Plasmid 90 (2017) 44–52

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Protein depletion using the arabinose promoter in *Xanthomonas citri* subsp. *citri*

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ARTICLE INFO

Keywords:
Citrus canker
Chromosome segregation
Bacterial gene knockout

ABSTRACT

Xanthomonas citri subsp. *citri* (*X. citri*) is a plant pathogen and the etiological agent of citrus canker, a severe disease that affects all the commercially important citrus varieties, and has worldwide distribution. Citrus canker cannot be healed, and the best method known to control the spread of *X. citri* in the orchards is the eradication of symptomatic and asymptomatic plants in the field. However, in the state of São Paulo, Brazil, the main orange producing area in the world, control is evolving to an integrated management system (IMS) in which growers have to use less susceptible plants, windshields to prevent bacterial spread out and sprays of cupric bactericidal formulations. Our group has recently proposed alternative methods to control citrus canker, which are based on the use of chemical compounds able to disrupt vital cellular processes of *X. citri*. An important step in this approach is the genetic and biochemical characterization of genes/proteins that are the possible targets to be perturbed, a task not always simple when the gene/protein under investigation is essential for the organism. Here, we describe vectors carrying the arabinose promoter that enable controllable protein expression in *X. citri*. These vectors were used as complementation tools for the clean deletion of *parB* in *X. citri*, a widespread and conserved gene involved in the essential process of bacterial chromosome segregation. Overexpression or depletion of ParB led to increased cell size, which is probably a resultant of delayed chromosome segregation with subsequent retard of cell division. However, ParB is not essential in *X. citri*, and in its absence the bacterium was fully competent to colonize the host citrus and cause disease. The arabinose expression vectors described here are valuable tools for protein expression, and especially, to assist in the deletion of essential genes in *X. citri*.

1. Introduction

The Gram-negative bacterium *Xanthomonas citri* subsp. *citri* is the causal agent of citrus canker, a severe disease that affects all the commercially important citrus plants all over the world (Brunings & Gabriel, 2003; Gottwald et al., 2002). The most effective preventive measure known to control the spread of *X. citri* within and among orchards is the eradication of infected plants, along with the elimination of asymptomatic trees that may be potential sources of inocula in the surroundings. This was the practice in the state of São Paulo, one of the biggest orange producers, from 1999 to 2009 (Belasque Jr. et al., 2009). However, changes in legislation starting from 2010 relaxed the control measures and citrus canker has increased enormously, resulting directly in losses in the orange producing market (FUNDECITRUS).

Due to its economic importance, *X. citri* had its genome sequenced in early 2000 (da Silva et al., 2002) unveiling the structure of one main chromosome of ~5 Mb and two rather large plasmids, pXAC33 and pXAC64, with 33 and 64 kb, respectively. Genome annotation showed that *X. citri* carries homologues of the *parAB* genes in the chromosome, which are involved in replicon partitioning in bacteria (Gerdes et al., 2010; Leonard et al., 2005). Among the forces that act on bacterial chromosome segregation, ParB is part of an active partitioning system involved in the organization of the bacterial centromere (Badrinarayanan et al., 2015; Pinho et al., 2013). ParB binds specifically to *parS* sites organized around the origin of replication of bacterial

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http://dx.doi.org/10.1016/j.plasmid.2017.03.005
Received 17 February 2017; Received in revised form 20 March 2017; Accepted 23 March 2017
Available online 24 March 2017
0147-619X/

chromosomes (Lin and Grossman, 1998; Livny et al., 2007). In addition to specific binding to *parS*, ParB also engages in a second mode of DNA interaction known as spreading, in which it uses *parS* as a nucleation point to form large nucleoprotein complexes (Murray et al., 2006). Following spreading, inter-complex bridging (the interaction among ParB/DNA complexes), and the recruitment of condensin have been suggested to act on the organization of the centromeric region for efficient chromosome segregation (Graham et al., 2014; Gruber & Errington, 2009). In some bacterial models (*Caulobacter crescentus* and *Vibrio cholera*), the ParB partner ParA has been directly implicated in origin/chromosome partitioning (Fogel and Waldor, 2006; Ptacin et al., 2010). Some segregation models, namely the diffusion-ratchet mechanism and DNA-relay mechanism (Hwang et al., 2013; Lim et al., 2014; Vecchiarelli et al., 2013), suggest that ParA, which is an ATPase and a molecular switch controlled by the ATP/ADP bound state (Leonard et al., 2005), would bind DNA nonspecifically in the form of ParA-ATP. The ParA-ATP/DNA nucleoprotein complex probably grows from the bacterial pole to which the centromere has to be oriented. Next, ParB bound to *parS* interacts with ParA-ATP and stimulates its ATPase activity, which leads to ParA/DNA dissociation and ParB/*parS* movement towards a ParA-ATP gradient, resulting in centromere segregation.

