Plant Pathology (2018) **67**, 1936–1943 Doi: 10.1111/ppa.12905

Xanthomonas vesicatoria virulence factors involved in early stages of bacterial spot development in tomato

V. Felipe[a], A. M. Romero[b], M. S. Montecchia[cd], A. A. Vojnov[e], M. I. Bianco[e] and P. M. Yaryura[af]*

[a]*Instituto Académico Pedagógico de Ciencias Básicas y Aplicadas (IAPCByA), Universidad Nacional de Villa María (UNVM), Villa María;* [b]*Facultad de Agronomía, Departamento de Producción Vegetal, Cátedra de Fitopatología, Universidad de Buenos Aires, Buenos Aires;* [c]*Facultad de Agronomía, Departamento de Biología Aplicada y Alimentos, Cátedra de Microbiología Agrícola, Universidad de Buenos Aires, Buenos Aires;* [d]*Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) – Universidad de Buenos Aires, Buenos Aires, Argentina;* [e]*Instituto de Ciencia y Tecnología Dr César Milstein, Fundación Pablo Cassará, CONICET, Ciudad Autónoma de Buenos Aires, Buenos Aires; and* [f]*Centro de Investigaciones y Transferencia de Villa María (CIT-VM), CONICET, Universidad Nacional de Villa María (UNVM), Villa María, Argentina*

Xanthomonas vesicatoria (Xv) is a member of a species complex that causes bacterial spot on tomato, one of the most important diseases of this crop worldwide. The objective of this investigation was to analyse several characteristics involved in Xv virulence in relation to strain aggressiveness. Motility, biofilm formation, adhesion and production of xanthan were evaluated in three local strains causing tomato bacterial spot in Argentina. The strains assayed presented differential swarming and twitching motilities, adhesion and biofilm formation abilities. The most aggressive strain, BNM 208, exhibited the greatest swarming and twitching motilities, and developed a mature biofilm with presence of defined cell clusters, a homogeneous and compact structure, and higher biomass and substratum coverage than the other two strains. Even though the three strains produced similar amounts of xanthan, BNM 208 produced the most viscous exopolysaccharide, which possibly relates to the better characteristics of its biofilm. Despite other differences, the three strains multiplied to similar levels when they were infiltrated into the leaf. The results suggest that the aggressiveness of Xv strains studied in this work was related to their ability to move by flagella or type IV pili, adhere to leaves and form well developed biofilms, factors that improve phyllosphere colonization. A better understanding of the factors involved in the Xv infection process at the early stages would contribute to developing new control strategies for this phytopathogen.

Keywords: bacterial spot of tomato, biofilm, swarming, twitching motility, xanthan, *Xanthomonas vesicatoria*

Introduction

Bacterial spot is one of the most destructive diseases of tomato (*Solanum lycopersicum*) in the world, especially in tropical and subtropical environments (Potnis *et al.*, 2015). It is caused by at least four species of *Xanthomonas*: *X. euvesicatoria*, *X. gardneri*, *X. perforans* and *X. vesicatoria* (Stall, 1995; Strayer *et al.*, 2016; Roach *et al.*, 2017). The disease is characterized by necrotic lesions on leaves, stems and fruits, resulting in a reduction of yield and fruit quality (Potnis *et al.*, 2015). Bacteria enter plant tissue through stomata and wounds, colonize the intercellular spaces and induce visible symptoms, which begin as water-soaked lesions that later become necrotic (Stall, 1995).

Disease control is difficult because of the lack of durable host-determined pathogen resistance in commercial tomato cultivars and the development of resistance to copper in *Xanthomonas* populations due to the extensive use of copper sprays in standard disease-control treatments (Obradovic *et al.*, 2004; Potnis *et al.*, 2015; Griffin *et al.*, 2017). In addition, these compounds can damage the environment. In Argentina, bacterial spot is present in all field-grown tomato-producing areas. In the horticultural belt of Buenos Aires–La Plata, the disease was related to *X. euvesicatoria* (Bouzar *et al.*, 1994) and more recently to *X. vesicatoria* (Xv; Romero *et al.*, 2003).

Epiphytic bacteria, such as Xv, are continually exposed to extreme conditions: lack of moisture, ultraviolet irradiation, strong winds and heat. Bacterial tolerance to these stressful conditions is often associated with the formation of biofilms. These structures have been related to virulence in different *Xanthomonas* species (Yaryura *et al.*, 2015; Bianco *et al.*, 2016).

The first step in biofilm formation is the attachment of bacteria to leaf surfaces, which is mediated by flagella and type IV pili (T4P; Malamud *et al.*, 2011; Burrows, 2012; Beaussart *et al.*, 2014). Then, microorganisms

*E-mail: yaryura@agro.uba.ar

Published online 16 July 2018

Plant Pathology

form microcolonies that can develop further into mature structured biofilms. The biofilm 'cycle' is completed when mature biofilms disperse to release motile cells (Lemon *et al.*, 2007). Besides attachment, flagella are important in determining the final structure of the biofilm and, in many bacteria, they are also necessary for swarming motility (Kaiser, 2007; Merritt *et al.*, 2007). T4P are cell surface structures involved in twitching motility that is independent of flagella and is important for host colonization in a wide range of plant pathogens (Taguchi & Ichinose, 2011; Nguyen *et al.*, 2012; Dunger *et al.*, 2014).

