinfestation treatments, seed health testing and chemical control in the field are limited in their ability to reduce the yield losses associated with BFB. In addition, to date, there are no reliable sources of BFB resistance. In many parts of the world, BFB occurs sporadically; however, because of its highly destructive potential, *A. citrulli* represents a major threat to the global watermelon and melon industries.

In this article, we provide a historical overview of BFB, discuss aspects related to its epidemiology and management, and examine the genetic diversity of *A. citrulli*. Finally, we review the current knowledge on the pathogenicity and virulence factors of this bacterium.

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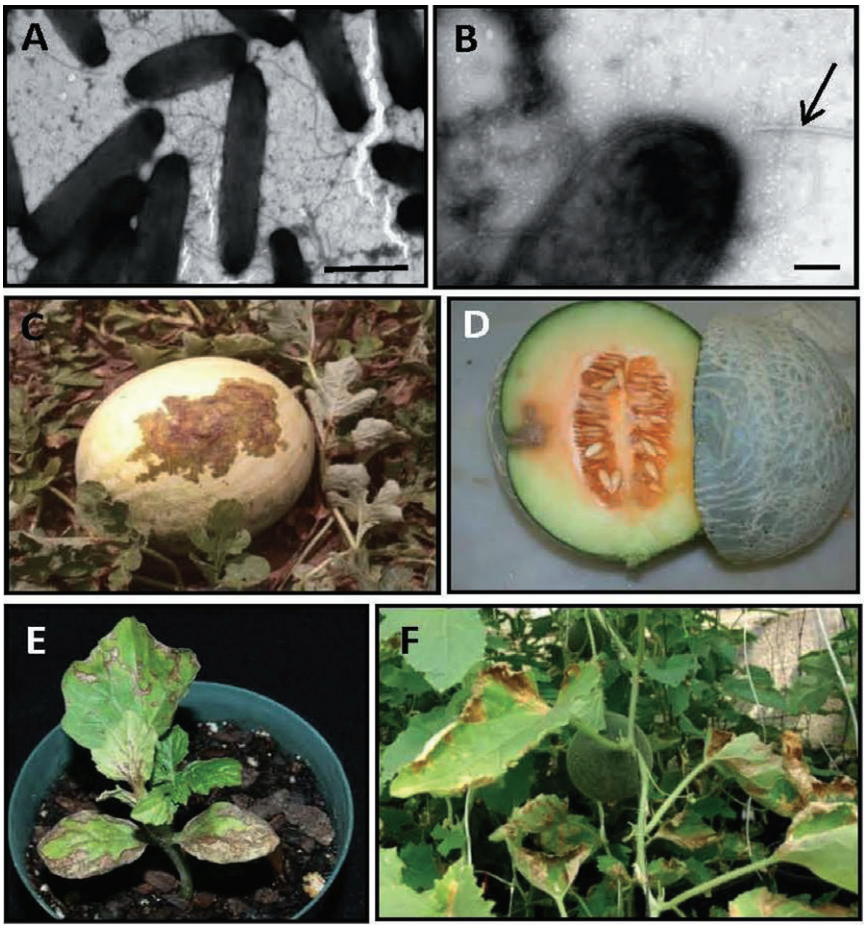

Fig. 1 *Acidovorax citrulli* and typical symptoms induced on host plants. (A, B) Transmission electron micrograph of *Acidovorax citrulli* M6 cells (bars: 1 μm in A and 0.12 μm in B). Arrow in (B) indicates polar flagellum. (C, D) Typical fruit blotch symptoms in watermelon and melon fruits, respectively. (E, F) Lesions on foliage of watermelon and melon, respectively.

EMERGENCE AND SPREAD OF BFB: HISTORICAL OVERVIEW

Most economically important plant diseases and their causal agents were described between the late 19th and early 20th centuries, with the emergence of modern phytopathology. In contrast, the documented history of BFB began in the 1960s, and only by the end of the 1980s was the economic impact of this disease realized. Therefore, relative to other important plant diseases, BFB is new.

In 1965, Webb and Goth reported an unidentified, seed-borne phytobacterium, isolated from necrotic watermelon cotyledons of different plant introductions from Turkey, at the US Department of Agriculture Plant Introduction Station in Griffin, GA, USA (Webb and Goth, 1965). The disease was reported to be restricted to seedlings, and its causal agent was subsequently classified as *Pseudomonas pseudoalcaligenes* ssp. *citrulli* by Schaad *et al*. (1978). The type strain, ATCC 29625, was reported to be nonfluorescent on King's B medium, negative for the hypersensitive response (HR) in tobacco and unable to induce watermelon fruit rot. It is likely that the type strain stored at the American Type Culture Collection (ATCC) lost its pathogenicity, as all *A. citrulli* strains tested thus far have been able to induce HR in nonhost tobacco and tomato leaves, and to induce watermelon fruit rot to some extent (Walcott *et al*., 2000).

Four years after the Webb and Goth report, Crall and Schenck (1969) described watermelon fruit blotch (WFB) symptoms. They reported the appearance of large, dark-green, water-soaked lesions on fruits, which were accompanied by leaf spots, at an experimental station in Leesburg, FL, USA, in 1967 and 1968. Nevertheless, until the late 1980s, the disease was considered to only affect seedlings, and to have a low damage potential on watermelon fruits in the field (Sowell and Schaad, 1979). It was not until 1987, when the first BFB outbreak occurred in the Mariana Islands (Wall and Santos, 1988) and entire fields of watermelon were lost as a result of fruit infection, that the destructive potential of this pathogen was appreciated. Additional outbreaks in commercial watermelon fields followed rapidly, including high yield losses in Florida, Indiana, Delaware and Texas (Black *et al*., 1994; Evans and Mulrooney, 1991; Latin and Rane, 1990; Somodi *et al*., 1991). BFB outbreaks in the USA in the mid-1990s led to numerous lawsuits filed by growers against vegetable seed companies. Seeds were identified as the primary inoculum source and seed companies were considered to be responsible for the out-