In *X. citri*, *parB* is also involved in chromosome segregation (Ucci et al., 2014). The lack of a normal ParB function in *X. citri* led to chromosome segregation defects, an increase in the number of anucleated cells, cell filamentation and an unexpected phenotype of loss of pathogenicity, which could be associated with the impairment of cell motility (Bartosik et al., 2009; Donovan et al., 2010; Ireton et al., 1994; Mohl et al., 2001). These observations were obtained by expressing truncated forms of ParB in *X. citri*, since previously we were unable to delete the *parB* gene from it (Ucci et al., 2014). Considering that essential genes are often characterized by protein depletion in many organisms, we decided to apply this technique to study *parB* in *X. citri*. However, the first obstacle to be overcome was the need for a protein expression vector carrying a tight/controllable promoter in *X. citri*.

Here, we describe the construction of two new protein expression vectors for *X. citri*, one replicative and another integrative, both based on the arabinose promoter of the pBAD series (Guzman et al., 1995). Our vectors were used to express ParB *in trans* in *X. citri*, which enabled us to obtain a clean deletion of *parB* in this plant pathogen. Fine modulation of ParB expression was achieved by using the arabinose promoter in *X. citri*, and that was explored for the characterization of the ParB function in this bacterium. Finally, we conclude that ParB is not essential in *X. citri*, and in its absence the bacterium is still able to colonize citrus and induce citrus canker symptoms.

2. Material and methods

2.1. Bacterial strains and growth conditions

The strains used in the present work are listed in Table 1. The wild-type *Xanthomonas citri* subsp. *citri* used was the sequenced strain 306 (da Silva et al., 2002; Schaad et al., 2006; Schaad et al., 2005). *X. citri* was cultivated in NYG/NYG-agar medium (Peptone 5 g/L, yeast extract 3 g/L and glycerol 20 g/L) at 30 °C, supplemented with D-Glucose (2%), L-Arabinose (0,05%) and Sucrose (3%) for the knockouts. For the cloning steps we used *E. coli* DH10B (Invitrogen), cultivated in LB/LB-agar at 37 °C (Sambrook et al., 1989). The antibiotics kanamycin, gentamicin and ampicillin were used at 20 μg/mL. Growth curves: wild type *X. citri* and mutants were cultivated in NYG for 14 h; next, cultures were diluted in fresh NYG medium to an O.D.600 nm of ~0.1 and a final volume of 1.5 mL. Cell cultures were distributed in wells of a 24-wells microtiter plate and incubated in a Synergy H1 (BioTek) at 30 °C with constant agitation and O.D.600 nm measures every 30 min. Plots of the growth curves were prepared using the software Graphpad Prism 6.