The formation of biofilms on abiotic or biotic surfaces in *Xanthomonas* is also related to the production and properties of xanthan, the main exopolysaccharide (EPS) synthesized by the majority of species of this genus. EPSs are needed to form an intricate and complex three-dimensional matrix, which provides structural support for bacterial biofilms. This contributes to epiphytic survival before colonization of the host plant and is essential for bacterial virulence and disease progression (Yun *et al.*, 2006; Aslam *et al.*, 2008; Bianco *et al.*, 2016).

The ability to form biofilms has been related to virulence in other *Xanthomonas* species (Darsonval *et al.*, 2009; Yaryura *et al.*, 2015; Bianco *et al.*, 2016). Biofilm formation and motility are well described for many plant pathogenic bacteria, but these virulence factors are understudied in Xv. Therefore, the objective of this study was to evaluate some bacterial characteristics involved in bacterial epiphytic growth and early stages of the infection process in relation to aggressiveness in Xv strains causing tomato bacterial spot in Argentina.

Materials and methods

Bacterial strains and growth conditions

Strains were originally isolated from leaves of tomato plants showing symptoms of bacterial spot from commercial field crops in General Belgrano (35°45′33.6″S, 58°28′16.0″W), Florencio Varela (34°54′02.7″S, 58°12′21.2″W) and La Plata (34°54′33.4″S, 58°02′02.4″W), Buenos Aires province, Argentina. These were deposited at Banco Nacional de Microorganismos (BNM, Argentina, WDCM number 938, GCM) culture collection. Xv strains used in this study were BNM 208, BNM 214 and BNM 216 (Romero *et al.*, 2003). The identity of the strains was confirmed by rep-PCR genomic fingerprinting and conventional PCR tests according to Koenraadt *et al.* (2009) using the species-specific primers Bs-XvF and Bs-XvR. Bacteria from −80 °C frozen stocks were grown on modified yeast dextrose calcium carbonate (YDC) agar plates (Ritchie & Dittapongpitch, 1991) for 48 h at 28 °C before use. For swarming motility assays, bacteria were grown on PYM culture medium (0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 1% glucose) plus 0.5% agar.

Disease evaluation and bacterial multiplication *in planta*

Experiments were conducted on fresh-market tomato cultivar ACE 55 (Asgrow). Plants were grown in individual 1 L pots with soil, peat and perlite (2:1:1 v/v/v) in a greenhouse under natural temperature (18–28 °C) and light (approx. 14 h) conditions. Plants were watered daily with tap water and were inoculated when they had six expanded leaves.

To prepare the inocula, bacteria from YDC plates were suspended in sterile distilled water (SDW) and the bacterial concentrations were adjusted with a spectrophotometer to 5×10^8 CFU mL^{-1} (OD_{600} = 0.3). For assessments of symptom development, suspensions were diluted in SDW to a final concentration of 5×10^6 CFU mL^{-1} and Silwet (0.01%) was added (Romero *et al.*, 2001). Plants were inoculated by immersing the aerial part in the inoculum suspension for 20 s. Control plants were immersed in SDW with Silwet. All plants were covered with plastic bags for 60 h to maintain relative humidity close to 100%. Plants were maintained in the greenhouse during the whole experiment. There were four plants per strain and the experiment was repeated twice. Ten days after inoculation, disease was estimated as the severity of bacterial spot (proportion of diseased tissue) on leaves 5 and 6 (leaf 1 was the first leaf above the cotyledons). In addition, the number of lesions on two leaflets of leaf 5 was counted and the diameter of 20–30 lesions on those leaflets was measured (10–15 per leaflet).

Bacterial multiplication *in planta* was evaluated in a different set of plants. In this case, a leaflet of leaf 4 of each plant was infiltrated with a suspension of bacteria (5×10^4 CFU mL^{-1}), using a needleless syringe. Four days later, the leaflet was removed and three 0.5 cm^2 disks were cut and macerated in 0.5 mL SDW. Bacterial population size was estimated by dilution plating on YDC. The experiment was repeated twice, with four plants per strain.

Motility assays

Swarming motility assays were carried out as previously described (Picchi *et al.*, 2016).

Motility was assessed qualitatively by examining the size of the circular halo formed by the bacterial cells growing in 0.5% agar PYM. The assays were performed in triplicate.

Twitching motility was assayed as previously described (Dunger *et al.*, 2014) with slight modifications. Sterile microscope slides were coated with King's B medium with 1% agar supplemented with 2 mM $CaCl_2$. Single colonies were inoculated by stabbing into the bottom of the slides, which were then incubated at 28 °C for 72 h. In order to observe the twitching halo, the agar was removed and slides were stained with 1% crystal violet (CV). After 10 min of staining, slides were washed with phosphate-buffered saline (pH 7.2) and observed using an inverted Nikon microscope (Eclipse TI-S) with NIS-Elements software.

Determination of xanthan production and viscosity

Xanthan production was quantified as described previously by Rigano *et al.* (2007) with some modifications. Briefly, Xv strains were cultured to the stationary growth phase at 28 °C in 100 mL of YDC medium supplemented with 2% glucose in 500 mL flasks, using an orbital shaker rotating at 200 rpm; after 72 h, cells were removed by centrifugation at 25 000 ***g*** for 1 h at 4 °C. Subsequently, xanthan was precipitated with two volumes of ethanol with 1% KCl and recovered by filtration on a stainless-steel sieve. Wet xanthan fibres were dried at room temperature and weighed to determine the yield of xanthan.