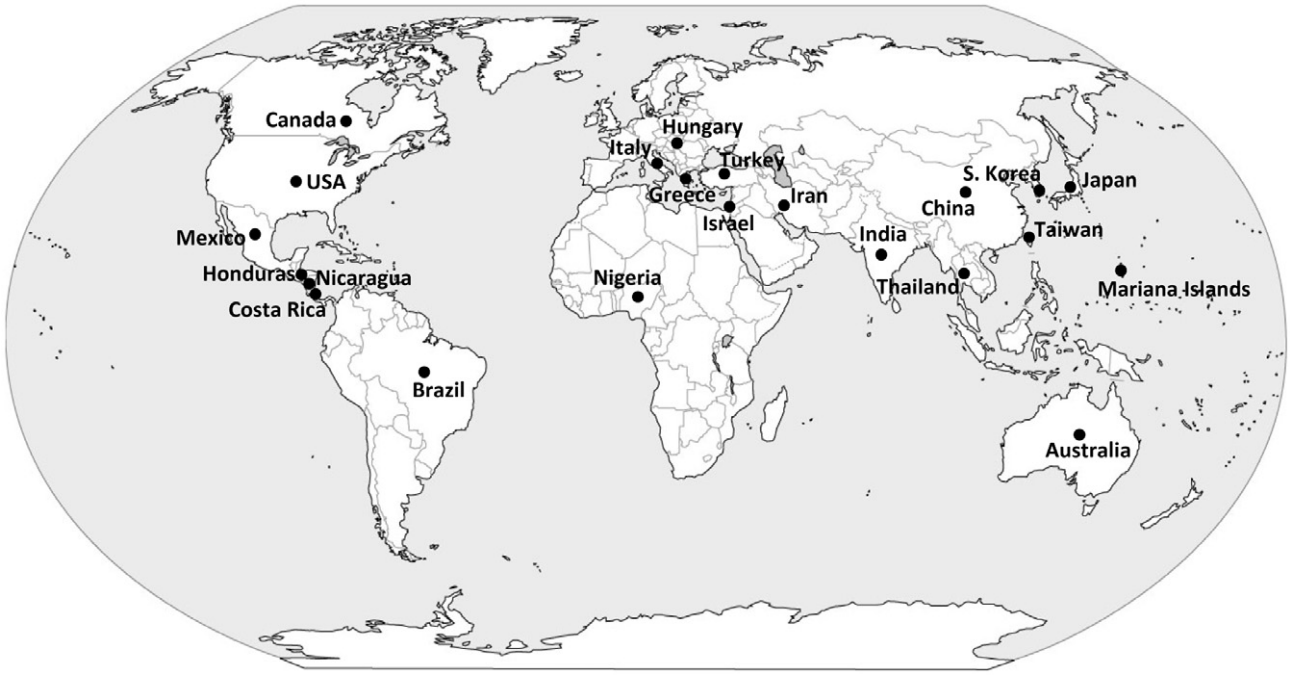

Fig. 2 Occurrences of bacterial fruit blotch (BFB) outbreaks around the world based on information in the literature and personal observations of the authors. After the first outbreaks in Florida and the Mariana Islands, which occurred in the late 1980s, the disease spread worldwide during the 1990s and 2000s (see references in the text).

breaks. As a result, many small seed companies went out of business and others required growers to sign contracts indicating that seeds had been tested, but that the risk and liability of BFB outbreaks would be assumed by the growers (Latin and Hopkins, 1995).

During the 1990s, the rapid spread of BFB progressed in two directions: broadening of the host range and global expansion. BFB has been reported in many cucurbits, such as honeydew, citron melon, cucumber, squash and pumpkin (Isakeit *et al*., 1997, 1998; Langston *et al*., 1999; Martin and O'Brien, 1999; Walcott *et al*., 2004). The pathogen has also spread worldwide (Fig. 2), mainly through international production and the sale of contaminated seeds. To date, BFB outbreaks have been reported in the Americas, Asia, Europe, Africa, the Middle and Far East, and Australia (Amadi *et al*., 2009; Black *et al*., 1994; Burdman *et al*., 2005; Evans and Mulrooney, 1991; Holeva *et al*., 2010; Latin and Rane, 1990; Mirik *et al*., 2006; O'Brien and Martin, 1999; Palkovics *et al*., 2008; Park *et al*., 2008; Ren *et al*., 2006; Schaad *et al*., 2003; Somodi *et al*., 1991; Walcott *et al*., 2004). Alarmingly, it is also possible that the pathogen can be spread via the seeds of nonhost plants. Assouline *et al*. (1997) reported that the bacterium was isolated in Israel from several shipments of tomato seeds imported into the country, as well as from eggplant seedlings produced from imported seeds. This report preceded the outbreaks of BFB in both melon and watermelon in Israel during the early 2000s (Burdman *et al*., 2005).

With regard to taxonomy, several phytopathogenic *Pseudomonas* species have been transferred to the genus *Acidovorax* as a result of high levels of phenotypic and rRNA cistron similarities to members of the already existing genus (Willems *et al*., 1992). As a result, *Pseudomonas pseudoalcaligenes* ssp. *citrulli* has been reclassified as *Acidovorax avenae* ssp. *citrulli*. In addition to ssp. *citrulli*, two other phytobacterial subspecies within *A. avenae*—*A. avenae* ssp. *avenae*, which is pathogenic to various species of the Poaceae family, including oat, corn, wheat, barley, rye, sorghum, sugarcane and rice seedlings, and *A. avenae* ssp. *cattleyae*, which is pathogenic to orchids—have been transferred to the genus *Acidovorax* (Willems *et al*., 1992). Recently, another reclassification has been proposed that elevates these subspecies to the species level, with the BFB-causing pathogen being renamed *A. citrulli* (Schaad *et al*., 2008).