2.2. Plasmids

The strategy used to construct the arabinose protein expression system for *X. citri* was essentially the one described by Sukchawalit et al. (1999), who ligated the Arabinose repressor/promoter cassette from the pBAD vector series (Guzman et al., 1995) into the backbone of a broad host range plasmid from the pBBR-MCS series (Kovach et al., 1995). Here, the cassette containing the arabinose repressor/promoter (pBAD), the *acp* gene of *E. coli* and the TAP1479 coding DNA (*araC-para-acp-tap1479*) was extracted by PCR from pEB304 (Gully et al., 2003) using the primers pARAF2/pARAR2 (Supplementary Table 1). The PCR product was ligated into pBBR1-MCS5 digested with *Hind*III/*Sma*I, giving rise to pLAL1 (Fig. 1). The replicative ParB expression vectors pLAC1 and pLAC2 were constructed by replacing the *acp* gene of pLAL1 with *X. citri parB*. The *parB* gene was PCR amplified from pAPU1 (Ucci et al., 2014) using the primer pairs ParBF20140220/ParBR20140220 or ParBF20140220/TAP1479-20140220, digested with *Hind*III/*Eco*RI, and ligated to pLAL1/*Hind*III/*Eco*RI, giving rise to pLAC1 and pLAC2, respectively (Fig. 1). To construct pLAL6, we first digested pGCD21 with *Bgl*II/*Sca*I in order to remove the markers for kanamycin and Ampicillin resistance. Next, we isolated the gentamicin coding DNA by PCR from pBBR1-MCS5 with primers 201410GmF/201409GmR, digested it with *Bam*HI, and ligated the product into pGCD21/*Bgl*II/*Sca*I, which gave rise to pGCD21-Gm (Fig. 1). Finally, *parB-tap1479* was isolated by PCR using pLAC2 as a template and the primers ParBF20141013/Tap1479-20141013. Then, *gfp* was removed from pGCD21-Gm using *Xho*I/*Xba*I, and replaced by the *parB-tap1479* cassette digested with the same enzymes, generating pLAL6 (Fig. 1). To construct the deletion plasmid pLAL7, two DNA fragments of approximately 1000 bp each (DNA1 and DNA2, corresponding to the genomic coordinates 4590802..4591785 and 4592668..4593677, respectively; da Silva et al. (2002)) were isolated by PCR from the *X. citri* genome using the primer pairs Delta-parBF1/Delta-parBR1 and Delta-parBF2/Delta-parBR2. Fragments DNA1 and DNA2 were digested with the enzymes *Eco*RI/*Sal*I and *Apa*I/*Eco*RI, respectively, and ligated to pNPTS138 linearized with *Apa*I/*Sal*I, giving rise to pLAL7 (Supplementary Fig. S1). All the PCR reactions were conducted using Pfu DNA polymerase (Thermo Scientific) and 1% formamide (Sigma F9037) was added to the reactions when amplifying *parB*. DNA constructs were checked by sequencing at Macrogen (Korea).

2.3. General molecular biology procedures

Standard protocols followed Sambrook et al. (1989). Electrotransformation of *X. citri* was carried out essentially as described in Ferreira et al. (1995). Analyses of southern and western blot were conducted according to the instructions contained in the DIG (Roche) and Westar Nova 2011 (Cyanagen) kits, respectively. Total proteins in cell extracts were quantified using the DC Protein assay kit (Bio-Rad 5000116). Before SDS-PAGE, protein samples were normalized to 0.8 mg/mL, and 3 μg of proteins were loaded per lane. The detection of the protein fusion ParB-TAP in western blot was done using the HRP-conjugated anti-horse (IgG) antibody raised in rabbits (Sigma-A6917) (Ucci et al., 2014). Knockout of *X. citri parB*: first, the complementation plasmids pLAC1 and pLAC2 (replicative) or pLAL6 (integrates into the *amy* locus of *X. citri*) were transformed into *X. citri*; secondly, the resultant strains, *X. citri*/pLAC1, *X. citri*/pLAC2 and *X. citri amy*::pLAL6, were transformed with pLAL7 and selected in NYG-agar supplemented with kanamycin. Only strains in which pLAL7 had integrated into the *X. citri* chromosome by a single crossover event involving either the DNA1 or the DNA2 region would be rescued (Supplementary Fig. S1). Single kanamycin resistant mutants (example: *X. citri*/pLAC1 *DNA1*::pLAL7 or *X. citri*/pLAC1 *DNA2*::pLAL7) were subsequently cultivated in NYG, without antibiotics, for 16 h. Next, cells were spread on NYG-agar supplemented with 3% sucrose and 0.05% arabinose (necessary to guarantee the supply of ParB/ParB-TAP) in order to select for those

Table 1
Strains and plasmids.