Low shear rate viscosity (LSRV) was determined for a 0.45% xanthan solution in synthetic tap water (16.2 mM NaCl,

0.95 mM $CaCl_2$) at room temperature using a Brookfield viscometer (RVTD II) with spindle no. 1 at 3 rpm. Intrinsic viscosity measurements of xanthan solutions in 0.05 M NaCl were performed using a viscoelasticity analyser (Vilastic Scientific).

Bacterial attachment to abiotic and biotic surfaces

In a preliminary assay, the CV technique was used to analyse bacterial adhesion on an abiotic surface. Briefly, bacterial strains were grown overnight in YDC medium and then inoculated into Y minimal medium (YMM) to a final OD_{600} of 0.1. Aliquots of 150 μL were used to fill different wells of a polystyrene 96-well microtitre plate and then incubated at 28 °C as previously described (Malamud *et al.*, 2011). After 72 h, the medium was removed, and the plate was washed using 0.9% NaCl and stained with 0.1% CV solution for 30 min; excess CV was removed with distilled water. The bound CV was solubilized with 200 μL of 33% (v/v) acetic acid and measured at OD_{570} in an ELISA reader (Thermo Scientific Multiskan FC). The adhesion value was normalized according to the number of cells at OD_{600} and expressed as (OD_{570}/OD_{600}). The assay was performed in triplicate.

Bacterial adhesion to tomato leaves was studied as described by Rigano *et al.* (2007) with slight modifications. Disks were cut from healthy leaves and placed in 24-well plates, adaxial side down. The disks were inoculated with each strain (OD_{600} = 0.1) and incubated at 28 °C. After 24 h, the attached bacteria were quantified using CV staining as described above.

In vitro biofilm assays

All strains were grown overnight in YDC medium as previously described (Yaryura *et al.*, 2015). Aliquots of 500 μL were diluted 1:1000 in YMM and then transferred to chambered coverglass slides with a 1 mm thick borosilicate glass base (Nunc) and incubated for 72 h at 28 °C. To allow visualization of biofilm structures, bacteria were stained with the BacLight LIVE/DEAD viability kit (Invitrogen). Confocal images of each fluorophore were collected sequentially using the FV1200 confocal laser scanning microscope (CLSM; Olympus) equipped with 473/559 nm diode lasers lines and a ×60 Super Corrected, oil-immersion objective lens (PLAPON60XOSC, NA 1.4). Images obtained were processed with ImageJ (available at https://imagej.nih.gov/ij/) and Imaris v. 6.3.1 software (Bitplane). COMSTAT analysis was performed using the image processing toolbox as previously described by Heydorn *et al.* (2000).

Statistical analysis

Data from experiments were analysed by one-way ANOVA and mean differences were determined by Tukey's test at a significance level of $P \leq 0.05$. All statistical analyses were performed using the software InfoStat (Di Rienzo *et al.*, 2017). Data from the experiments were expressed as mean ± the standard error (SE).

Results

Aggressiveness of Xv strains on tomato plants

The strains evaluated in this study varied in aggressiveness on inoculated tomato plants. BNM 208 caused the highest level of disease severity, with lesions that were greater in number and larger than those induced by BNM 214 and BNM 216 (Table 1). No difference was observed between strains in the multiplication of bacteria *in planta* after infiltration of leaves (Table 1).

Swarming and twitching motilities

BNM 208 showed a higher swarming motility than BNM 214 and BNM 216 strains in 0.5% agar (Fig. 1a,b). Twitching motility (Fig. 1c) was observed at the interstitial surface between nutrient agar medium and the slide glass surfaces. The linear nature of the colony edge of BNM 208 strain was interrupted by dozens or a few hundred cells that appear to be budding from the main body of the colony, indicative of the spreading morphology of bacteria capable of twitching motility. With strain BNM 214, cells emerged from the edge of the colony and moved away from it, but to a lesser extent than BNM 208. In contrast, the edges of the colony of BNM 216 were whole or, in a few sections, slightly wavy, characteristic of strains defective in twitching motility (Fig. 1c).

Xanthan production and viscosity

The three Xv strains considered in this study produced similar amounts of xanthan (Fig. 2a); however, determinations of LSRV showed differences among the xanthan samples. The value of LSRV determined for xanthan produced by BNM 208 was significantly higher than values determined for the EPS produced by BNM 214 and BNM 216 (Fig. 2b).

Bacterial cell attachment to different surfaces

The adhesion capability of the strains was evaluated on abiotic and biotic surfaces. The first was performed with cells grown in polystyrene microtitre plates stained with crystal violet. BNM 208 and BNM 214 had a higher

Table 1 Agressiveness of *Xanthomonas vesicatoria* strains on tomato plants and multiplication of bacteria *in planta*.

Strain	Disease severity	Number of lesions per leaflet	Lesion size (mm)	Bacteria *in planta* (CFU mL^{-1} ($\times 10^8$))
BNM 208	43.75 ± 8.24 a	97.25 ± 13.30 a	4.44 ± 0.13 a	2.94 ± 0.87 a
BNM 214	17.50 ± 8.24 ab	58.63 ± 13.30 ab	3.08 ± 0.13 b	3.71 ± 0.87 a
BNM 216	10.00 ± 8.24 b	25.38 ± 13.30 b	3.02 ± 0.13 b	5.86 ± 0.87 a

Values represent means ± standard errors (SE). For each variable evaluated means followed by different letters within the columns indicate significant difference at $P \leq 0.05$ according to Tukey's test.