BFB EPIDEMIOLOGY

Acidovorax citrulli is seed borne and seed transmitted, and contaminated seeds represent the most important source of primary inoculum for BFB outbreaks. Seed-to-seedling transmission of BFB has been demonstrated for a range of cucurbits, including watermelon, muskmeklon, honeydew, acorn squash, butternut squash, zucchini squash, cucumber and pumpkin, even though fruit symptoms do not occur for all hosts (Hopkins and Thompson, 2002a). Volunteer cucurbit seedlings, noncucurbit and cucurbit weeds,

such as citron melon and wild bur gherkin (*Cucumis anguria* var. anguria) (Isakeit *et al*., 1998), and infected plant debris are also potential inoculum sources; however, these are not important in all environments.

When infested seeds are planted directly into the soil, a proportion of the emerging seedlings may develop BFB symptoms by 6 and 10 days after germination. The exact time of symptom development depends on the environmental conditions [primarily temperature and relative humidity (RH)] and the *A. citrulli* population per seed. High temperatures and RH, and high seed inoculum loads of *A. citrulli*, will lead to more rapid BFB symptom expression. Because environmental conditions in the field vary, BFB seed-to-seedling transmission and BFB outbreaks are generally more sporadic with direct seeding.

Increasingly, seedling transplants are being used for commercial cucurbit production to improve stand establishment and production efficiency. Seeds are planted under greenhouse conditions and, after 3–4 weeks, seedlings are transplanted into the field. Typical transplant house conditions, including high temperatures and RH, dense plant populations and overhead irrigation, are highly conducive to BFB development and spread amongst seedlings. Particularly with overhead irrigation, *A. citrulli* can be splash dispersed throughout a transplant house, where it rapidly infects healthy seedlings. Because of splash dispersal and aerosol generation, seed lots with low proportions of *A. citrulli*-infested seeds can result in 100% seedling infection within a transplant house. Recent studies have indicated that a single seed containing 10 *A. citrulli* colony-forming units (cfu) within a seed lot can lead to BFB transmission under greenhouse conditions (Dutta *et al*., 2012b). Even if cucurbit seedlings do not develop BFB symptoms in the transplant house, epiphytic *A. citrulli* populations can lead to BFB outbreaks when transplanted to the field under conducive environmental conditions. Because of these factors, there is zero tolerance for *A. citrulli* in commercial cucurbit seed lots.

As in the greenhouse, high RH and high temperatures favour BFB development under field conditions. *Acidovorax citrulli* is spread by wind-driven rain and overhead irrigation, and the bacterium penetrates through stomata and wounds to establish infections that result in foliar lesions and blight. In general, however, infected plants are not killed by infection and lesions on mature foliage may be restricted. Foliar lesions and epiphytic populations serve as *A. citrulli* reservoirs and contribute to BFB development on fruit. Frankle *et al*. (1993) demonstrated that watermelon fruit infection occurs with bacterial penetration of fruit stomata 2–3 weeks after anthesis. After this period, waxy deposits on the surface of the fruit block stomata and prevent invasion. During the early stages of fruit development, BFB symptoms are absent, but characteristic water-soaked lesions develop shortly before harvest maturity. Fruits may eventually rot in the field and bacteria in the decaying tissue or contaminated seeds within can serve as inoculum for the subsequent season.

BIOLOGY OF SEED INFECTION

Although infested seeds are important sources of primary inoculum for BFB epidemics, little is known about the seed infection process. Rane and Latin (1992) reported that seeds harvested from symptomatic fruits were infested and transmitted the disease to seedlings, whereas those from asymptomatic fruits were not infested. However, in commercial seed production, only symptomless fruits are harvested. Hence, it is unlikely that seeds produced in symptomatic fruits account for natural commercial seed infection. To explore how seeds become infested with *A. citrulli*, Walcott *et al*. (2003) demonstrated that pollination and inoculation of female watermelon blossoms led to seed infestation within symptomless fruits. Seeds produced in this manner transmitted BFB to seedlings once planted. In agreement with these findings, Bahar *et al*. (2009b) reported that, under unfavourable conditions for BFB development, over 50% of seeds within asymptomatic melon fruits were infested with *A. citrulli*.

Lessl *et al*. (2007) reported that *A. citrulli* rapidly colonizes watermelon stigmas, and that there is a strong linear relationship between blossom inoculum dose and seed infection. It has also been shown that the bacterium penetrates through the style via the transmitting tract tissues and enters the ovary by 24 h after pollination (Dutta, 2011). Dutta *et al*. (2012a) demonstrated that, in seeds infested via blossom inoculation, *A. citrulli* cells are deposited deep within the seed (under the perisperm–endosperm layer), when compared with seeds that are infested within symptomatic fruits (bacteria just under the seed coat). These studies indicate that blossom invasion by *A. citrulli* can lead to seed infection, even in the absence of BFB fruit symptoms. Despite these findings, the epidemiological significance of blossom invasion in seed infection remains to be determined under seed production field conditions.

BFB MANAGEMENT

At present, there are no commercial cucurbit cultivars with resistance to BFB. Hence, effective BFB management requires the integration of a range of approaches in seed, transplant and fruit production. As seeds are the most important source of primary inoculum, efforts to prevent seed infection are critical. Cucurbit seeds are generally produced in regions of countries with cool dry climates, or during dry periods. Only *A. citrulli*-free stock seeds should be used for commercial seed production and seed fields should be physically separated from other cucurbit fields to avoid contamination. Seed fields should be visually inspected for fruit and foliar symptoms of BFB, and fields with symptomatic plants should not be used for seed production.