	Relevant characteristics	Reference
Plasmids		
pBBR1-MCS5	Broad host range vector; Gm^R	Kovach et al. (1995)
pEB304	pBAD derivative and source of the *araC-para-acp-tap1479* cassette; Ap^R	Gully et al. (2003)
pNPTS138	*Bacillus subtilis sacB* gene; Km^R; suicide vector in *X. citri*	Prof. L. Shapiro (Stanford University, USA)
pGCD21	Derivative of pHF5Ca (Ucci et al., 2014); *araC-para-gfpmut1*; *amy106-912*; Ap^R Km^R; integrative vector in *X. citri*	GenBank KU678206 (G. C. Dantas and H. Ferreira, unpublished)
pAPU1	ParB-TAP expression vector; *xylR-pxyl-parB-tap1479*; Ap^R Km^R	Ucci et al. (2014)
pGCD21-Gm	Derivative of pGCD21; Gm^R	This work
pLAL1	*araC-para-acp-tap1479*; Gm^R; replicative vector in *X. citri*	GenBank KP696472 This work
pLAC1	Derivative of pLAL1; *araC-para-parB*; Gm^R	This work
pLAC2	Derivative of pLAL1; *araC-para-parB-tap1479*; Gm^R	This work
pLAL6	Derivative of pGCD21-Gm; *araC-para-parB-tap1479*; Gm^R; integrative vector in *X. citri*	This work
pLAL7	Derivative of pNPTS138; carries DNA fragments 1, genomic coordinates 4590802..4591785, and 2, 4592668..4593677	This work
Strains		
X. citri	Wild-type strain 306; Ap^R	IBSBF-1594* (Schaad et al., 2006; Schaad et al., 2005)
E. coli DH10B	Cloning strain	Invitrogen
X. citri Δ*parB*	Carries a deletion between the genomic coordinates 4591785..4592668	This work
X. citri Δ*parB amy*::pLAL6	Expresses ParB-TAP; Gm^R	This work

*IBSBF- Instituto Biológico, Seção de Bacteriologia Fitopatológica, Campinas, SP, Brazil; Ap^R, km^R, Gm^R, ampicillin, kanamycin, and gentamicin resistance, respectively.