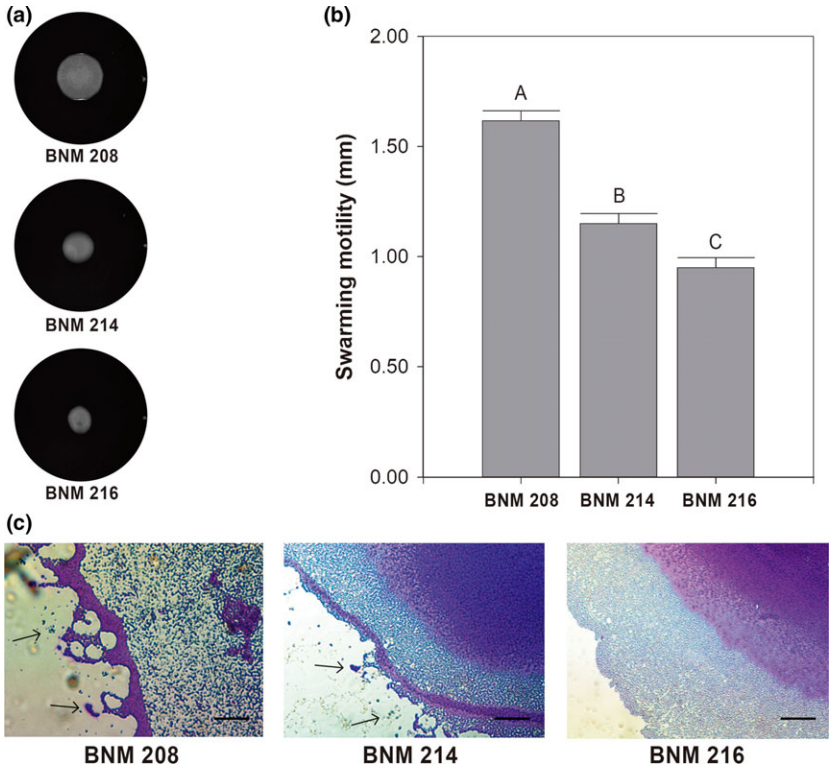

Figure 1 Swarming and twitching motilities of different strains of *Xanthomonas vesicatoria*. (a) Representative images showing the halos formed during swarming motility assays in plates. (b) Diameters of the halos formed by swarming motility of the growing bacterial cells. Each bar represents the mean ± standard error (SE) of three independent experiments. Different letters indicate significant differences at $P \leq 0.05$ according to Tukey's test. (c) Light microscopy of twitching halos from cultures stained with crystal violet (CV). The arrows indicate the mobile cells produced at the edge of the colonies. Images were obtained using an inverted microscope. Scale bars = 20 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

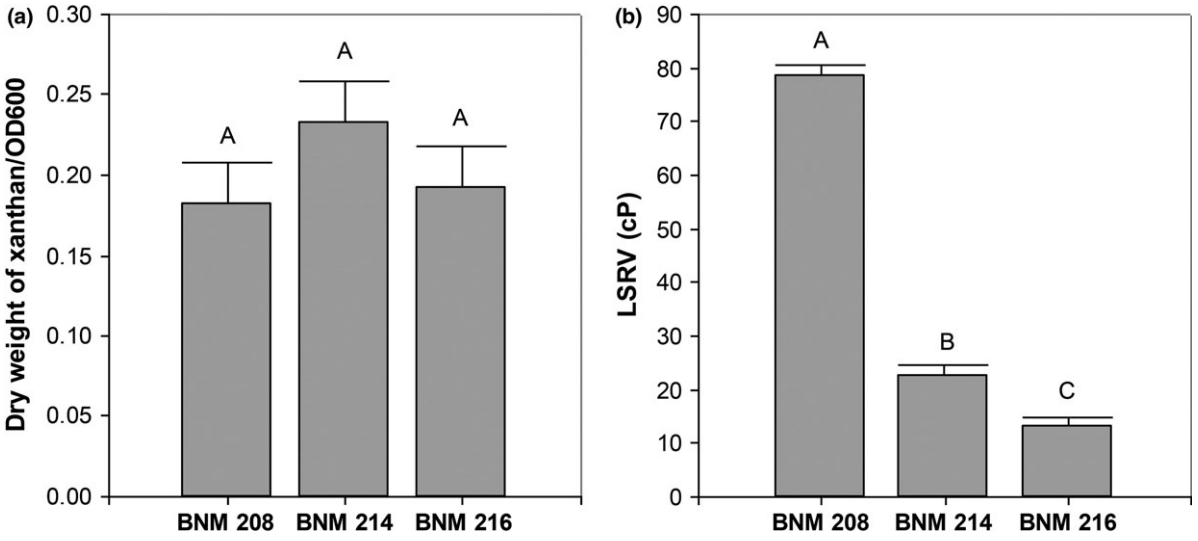

Figure 2 Xanthan production and viscosity of different strains of *Xanthomonas vesicatoria*. (a) Xanthan production relative to bacterial growth (OD_{600}). (b) Viscosity of xanthan determined as low shear rate viscosity (LSRV). Each bar represents the mean ± standard error (SE) of three independent experiments. Different letters indicate significant differences at $P \leq 0.05$ according to Tukey's test.

adherence to abiotic surfaces than BNM 216 (Fig. 3a). To investigate attachment to biotic surfaces, adhesion was evaluated on the abaxial surface of tomato leaves. In line with the findings *in vitro*, it was observed that BNM 208 had better adhesion to leaf surfaces than BNM 214 and BNM 216, shown by the higher level of CV present on the leaves (Fig. 3b).