A range of treatments have been suggested to decontaminate cucurbit seeds; however, to date, none are 100% effective. Factors that influence the effectiveness of seed treatments include: (i) the

ability of seed treatments to penetrate the seed coat; and (ii) the location of bacteria on/in the seed. Although seed treatments, including streptomycin sulphate and NaOCl, have been reported to reduce BFB seedling transmission, they generally fail to eradicate the bacterium from within the seed (Sowell and Schaad, 1979). Hopkins *et al*. (1996) reported that fermentation of seeds in watermelon juice for 24–48 h, followed by treatment with 1% HCl for 15 min, eliminated BFB seedling transmission. However, this can adversely affect seed quality parameters of certain hybrids (Hopkins *et al*., 1996). Hopkins *et al*. (2003) also showed that treatment with peroxyacetic acid at 1600 μg/mL for 30 min eliminated *A. citrulli* and other pathogens, including *Didymella bryoniae* and *Fusarium oxysporum*, from watermelon and melon seeds. However, despite the routine use of peroxyacetic acid in commercial watermelon seed production, BFB outbreaks continue to occur, which suggests that these treatments are not 100% effective. Other seed treatments have also been proposed, including 9 h of exposure to chlorine gas (Stephens *et al*., 2008) and acidic electrolysed water (Feng *et al*., 2009a); however, it is unlikely that seed treatments alone will control BFB, as the pathogen may exist deep within the seed (Dutta *et al*., 2012a). In addition, the risk of BFB outbreaks in transplant houses is high for seed lots with low levels of *A. citrulli* contamination.

With zero tolerance for BFB in seedling transplant facilities, seed health testing is critical for disease management. Many polymerase chain reaction (PCR)-based assays have been reported for the testing of seeds for *A. citrulli* (Bahar *et al*., 2008; Fessehaie *et al*., 2005; Park *et al*., 2008; Song *et al*., 2003; Walcott and Gitaitis, 2000). However, as cucurbit seeds contain PCR inhibitors, a range of techniques, including BIO-PCR, immunomagnetic separation and PCR (IMS-PCR), and magnetic capture hybridization and PCR, have been developed to improve detection accuracy and efficiency (Ha *et al*., 2009; Walcott *et al*., 2006; Walcott and Gitaitis, 2000; Zhao *et al*., 2009). Despite their improved sensitivity and efficiency, however, PCR-based techniques are not routinely employed for commercial seed health testing for *A. citrulli*. In contrast, most commercial seed lots are assayed using greenhouse or sweatbox seedling grow-out bioassays, which rely on the planting of seed samples (n = 10 000–50 000 seeds/lot) under conditions that promote the disease and observing the resulting seedlings for BFB symptoms (http://www.worldseed.org/isf/ishi_vegetable.html).

As with other phytobacterial diseases, host resistance represents the most effective approach for the management of BFB. Hopkins and Thompson (2002b) reported that five self-crossed populations of watermelon accessions from Zimbabwe and Zambia displayed high levels of resistance to BFB. More recently, Wechter *et al*. (2011) screened 332 *Cucumis* sp. accessions and reported that four *C. melo* and one *C. ficifolius* plant introductions showed high levels of BFB resistance. Bahar *et al*. (2009b) also reported BFB tolerance in cultivated and wild melons. They also reported that there was a difference in tolerance depending on the inoculation assay used, and that three cultivars/plant introductions—6401, BLB-B and EAD-B—were tolerant in seedling transmission assays, whereas cv. ADIR was tolerant in all assays used. Despite these observations, at present, no commercial watermelon or melon cultivars have significant levels of BFB resistance.

Despite the availability of a wide range of antimicrobial compounds that are effective against *A. citrulli*, the most widely used foliar treatment is copper-based compounds, such as Kocide and Mankocide. For maximum efficacy, copper-based compounds should be applied in a preventative/protectant manner.

GENETIC AND PHYTOPATHOGENIC DIVERSITY

Initially, *A. citrulli* strains were thought to comprise a homogeneous population, and most of the initial BFB outbreaks in the USA occurred on watermelon. However, with the BFB outbreaks in Florida, USA in the late 1980s, it became clear that there was genetic variability amongst *A. citrulli* strains. Somodi *et al*. (1991) reported that the *A. citrulli* strains recovered from BFB outbreaks in watermelon in Florida were related to the pathogen recovered from the USDA plant introduction station in Georgia, USA (Webb and Goth, 1965). However, unlike the Georgia strain, the Florida strain induced an HR on tobacco. It was not until 1999 that O'Brien and Martin (1999) reported significant differences in populations of *A. citrulli* in Australia. *Acidovorax citrulli* strains from North Queensland were severe on watermelon and melon cultivars, but strains from South Queensland were more aggressive on watermelon than on melon cultivars. In addition, the strains from North Queensland displayed reduced virulence on *Cucumis myriocarpus* and failed to utilize L-leucine as a sole carbon source. In contrast, the South Queensland strains did not utilize 2-aminoethanol, whereas the North Queensland strains did (O'Brien and Martin, 1999).

Using pulse field gel electrophoresis (PFGE) DNA fingerprinting and gas chromatography-fatty acid methyl ester (GC-FAME) to analyse a global population of 121 *A. citrulli* strains from a range of cucurbit hosts, Walcott *et al*. (2000) provided evidence for two genetically and physiologically distinct groups. Group II strains were isolated mainly from watermelon, whereas group I included the ATCC type strain, as well as strains recovered from nonwatermelon cucurbit hosts. In a subsequent study, Walcott *et al*. (2004) confirmed the existence of two genetically distinct groups of *A. citrulli* strains using repetitive PCR. They also confirmed that the group I strains did not utilize L-leucine and were moderately aggressive on a range of cucurbit hosts. In contrast, the group II strains, isolated primarily from watermelon, were highly aggressive on watermelon, but only mildly aggressive on nonwatermelon hosts. Interestingly, *A. citrulli* strains from North Queensland, Australia clustered with group I by PFGE analysis (Walcott *et al*., 2000). Independent confirmation of the two genetically distinct populations of *A. citrulli* was provided by Burdman *et al*. (2005)

using PFGE typing on an Israeli population of strains, and by Feng *et al*. (2009b) using multilocus sequence typing (MLST) on a Chinese population of strains. At present, it is accepted that there are at least two genetically distinct populations of *A. citrulli* strains with varying host range profiles. Efforts are currently underway to understand the genetic determinants of the host range and virulence of these distinct groups.