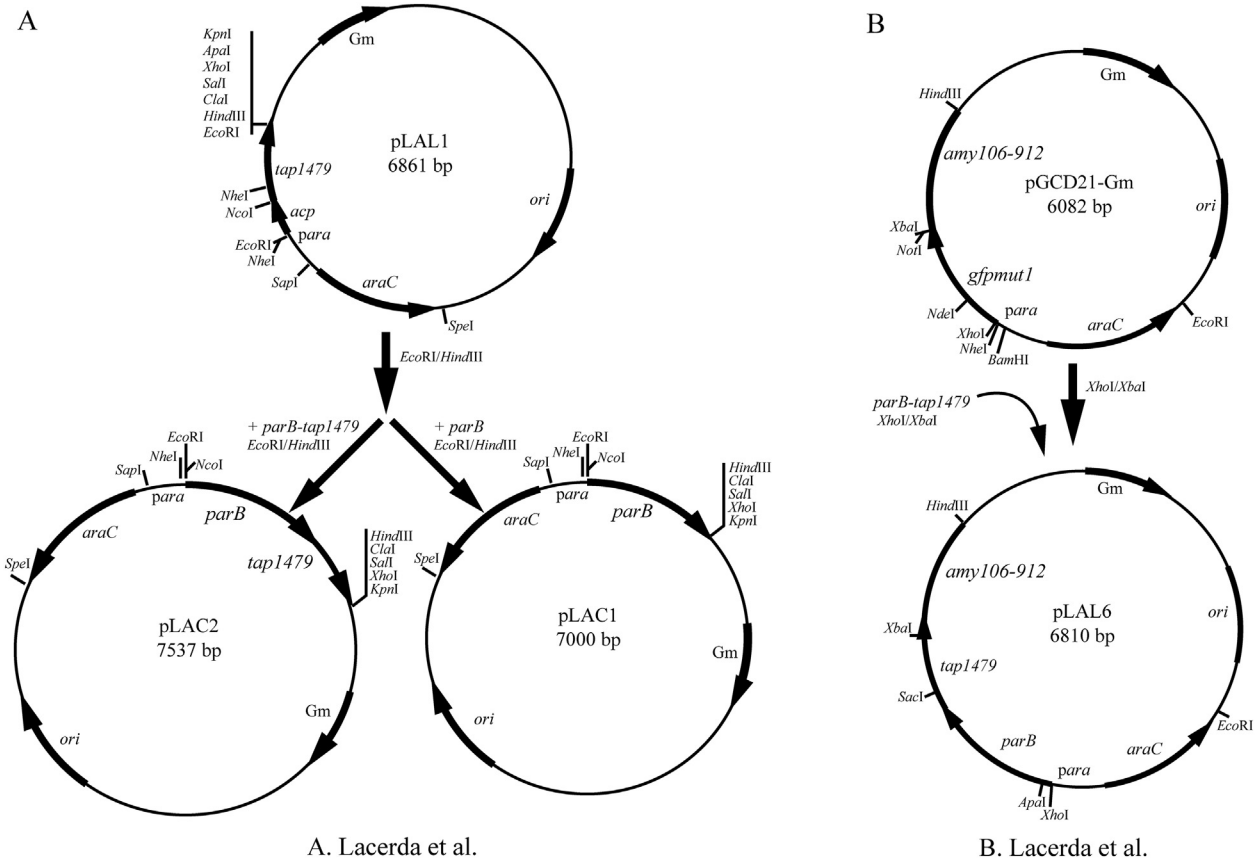

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Fig. 1. The arabinose protein expression vectors for *X. citri*. Maps of the replicative (A) and integrative (B) vectors showing their relevant features and available restriction sites. A) *ori* and Gm, origin of replication and gentamycin resistance cassettes, respectively, derived from pBBR1-MCS5. The DNA fragment containing *araC*, the arabinose repressor, p*ara*, arabinose promoter, *acp*, which encodes the acyl carrier protein of *E. coli*, and *tap1479*, encoding the TAP1479 tag (Puig et al., 2001), were extracted from pEB304 (Gully et al., 2003) (the *araC*-p*ara* region of pEB304, which includes the Ribosome Binding Site, derives from pBAD24 (Guzman et al., 1995)); *parB*, encodes the chromosome segregation protein ParB of *X. citri* (Ucci et al., 2014). B) pGCD21-Gm and pLAL6 carry the DNA fragment *amy*106-912 of *X. citri*, which allows the integration of the plasmids into the *amy* locus of the bacterial chromosome by a single crossover event. The complete nucleotide sequences of the main vectors pCGD21 and pLAL1 are available in the GenBank, under accession numbers KU678206 and KP696472, respectively.

mutants that had undergone the second crossover event involving the DNA region (DNA1 or DNA2) that had not been used in the first crossover. Deletion of *parB* was detected by PCR using primers Bup/Bdown (Supplementary Table 1 and Supplementary Fig. S1), and confirmed by Southern blot using *parB* as a probe (data not shown). Diagnostic PCR to *X. citri* was conducted according to the PCR protocol of Coletta-Filho et al. (2006).

2.4. Microscopy

X. citri and mutants were cultivated in NYG (supplemented with arabinose (0.05%) or glucose (2%) when needed) from the starting O.D.600 nm of ~0.1 at 30 °C. Upon reaching the O.D.600 nm of ~0.4, cells were immobilized on agarose-covered slides as described in Martins et al. (2010) and immediately visualized using a BX-61 Olympus Microscope equipped with an Orca-Flash2.8 monochromatic camera (Hamamatsu). Chromosome staining was performed by exposing the cells to SYTO 9, according to the instructions contained in the Live/Dead BacLight bacterial viability kit (Molecular Probes L7007). Image capture and processing was done via the software Cell Sens Dimension ver.11 (Olympus).

2.5. Pathogenicity tests

The host used was sweet orange Pera (*Citrus sinensis* L. Osbeck), cultivated under greenhouse conditions with temperatures ranging from at 25–35 °C. Strains to be tested were cultivated in NYG medium from the starting O.D.600 nm of ~0.1. Before inoculation, cultures were adjusted to the O.D.600 nm of ~0.4 (10^8 CFU/mL) using saline (0.9% NaCl), and cells were sprayed on leaves (between the stages V4-V6: leaves with 5–7 cm; FUNDECITRUS) until runoff. After inoculation, plants were covered with plastic bags for 24 h to help infection. Citrus canker symptoms were scored over a period of 30 days.