Structural development of biofilms in Xv

BNM 208 showed a better-developed biofilm than the other two strains evaluated, which exhibited biofilms with similar characteristics to each other (Fig. 4). Accordingly, biomass and surface coverage values for BNM 214 and BNM 216 biofilms were significantly

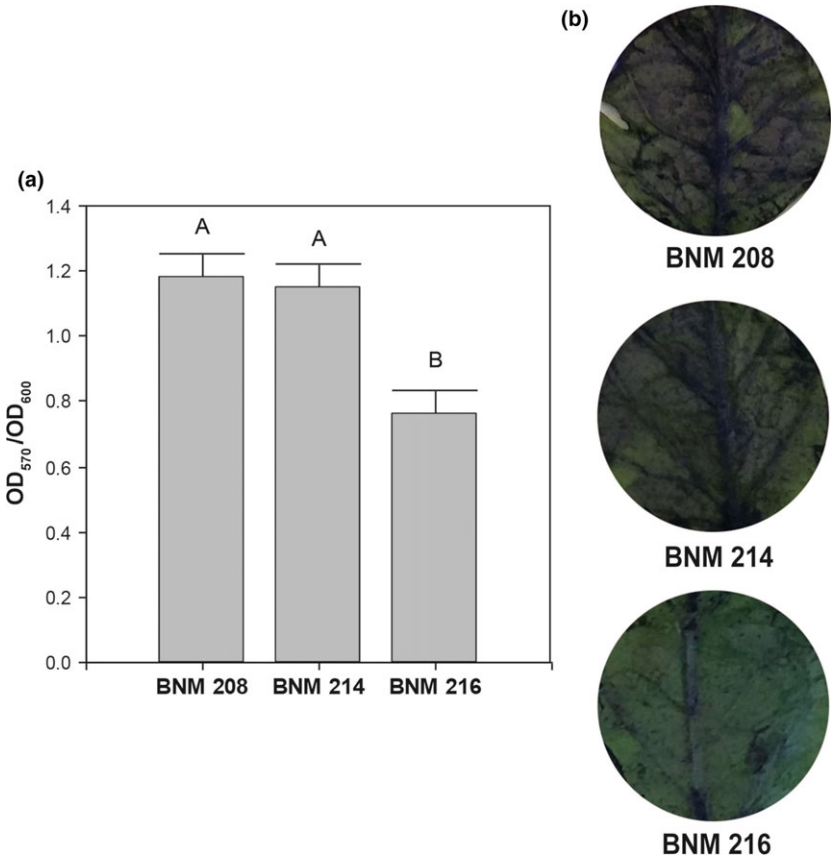

Figure 3 Adhesion of different strains of *Xanthomonas vesicatoria* to abiotic and biotic surfaces. (a) Adhesion to polystyrene visualized by crystal violet (CV) technique, determined after 72 h of incubation. Each bar represents the mean ± standard error (SE) of two independent experiments, with 24 replicates each time. Data represents the proportion of adhered to non-adhered bacteria (OD_{570}/OD_{600}). Different letters indicate significant difference at $P \leq 0.05$ according to Tukey's test. (b) Representative images of bacterial adhesion on abaxial surfaces of tomato leaves quantified by CV staining. [Colour figure can be viewed at wileyonlinelibrary.com].

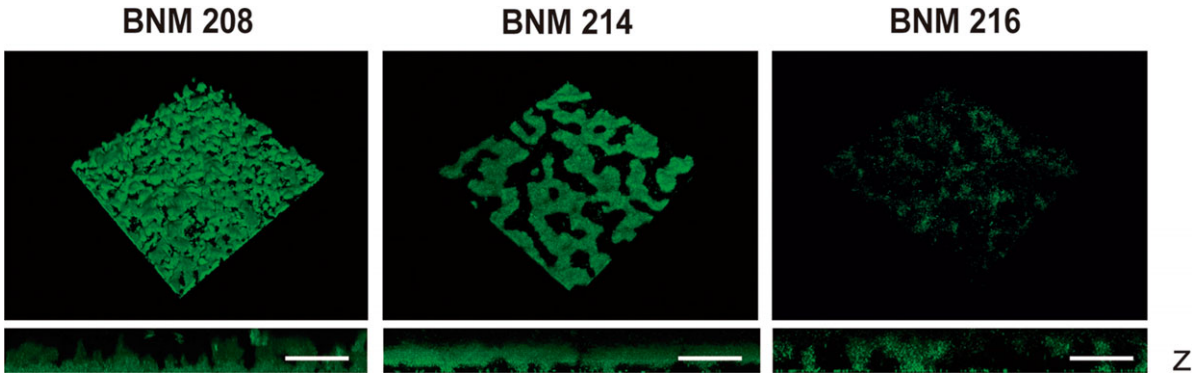

Figure 4 Biofilm architecture of *Xanthomonas vesicatoria* strains. Three-dimensional reconstruction of biofilms visualized under confocal laser scanning microscopy (CLSM) and projected images of the *z*-axis. Scale bars = 10 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

smaller than those of BNM 208 (Table 2). The surface coverage reflects how efficiently the abiotic surface is colonized by bacteria and corresponds to the area coverage in the first image of the stack. The biofilm of BNM 216 was the most heterogeneous, with a great variation in thickness, as indicated by its high roughness coefficient (Table 2). Also, the biofilm produced by BNM 208 presented the largest average diffusion distances, indicating that it produces large aggregates of biomass and cell clusters and, hence, a more confluent and compact biofilm. Finally, the average thickness of the biofilm of BNM 216 was significantly higher than those of BNM 208 and BNM 214.