PATHOGENICITY AND VIRULENCE FACTORS

Despite the economic importance of BFB, little is known about the basic aspects of the biology and pathogenesis of *A. citrulli*. The genome sequence of a group II strain, AAC00-1, was released in 2007 by the Joint Genome Institute (JGI; GenBankNC_008752), providing a great contribution to the investigation of basic aspects of BFB. The AAC00-1 genome comprises a single circular chromosome of about 5.3 Mb and putatively encodes 4858 genes (Table 1). Recently, we have sequenced the genome of *A. citrulli* M6, a group I strain (S. Burdman and R. Walcott, unpublished data). It is expected that comparative analyses between group I and II genomes, combined with suitable experimental approaches, will enable the detection of genetic factors that govern the host preferences of the two *A. citrulli* groups. Below, we provide an overview of the current knowledge of *A. citrulli* pathogenesis.

Type III secretion

Many Gram-negative plant-pathogenic bacteria secrete protein effectors directly into the host cell via a specialized type III secretion (T3S) system (Alfano and Collmer, 2004; Bogdanove *et al*., 1996; Mansfield, 2009). The exact role of most T3S effectors still remains unclear, although some have been shown to contribute to virulence through the modulation of host cellular processes and the suppression of host defence responses (Block *et al*., 2008; Grant *et al*., 2006; Mansfield, 2009; Mudgett, 2005). Many T3S effectors were first identified as products of avirulence (*avr*) genes, which are specifically recognized in resistant plants by the action of corresponding resistance (*R*) gene products. This recognition event results in effector-triggered immunity (ETI), which is generally associated with the induction of HR, a localized programmed cell death of infected tissue that ultimately arrests the growth of biotrophic pathogens (Jones and Dangl, 2006; Mansfield, 2009).

Table 1 Characteristics of the *Acidovorax citrulli* AA C00-1 genome (sequenced and annotated by the Joint Genome Institute; GenBank NC_008752).

Feature*	
Sequencing centre	DOE Joint Genome Institute
Release date	1st March 2007
Sequencing method	Sanger
Sequencing depth	11X
Distribution	One single chromosome
Size (bp)	5 352 772
GC (%)	68.53
DNA coding regions (%)	89.47
Total number of genes	4868
Protein coding genes	4793
Protein coding genes with predicted functions	3376 (69.35%)
Number of open reading frames	4709

*Sources: JGI Integrated Microbial Genomes (IMG; http://img.jgi.doe.gov) and National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

As in many Gram-negative plant-pathogenic bacteria, the T3S system is required for pathogenicity and HR induction in susceptible and resistance plants, respectively. The genes encoding the components of this secretion apparatus are named *hrp* genes (for hypersensitive response and pathogenicity). *hrp* genes are located in large clusters, generally of 20–25 kb (Buttner and Bonas, 2002). On the basis of gene organization, sequence analysis and regulation, *hrp* clusters are divided into two classes: class I contains the clusters of *Pseudomonas syringae* and enteric plant-pathogenic bacteria, whereas class II contains the *hrp* clusters of *Xanthomonas* species and *Ralstonia solanacearum* (Bogdanove *et al*., 1996; Buttner and Bonas, 2002). The genome sequence of the *A. citrulli* group II strain AAC00-1 revealed the existence of an Hrp-T3S system. On the basis of sequence analysis and cluster organization, the *A. citrulli hrp* cluster belongs to class II. The generation and characterization of *hrp* mutants of both group I and II strains have revealed that, in *A. citrulli*, a functional Hrp-T3S system is required for both pathogenicity on cucurbit hosts and HR-inducing ability on nonhost tobacco and tomato (Bahar and Burdman, 2010; Johnson *et al*., 2011).

Annotation of the AAC00-1 genome has also revealed at least 11 putative T3S effectors homologous to known effectors from *Xanthomonas* species, *P. syringae* and *R. solanacearum*. In collaboration with others, we are currently assessing the distribution of these putative effectors in a global population of *A. citrulli*, and characterizing their role in pathogenicity. Initial findings from these studies have revealed that groups I and II differ significantly from each other in the sequence of most effectors. Moreover, some effectors that are present in strain AAC00-1 and all assessed group II strains seem to be absent or nonfunctional in group I strains (N. Levi, T. Zimmermann, A. Castro-Sparks, J. Sikorski, B. Zhao, G. Welbaum, R. Walcott and S. Burdman, unpublished data). We hypothesize that differences in the arsenal of T3S effectors are responsible, at least in part, for the observed differences in host preference among isolates from these groups.

Type II secretion

In many Gram-negative bacteria, the type II secretion (T2S) system is responsible for the translocation of several pathogenesis-related proteins from the bacterial cytoplasm to the extracellular environment (Douzi *et al*., 2012). T2S proteins include toxins and hydrolytic enzymes, such as lipases, cellulases, pectate lyases and

proteases, and have been shown to be virulence factors in plant-pathogenic bacteria, including *Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *vesicatoria* and *R. solanacearum* (Hu *et al*., 2007; Liu *et al*., 2005; Szczesny *et al*., 2010).