3. Statistics

All the data analyses were conducted using the software Graphpad Prism 6. Averages of cell length from different groups under study were compared with internal experimental controls (*X. citri* wild type) applying one-way ANOVA and Tukey post hoc test ($P < 0.05$).

Results

3.1. Responsiveness of the arabinose promoter in X. citri

Before we could start protein depletion studies of factors suspected to be essential in *X. citri*, we constructed two expression vectors for this plant pathogen (pLAL1 and pLAL6; Fig. 1A and B) based on the pBAD series of Guzman et al. (1995), which is considered a tightly regulated protein expression system in *E. coli*. The arabinose promoter governs transcription in both vectors, and they also allow the production of C-terminal TAP1479 tag fusions to polypeptides of interest (Puig et al., 2001). These vectors differ in the way they are kept within *X. citri*: pLAL1 is replicative, since it carries the origin of replication of the broad host range plasmid pBBR1-MCS5 (Kovach et al., 1995), while pLAL6 is integrative, which is intended to deliver a protein expression platform as a single copy into the bacterial chromosome.

The responsiveness of the arabinose promoter in *X. citri* was subsequently evaluated by western blot using strains deleted for *parB* and carrying the complementation plasmids pLAC2 (replicative) or pLAL6 (integrative). Both, pLAC2 and pLAL6, encode ParB-TAP (Fig. 1A and B). The expression levels of ParB-TAP in these strains were monitored over the course of 4 h after the promoter inducer arabinose was added (Fig. 2). First, untreated *X. citri* Δ*parB*/pLAC2 (cultivated without arabinose) exhibited a basal level of promoter leakage, which appeared as very faint bands at all the time points tested from 0 to 4 h

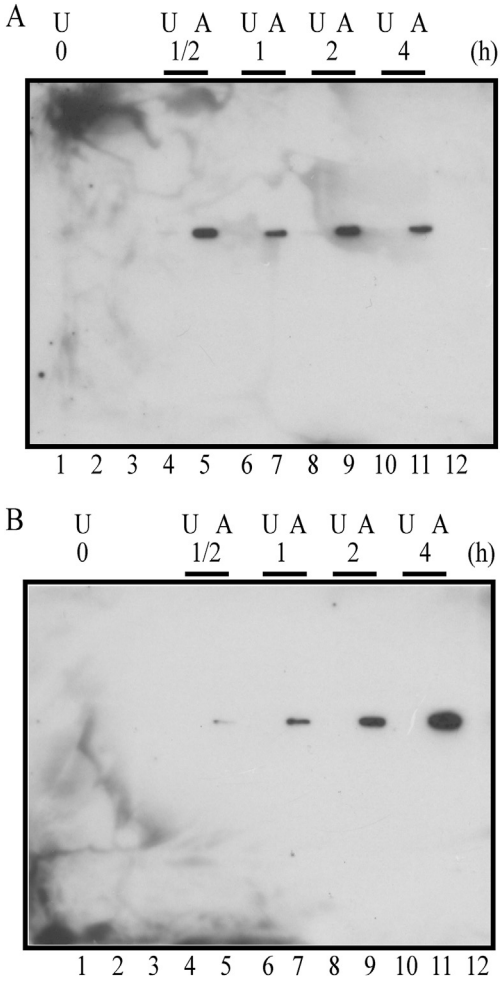

Fig. 2. Responsiveness of the arabinose promoter in *X. citri*. *X. citri* Δ*parB*/pLAC2 (A) and *X. citri* Δ*parB amy*::pLAL6 (B), both expressing ParB-TAP, were cultivated in NYG at 30 °C from the starting O.D.600 nm of ~0.1. The inducer arabinose was added to the media after 3 h of growth. Samples were collected at the time points indicated for the immunodetection of ParB-TAP in western blot. Lanes: 1, 4, 6, 8, and 10, untreated (U); 2, 3, protein ladder (see Fig. S2 for the correspondent SDS-PAGE); 5, 7, 9, and 11, arabinose 0.05% (A). The unique band signal shown corresponds to ParB-TAP of ~54 kDa.Responsiveness of the arabinose promoter in *X. citri*. *X. citri* Δ*parB*/pLAC2 (A) and *X. citri* Δ*parB amy*::pLAL6 (B), both expressing ParB-TAP, were cultivated in NYG at 30 °C from the starting O.D.600 nm of ~0.1. The inducer arabinose was added to the media after 3 h of growth. Samples were collected at the time points indicated for the immunodetection of ParB-TAP in western blot. Lanes: 1, 4, 6, 8, and 10, untreated (U); 2, 3, protein ladder (see Fig. S2 for the correspondent SDS-PAGE); 5, 7, 9, and 11, arabinose 0.05% (A). The unique band signal shown corresponds to ParB-TAP of ~54 kDa.