Discussion

The data presented here improve the knowledge of virulence factors that contribute to aggressiveness in strains of Xv causing tomato bacterial spot in Argentina. Previous studies on this pathogen have focused mainly on the type III secretion system and its effectors, which have been widely studied as virulence determinants (Thieme

Table 2 Evaluation of biofilm properties of strains of *Xanthomonas vesicatoria*.

Strain	Biomass ($\mu m^3\ \mu m^{-2}$)	Average thickness (μm)	Roughness coefficient	Average diffusion distance (μm)	Surface coverage (%)
BNM 208	6.71 ± 0.29 a	7.87 ± 0.16 b	0.49 ± 0.03 b	0.80 ± 0.01 a	0.83 ± 0.04 a
BNM 214	4.44 ± 0.12 b	8.80 ± 0.20 b	0.57 ± 0.03 b	0.44 ± 0.05 b	0.47 ± 0.005 b
BNM 216	3.24 ± 0.24 b	12.60 ± 0.40 a	0.78 ± 0.03 a	0.24 ± 0.04 c	0.07 ± 0.003 c

Values represent means ± standard errors (SE). For each variable different letters within the columns indicate significant differences at $P \leq 0.05$ according to Tukey's test.

et al., 2005; Lorenz *et al.*, 2008); however, other factors that occur before infection are also involved in the aggressiveness of this species.

The results of the present investigation suggest that factors that determine epiphytic growth and biofilm formation are important determinants of strain aggressiveness in Xv. Among the strains assayed, BNM 208 caused the highest level of disease on tomato. However, when infiltrated into leaf tissues, surpassing the cuticle, it multiplied to levels similar to the other strains. Upon arrival to the phyllosphere, pathogenic xanthomonads must have the ability to colonize the leaf surface before entering the plant tissues through stomata and wounds (Thieme *et al.*, 2005; Potnis *et al.*, 2015). The development of bacterial biofilms is generally a multistep process, which is initiated when the bacteria reach a surface. The bacteria attach to a surface reversibly, where they can move freely across it until they become immobilized. Specialized surface exposed proteins called adhesins, such as flagella and pili, mediate bacterial adhesion to accomplish this critical step (Petrova & Sauer, 2012).

The high aggressiveness of strain BNM 208 could be related to its high potential to attach and move over surfaces (swarming and twitching motilities), and to develop well-structured biofilms.

The capability for swarming motility is associated with flagella number and/or placement, quorum sensing, and the production of wetting agents (Harshey, 2003; Kaiser, 2007; Overhage *et al.*, 2007). This bacterial movement promotes the entry into stomata or wounds and allows bacteria to locate specific sites for adhesion. It is also involved in the first step of biofilm formation on leaf surfaces, which is a key factor for bacterial growth and development of disease in *Xanthomonas* (Rigano *et al.*, 2007; Sena-Vélez *et al.*, 2015).

The other type of bacterial movement evaluated, twitching motility, is dependent on T4P and independent of flagella (Burrows, 2012; Dunger *et al.*, 2016). T4P are involved in surface attachment, biofilm formation, and cell-to-cell aggregation in several *Xanthomonas* species (Caserta *et al.*, 2010; Dunger *et al.*, 2014). In Xv, mutations in a gene involved in T4P structure dramatically reduced cell aggregation and tolerance to UV light (Ojanen-Reuhs *et al.*, 1997). Some authors have suggested that flagella and T4P contribute to swarming motility in bacteria (Kohler *et al.*, 2000; Rashid & Kornberg, 2000; Overhage *et al.*, 2007). Kohler *et al.* (2000) observed that in *P. aeruginosa*, T4P assist the flagellum in surface propagation and, alternatively, the pili could be involved in sensing the viscosity of the surface and sending a signal for initiation of swarming. Thus, the greater swarming motility observed in the BNM 208 strain could be related to its higher twitching motility.

During their epiphytic life many plant pathogenic bacteria form biofilms, a strategy that allows them to cope with harsh environments and establish pathogenic interactions with their hosts (Castiblanco & Sundin, 2016). In other *Xanthomonas* species, biofilm establishment requires, among other factors, the production of xanthan (Rigano *et al.*, 2007; Bianco *et al.*, 2016), and the presence of flagella (Malamud *et al.*, 2011) and T4P (Dunger *et al.*, 2016). In this study, the most aggressive strain, BNM 208, developed a biofilm with mature characteristics, with defined cell clusters, a homogeneous and compact structure, high biomass and substratum coverage. In contrast, BNM 216 exhibited a biofilm practically unstuck and without any characteristic of maturation, while BNM 214 developed a biofilm with an intermediate robustness and attachment to the surface. Despite this, BNM 216 showed the greatest average thickness values. However, this parameter does not reflect all of the structural heterogeneity of the biofilm; thickness represents the distance between the substratum and the outermost cell, but internal features of the biofilm, such as voids or cell clusters, are not captured by this measurement. Interestingly, BNM 208 produces a highly viscous xanthan, which could also help explain its high swarming motility. Surfactants like xanthan are amphipathic molecules that reduce tension between the substrate and the cell, enhancing the spread of bacteria over solid surfaces (Kearns, 2010). Furthermore, the EPS xanthan enables bacterial cells to stick together in a biofilm and is a key element in the development of complex, three-dimensional attached communities (Sauer *et al.*, 2007). Therefore, the differences observed in the biofilm structure of each strain might be related to the differences in viscosity of the xanthan they produced.