The T2S system is conserved and generally comprises 12–16 proteins that form a complex (secreton) that spans the bacterial envelope. The subunits of the secreton are encoded by general secretion pathway (*gsp*) genes that are often organized into large operons. A complete secreton operon includes *gspC, D, E, F, G, H, I, J, K, L, M, O* and, in some cases, *gspAB and gspN/S* (Douzi *et al*., 2012). However, in some bacterial species, *gsp* genes may be missing or located outside the operon (Cianciotto, 2005). In addition, some bacterial species, such as *Pseudomonas aeruginosa*, *R. solanacearum* and *X. campestris*, have multiple operons encoding the secreton (Cianciotto, 2005).

There are three major components of the secreton: an inner membrane platform, a piston-like pseudopilus and an outer membrane secretin. The inner membrane platform comprises the proteins Gsp C, F, L, M and E. The pseudopilus comprises five pseudopilin proteins, GspG, H, I, J and K, which have N-terminal sequence homology to the pilins involved in type IV pilin biogenesis (Hansen and Forest, 2006; Nunn, 1999). These pseudopilins are synthesized as prepilins with short leader sequences that are cleaved by prepillin peptidase (GspO). The final component of the secreton, the outer membrane secretin, is encoded primarily by *gspD* and forms a pore through which proteins are translocated.

Acidovorax citrulli AAC00-1 possesses two sets of T2S gene clusters. One cluster, *gsp1*, lacks *gspA, S, B* and *N*, whereas the second cluster, *gsp2*, lacks *gsp A, B, S* and *F*. Deletion of both copies of *gspG* (major pseudopilin) in *A. citrulli* AAC00-1 resulted in the loss of the ability to secrete endoglucanase, which confirmed the existence of a functional T2S system (Johnson, 2010). This mutant also displayed a significant reduction in ability to colonize watermelon seedling cotyledons relative to the wild-type strain, suggesting that T2S enzymes may be virulence factors. Using a T3S system AAC00-1 mutant, Johnson *et al*. (2011) reported that, during the first 6 days of seed germination, *A. citrulli* grows as a saprophyte, as it does not require effector proteins to colonize watermelon seeds. On the basis of this observation, it was hypothesized that other factors, possibly T2S enzymes, might be involved in watermelon seed colonization by *A. citrulli*.

In watermelon seed colonization and BFB seedling transmission assays, the *gspG1/G2* deletion (T2S) mutant of AAC00-1 displayed a significant reduction in seed colonization 96 h after planting, and a significant reduction in seed-to-seedling BFB transmission (Johnson, 2010). These observations suggest that T2S proteins contribute to the bacterium's ability to colonize germinating watermelon seeds prior to the infection of seedling tissue. Genomic analysis of AAC00-1 revealed the presence of three putative cell wall-degrading enzymes, namely endoglucanase, xylanase and pectate lyase. Although there is evidence that *A. citrulli* produces and secretes endoglucanase, no such evidence exists for xylanase or pectate lyase. To date, assays with individual xylanase, pectate lyase and endoglucanse deletion mutants of AAC00-1 have indicated that only endoglucanase contributes minimally to watermelon seed colonization (Johnson, 2010). As *A. citrulli* has a functional T2S system that contributes to BFB seed-to-seedling transmission, there is a need to further characterize the role of other T2S proteins in the pathogenicity and seed-to-seedling transmission of BFB.

Type IV pili

Type IV pili (T4P) are hair-like appendages found on the surface of a wide range of bacteria. They constitute an efficient device for a particular type of flagellum-independent surface motility, named twitching motility, and are involved in several other bacterial activities, including adherence, colonization, biofilm formation, uptake of genetic material and virulence (Craig *et al*., 2004; Nudleman and Kaiser, 2004). Despite the multiple functionality of T4P and their well-established role in the pathogenicity of animal-pathogenic bacteria, relatively little attention has been given to the role of T4P in plant-pathogenic bacteria (Burdman *et al*., 2011). With regard to the latter, the contributions of T4P to virulence have been mainly demonstrated in vascular plant-pathogenic bacteria (e.g. those possessing the ability to colonize and spread via the plant xylem vessels), such as *R. solanacearum* (Kang *et al*., 2002; Liu *et al*., 2001) and *Xylella fastidiosa* (Cursino *et al*., 2011; De La Fuente *et al*., 2007; Meng *et al*., 2005). It has been proposed that T4P may contribute to bacterial colonization and spread in the xylem through cell attachment, biofilm formation and twitching motility.

Recently, a random mutagenesis approach combined with virulence screens has revealed that T4P contribute significantly to the virulence of the *A. citrulli* group I strain M6 on melon seedlings. A transposon M6 mutant impaired in *pilM*, which encodes a protein required for T4P assembly, was shown to be impaired in twitching motility, biofilm formation and virulence following seed transmission and stem inoculation assays (Bahar *et al*., 2009a; 2010) (Fig. 3). A marker exchange mutant impaired in *pilT* showed similar phenotypes to the *pilM* mutant, although the reduced virulence in the former was more accentuated than in the latter (Bahar *et al*., 2009a). *pilT* encodes an ATPase that is required for T4P retraction, a process that drives twitching motility. The *A. citrulli* M6 *pilT* mutant produces T4P, and is even hyperpiliated relative to the wild-type, indicating that functional T4P (and not their presence *per se*) are required for the virulence of this bacterium on melon seedlings. Moreover, the *pilT* mutant was also severely affected in swimming motility, which could also add to the reduction in virulence observed for this mutant (Bahar *et al*., 2009a). It is possible that pili in excess might mechanically interfere with polar flagellum function, thus leading to these phenotypes.

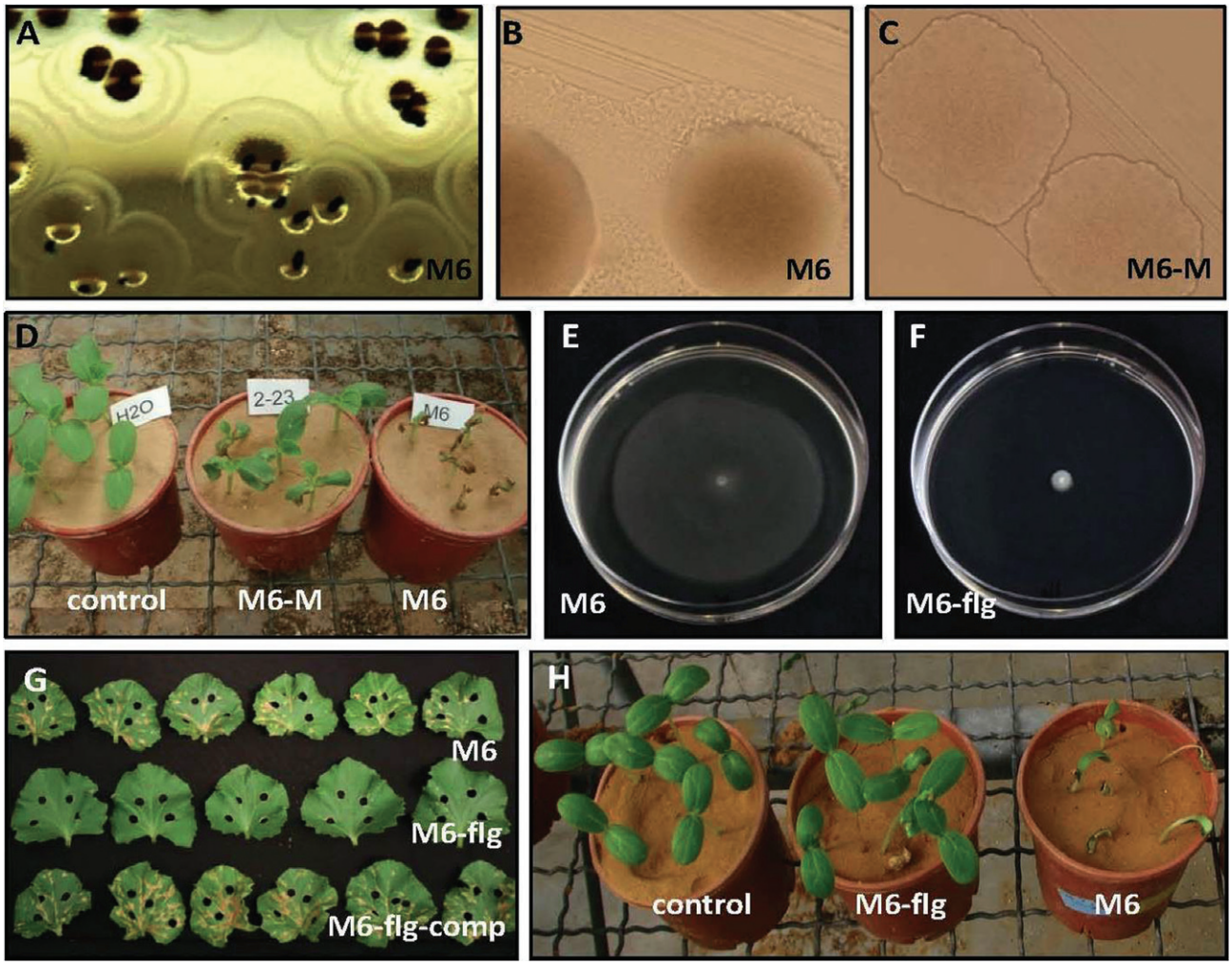

Fig. 3 Type IV pili (T4P) and the polar flagellum (PF) are virulence factors of *Acidovorax citrulli*. (A, B) Typical haloes surrounding colonies of wild-type strain M6, as a result of T4P-mediated twitching motility, as seen by the naked eye and through light microscopy, respectively (after 96 h of growth on nutrient agar plates). (C) M6-M, an M6 mutant impaired in *pilM*, a gene required for T4P assembly, is unable to perform twitching motility (note the lack of twitching haloes). (D) The M6-M mutant possesses reduced virulence relative to the wild-type M6 in melon seed transmission assays (photograph taken 10 days after inoculation and sowing). (E, F) Swimming motility assays in soft agar plates showing that M6-flg, an M6 mutant impaired in the *fliC* gene (encoding flagellin), is unable to perform swimming motility. (G) Leaves of melon plants showing disease symptoms 11 days after vacuum inoculation of plants with strains M6, M6-flg and M6-flg mutant complemented with a plasmid carrying the functional *fliC* gene (M6-flg-comp). Note the reduced virulence of mutant M6-flg relative to the wild-type and complemented strains. (H) The M6-flg mutant possesses reduced virulence relative to the wild-type M6 in melon seed transmission assays (photograph taken 10 days after inoculation and sowing). Most photographs were taken or modified from Bahar *et al*. (2009a; 2011).

On the basis of the characterization of *pilM* and *pilT* mutants of *A. citrulli* and the notion that T4P is important for the virulence of vascular plant-pathogenic bacteria, it was hypothesized that *A. citrulli* possesses the ability to colonize and spread through xylem vessels of melon seedlings, a hypothesis that was further confirmed (Bahar *et al*., 2009a). In contrast with these results, no significant differences in symptom induction ability and growth *in planta* have been observed between *pilM* mutant and wild-type strains following foliage inoculation of mature melon plants (O. Bahar, R. Kumar Shrestha and S. Burdman, unpublished data). These results suggest that, in contrast with the importance of T4P for vascular infection of seedlings, T4P and twitching motility may not play a crucial role in local, foliar infection of mature plants by *A. citrulli*. Nevertheless, it is important to mention that vascular infection of *A. citrulli* has been reported recently in leaves of squash plants (Makizumi *et al*., 2011). Whether T4P contribute significantly to fruit infection by this pathogen must be investigated.