The results of the present study are consistent with those reported by other authors who highlight that biofilm formation facilitates bacterial establishment around the stomata and support the role of these structures as the initial step of the infection process (Rigano *et al.*, 2007).

To the best of the authors' knowledge, this is the first report that describes biofilm formation in relation to aggressiveness in strains of Xv. A better understanding

of the importance of biofilm formation in Xv virulence would help the development of new control methods for bacterial spot based on the disruption of biofilms or interference in their formation. As an example, 2-aminoimidazole, an analogue of the marine sponge natural product oroidin, decreases biofilm formation and bacterial spot symptoms induced by *X. euvesicatoria* on pepper (Worthington *et al.*, 2012). Further studies are needed to analyse other virulence factors implicated in the infection process of Xv. Such knowledge may provide a rational basis for the development of crop protection methods based on interference with key processes in the epiphytic survival and disease phases of bacterial pathogenesis.

Acknowledgements

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional de Villa María, project PIO-UNVM (grant number 20320150100008CO) and PIC-UNVM (grant number 053/2016). The authors thank the following people for technical assistance: Pilar María Crespo and Carlos Mas for confocal microscopy, and Jorge N. Gastón for bacterial multiplication and greenhouse experiments.

References

Aslam SN, Newman MA, Erbs G *et al.*, 2008. Bacterial polysaccharides suppress induced innate immunity by calcium chelation. *Current Biology* **18**, 1078–83.

Beaussart A, Baker AE, Kuchma SL, El-Kirat-Chatel S, O'Toole GA, Dufrene YF, 2014. Nanoscale adhesion forces of *Pseudomonas aeruginosa* type IV pili. *ACS Nano* **8**, 10723–33.

Bianco MI, Toum L, Yaryura PM *et al.*, 2016. Xanthan pyruvilation is essential for the virulence of *Xanthomonas campestris* pv. *campestris*. *Molecular Plant–Microbe Interactions* **29**, 688–99.

Bouzar H, Jones J, Stall R *et al.*, 1994. Physiological, chemical, serological, and pathogenic analyses of a worldwide collection of *Xanthomonas campestris* pv. *vesicatoria* strains. *Phytopathology* **84**, 663–71.

Burrows LL, 2012. *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annual Review of Microbiology* **66**, 493–520.

Caserta R, Takita M, Targon M *et al.*, 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. *Applied and Environmental Microbiology* **76**, 4250–9.

Castiblanco LF, Sundin GW, 2016. New insights on molecular regulation of biofilm formation in plant-associated bacteria. *Journal of Integrative Plant Biology* **58**, 362–72.

Darsonval A, Darrasse A, Durand K, Bureau C, Cesbron S, Jacques M-A, 2009. Adhesion and fitness in the bean phyllosphere and transmission to seed of *Xanthomonas fuscans* subsp. *fuscans*. *Molecular Plant–Microbe Interactions* **22**, 747–57.

Di Rienzo J, Casanoves F, Balzarini M, Gonzalez L, Tablada M, Versión C, 2017. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Córdoba, Argentina. [http://www.infostat.com.ar]. Accessed 28 June 2017.

Dunger G, Guzzo CR, Andrade MO, Jones JB, Farah CS, 2014. *Xanthomonas citri* subsp. *citri* type IV pilus is required for twitching motility, biofilm development, and adherence. *Molecular Plant–Microbe Interactions* **27**, 1132–47.

Dunger G, Llontop E, Guzzo CR, Farah CS, 2016. The *Xanthomonas* type IV pilus. *Current Opinion in Microbiology* **30**, 88–97.

Griffin K, Gambley C, Brown P, Li Y, 2017. Copper-tolerance in *Pseudomonas syringae* pv. *tomato* and *Xanthomonas* spp. and the control of diseases associated with these pathogens in tomato and pepper. A systematic literature review. *Crop Protection* **96**, 144–50.

Harshey RM, 2003. Bacterial motility on a surface: many ways to a common goal. *Annual Review of Microbiology* **57**, 249–73.

Heydorn A, Nielsen AT, Hentzer M *et al.*, 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146**, 2395–407.

Kaiser D, 2007. Bacterial swarming: a re-examination of cell-movement patterns. *Current Biology* **17**, R561–70.

Kearns DB, 2010. A field guide to bacterial swarming motility. *Nature Reviews Microbiology* **8**, 634–44.

Koenraadt H, Van Betteray B, Germain R, Hiddink G, Jones J, Oosterhof J, 2009. Development of specific primers for the molecular detection of bacterial spot of pepper and tomato. *Acta Horticulturae* **808**, 99–102.