Polar flagellum

Flagella are found on the surface of many bacteria, where they mediate motility and are involved in various processes, such as

adhesion to and colonization of biotic and abiotic surfaces, and virulence on both animal and plant hosts (Macnab, 2003; Moens and Vanderleyden, 1996). In the same virulence screens with the *A. citrulli* M6 transposon library that led to the identification of T4P as a virulence factor, a mutant impaired in *fliR* displayed reduced virulence in seed transmission assays. *fliR* encodes a flagellar biosynthetic protein involved in flagellin secretion. Further generation and characterization of an M6 mutant impaired in *fliC*, encoding flagellin, confirmed the polar flagellum as a virulence factor of *A. citrulli*. The *fliC* mutant was more reduced in virulence than the wild-type in seed transmission assays and following both stem and foliage inoculations (Bahar *et al*., 2011) (Fig. 3).

In stem inoculation assays, the *fliC* mutant displayed reduced ability to colonize the xylem vessels of melon seedlings. Foliage inoculation experiments indicated that the polar flagellum contributes to *A. citrulli* virulence at both pre- and post-host tissue penetration (Bahar *et al*., 2011). This was an interesting observation as, in most studies involving biotrophic plant-pathogenic bacteria, polar flagella have been proposed to be important for virulence only at the early stages of infection, when flagellum-mediated motility is needed for the penetration of the host tissue (Bayot and Ries, 1986; Hattermann and Ries, 1989; Lee *et al*., 2003; Panopoulos and Schroth, 1974). More importantly, the contribution of polar flagella for *A. citrulli* virulence at post-penetration stages could be detected in experiments in which foliage inoculation was performed with vacuum, using relatively low inoculum concentrations (10^2 cfu/mL), whereas studies with other bacteria generally involved highly concentrated inocula (~10^7–10^8 cfu/mL). This is a crucial difference, as the infiltration of bacteria using high inoculum concentrations probably leads to rapid saturation of the plant tissue with bacteria and may mask the contribution of polar flagella to the further spread and establishment of invading bacteria inside the plant tissue (Bahar *et al*., 2011).

It appears that the major contribution of polar flagella to *A. citrulli* virulence is via swimming motility. In contrast with T4P mutants, polar flagellum mutants did not appear to be affected in adhesion and biofilm formation abilities in several assays (Bahar *et al*., 2010, 2011). Interestingly, both *fliR* and *fliC* mutations negatively affected T4P-mediated twitching motility of *A. citrulli* (Bahar *et al*., 2011), which could also contribute to the reduction in virulence of mutants lacking polar flagella.

Quorum sensing

Quorum sensing (QS) is a form of cell density-dependent communication used by bacteria to coordinate the expression of several genes and their behaviour (Bassler, 2002). QS is achieved through the response to a stimulatory concentration of extracellular autoinducers. In Gram-negative bacteria, typical autoinducers are acylated homoserine lactones (AHLs) (Bassler, 2002; Ng and Bassler, 2009; Withers *et al*., 2001). The role of QS in the virulence of plant-pathogenic bacteria has been studied extensively in several species, including *Agrobacterium tumefaciens*, *P. syringae*, *Pantoea stewartii*, *R. solanacearum*, *Pectobacterium* sp. and *Xanthomonas* sp. Among the traits that were found to be regulated by QS in these bacteria were the production of extracellular polysaccharides, degradative enzymes, antibiotics and siderophores, T3S, motility, biofilm formation and epiphytic fitness. As QS is generally associated with pathogenesis, the development of approaches that aim to interfere with QS signalling may be helpful for the control of phytobacterial diseases (Fray, 2002; van Bodman *et al*., 2003).

The genome of *A. citrulli* AAC00-1 contains genes homologous to *luxI* and *luxR*, encoding the AHL synthase and the AHL-dependent transcriptional protein, respectively. Chen *et al*. (2009) generated a mutant strain of *A. citrulli* impaired in the *luxI* homologous gene, which they named *aacI*. Pathogenicity tests on watermelon fruits revealed that the *aacI* mutant possessed reduced virulence relative to the wild-type, thus confirming that QS is important for *A. citrulli* pathogenicity (Chen *et al*., 2009). The authors also reported that the autoinducer molecule of *A. citrulli* is *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL). In a recent study, Fan *et al*. (2011) also showed that 3-oxo-C8-HSL is the QS signalling molecule of *A. citrulli* and that QS is involved in the pathogenicity of this bacterium. The authors generated an *aacI* mutant in the background of a different *A. citrulli* strain, and showed that this mutant was unable to produce AHL molecules. The mutant showed a significant reduction in virulence in watermelon fruits and in melon seedlings, and was also affected in swimming motility. In contrast, under the tested conditions, the *aacI* mutant did not differ from the wild-type in biofilm formation, the production of extracellular polysaccharides and the induction of HR in tobacco (Fan *et al*., 2011).

CONCLUSIONS

Acidovorax citrulli has emerged as a serious seed-borne threat to the global cucurbit industry. Despite advances in recent years, the effectiveness of seed disinfestation and pathogen exclusion by seed health testing remains limited in minimizing the losses to BFB. In addition, to date, there are no reliable sources of BFB resistance and the disease is difficult to manage under conducive environmental conditions.

Despite the economic importance of BFB and the availability of the *A. citrulli* genome, little is known about the basis of *A. citrulli* pathogenesis. This knowledge is crucial to the development of efficient strategies to mitigate the economic impact of this disease. For instance, the development of resistant watermelon and melon cultivars is one of the most important strategies for BFB management. Advances in our understanding of the role played by *A. citrulli* T3S effectors may help in the identification of suitable resistance genes, in the cucurbit germplasm or in other plant species, which could facilitate the development of reliable

resistance in commercial cucurbit cultivars. In addition, a more detailed understanding of the molecular mechanisms involved in seed-to-seedling transmission of BFB may lead to effective seed treatments to eliminate seed-borne inoculum.

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