Kohler T, Curty LK, Barja F, Van Delden C, Pechere JC, 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *Journal of Bacteriology* **182**, 5990–6.

Lemon KP, Higgins DE, Kolter R, 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *Journal of Bacteriology* **189**, 4418–24.

Lorenz C, Kirchner O, Egler M, Stuttmann J, Bonas U, Buttner D, 2008. HpaA from *Xanthomonas* is a regulator of type III secretion. *Molecular Microbiology* **69**, 344–60.

Malamud F, Torres PS, Roeschlin R *et al.*, 2011. The *Xanthomonas axonopodis* pv. *citri* flagellum is required for mature biofilm and canker development. *Microbiology* **157**, 819–29.

Merritt JH, Brothers KM, Kuchma SL, O'Toole GA, 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *Journal of Bacteriology* **189**, 8154–64.

Nguyen LC, Taguchi F, Tran QM *et al.*, 2012. Type IV pilin is glycosylated in *Pseudomonas syringae* pv. *tabaci* 6605 and is required for surface motility and virulence. *Molecular Plant Pathology* **13**, 764–74.

Obradovic A, Jones J, Momol M, Balogh B, Olson S, 2004. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. *Plant Disease* **88**, 736–40.

Ojanen-Reuhs T, Kalkkinen N, Westerlund-Wikström B *et al.*, 1997. Characterization of the fimA gene encoding bundle-forming fimbriae of the plant pathogen *Xanthomonas campestris* pv. *vesicatoria*. *Journal of Bacteriology* **179**, 1280–90.

Overhage J, Lewenza S, Marr AK, Hancock RE, 2007. Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn*5-lux* mutant library. *Journal of Bacteriology* **189**, 2164–9.

Petrova OE, Sauer K, 2012. Sticky situations: key components that control bacterial surface attachment. *Journal of Bacteriology* **194**, 2413–25.

Picchi S, Takita M, Coletta-Filho H, Machado M, Souza A, 2016. N-acetylcysteine interferes with the biofilm formation, motility and epiphytic behaviour of *Xanthomonas citri* subsp. *citri*. *Plant Pathology* **65**, 561–9.

Potnis N, Timilsina S, Strayer A *et al.*, 2015. Bacterial spot of tomato and pepper: diverse *Xanthomonas* species with a wide variety of virulence factors posing a worldwide challenge. *Molecular Plant Pathology* **16**, 907–20.

Rashid MH, Kornberg A, 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 4885–90.

Rigano LA, Siciliano F, Enrique R *et al.*, 2007. Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv. *citri*. *Molecular Plant–Microbe Interactions* **20**, 1222–30.

Ritchie DF, Dittapongpitch V, 1991. Copper- and streptomycin-resistant strains and host differentiated races of *Xanthomonas campestris* pv. *vesicatoria* in North Carolina. *Plant Disease* **75**, 733–6.

Roach R, Mann R, Gambley C, Shivas R, Rodoni B, 2017. Identification of *Xanthomonas* species associated with bacterial leaf spot of tomato, capsicum and chilli crops in eastern Australia. *European Journal of Plant Pathology* **150**, 595–608.

Romero A, Kousik C, Ritchie D, 2001. Resistance to bacterial spot in bell pepper induced by acibenzolar-*S*-methyl. *Plant Disease* **85**, 189–94.

Romero A, Correa O, Moccia S, Rivas J, 2003. Effect of *Azospirillum*-mediated plant growth promotion on the development of bacterial diseases on fresh-market and cherry tomato. *Journal of Applied Microbiology* **95**, 832–8.

Sauer K, Rickard AH, Davies DG, 2007. Biofilms and biocomplexity. *Microbe* **2**, 347.

Sena-Vélez M, Redondo C, Gell I *et al.*, 2015. Biofilm formation and motility of *Xanthomonas* strains with different citrus host range. *Plant Pathology* **64**, 767–75.

Stall RE, 1995. *Xanthomonas campestris* pv. *vesicatoria*. *Prokaryotes* **1**, 167–84.

Strayer AL, Jeyaprakash A, Minsavage GV *et al.*, 2016. A multiplex real-time PCR assay differentiates four *Xanthomonas* species associated with bacterial spot of tomato. *Plant Disease* **100**, 1660–8.

Taguchi F, Ichinose Y, 2011. Role of type IV pili in virulence of *Pseudomonas syringae* pv. *tabaci* 6605: correlation of motility, multidrug resistance, and HR-inducing activity on a nonhost plant. *Molecular Plant–Microbe Interactions* **24**, 1001–11.

Thieme F, Koebnik R, Bekel T *et al.*, 2005. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* revealed by the complete genome sequence. *Journal of Bacteriology* **187**, 7254–66.

Worthington RJ, Rogers SA, Huigens RW III, Melander C, Ritchie DF, 2012. Foliar-applied small molecule that suppresses biofilm formation and enhances control of copper-resistant *Xanthomonas euvesicatoria* on pepper. *Plant Disease* **96**, 1638–44.

Yaryura PM, Conforte VP, Malamud F *et al.*, 2015. XbmR, a new transcription factor involved in the regulation of chemotaxis, biofilm formation and virulence in *Xanthomonas cit*ri subsp. *citri*. *Environmental Microbiology* **17**, 4164–76.

Yun MH, Torres PS, El Oirdi M *et al.*, 2006. Xanthan induces plant susceptibility by suppressing callose deposition. *Plant Physiology* **141**, 178–87.