(Fig. 2A, lanes 1, 4, 6, 8, and 10). Note that the time points for the untreated samples are just a reference to the period of exposure to arabinose. Noteworthy, leakage from pLAC2 was also observed in pilot experiments using *E. coli* DH10B as a host (data not shown). When arabinose (0.05%) was added to this culture we documented a rapid increase in the amount of ParB-TAP (Fig. 2A, compare lanes 1 and 5). The expression here was investigated after 30 min of induction; however, in pilot experiments, we could detect ParB-TAP as fast as 5 min after the addition of arabinose (the shortest time evaluated), which resembled the fast induction in *E. coli* (Guzman et al., 1995). As the exposure to the inducer increased, we saw a continuous production of ParB-TAP with a slight oscillation among time points (compare lanes 5, 7, 9, and 11). This oscillation resembled a typical dynamics of synthesis/degradation of inducible protein factors, which impose some degree of toxicity within the cells (see below).

A noticeable difference was observed when we analyzed *X. citri* Δ*parB amy*::pLAL6, which carries the integrative system (Fig. 2B). First,

we did not detect any promoter leakage over the period evaluated in comparison with the basal level of expression observed with the replicative vector pLAC2 (compare lanes 1, 4, 6, 8, and 10 in Fig. 2B with the same lanes in Fig. 2A). Secondly, upon arabinose addition, expression of ParB-TAP could also be detected as quickly as 30 min (lane 5), however, in a much lower amount than that observed for the replicative system (compare lanes 5 in Fig. 2A and B). This result is consistent with the single copy status of pLAL6 integrated into the *amy* locus of *X. citri*. As the incubation time with arabinose increased, we saw a gradual accumulation of ParB-TAP (Fig. 2B, compare lanes 5, 7, 9 and 11), which differs markedly from the rapid and high accumulation of the fusion in *X. citri* Δ*parB*/pLAC2. Taken together, the rapid/high accumulation of ParB-TAP in *X. citri* Δ*parB*/pLAC2, if toxic, may hamper the development of the culture, which is reflected by the oscillation in the production of ParB-TAP followed by a decline in the culture (see next section). Finally, considering that equal amounts of protein extracts were used in each lane, the variation detected in the protein expression levels of untreated and treated cultures reflects the responsiveness of the arabinose promoter in *X. citri*.

3.2. Depletion of ParB in X. citri

In our previous report of Ucci et al. (2014), we could not obtain a clean deletion of the *X. citri parB* gene. This result, allied to the disruptive phenotype exhibited by *X. citri* strains carrying only truncated forms of ParB, suggested that *parB* is essential in this plant pathogen. However, as mentioned above, in the present work we succeeded in deleting *parB* from *X. citri* strains carrying the complementation plasmids pLAC1 and pLAC2 (both replicative, encoding ParB and ParB-TAP, respectively), and pLAL6 (integrative, encoding ParB-TAP) (Fig. 1A and B). The rationale for this was that deletion could be facilitated if ParB were supplied *in trans* (pLAC1 and pLAC2) or ectopically (pLAL6).

In order to find out what would be the best conditions to achieve depletion of ParB, we started by analyzing the growth patterns of the three mutants *X. citri* Δ*parB*/pLAC1, *X. citri* Δ*parB*/pLAC2, and *X. citri* Δ*parB amy*::pLAL6 in comparison with the wild type strain (Fig. 3A–D). Cultures were also exposed to arabinose to evaluate the effects of overexpressing ParB. Wild type *X. citri* and mutants were initially cultivated for one and a half generation (3 h, which for the wild type *X. citri* corresponds to the start of the exponential phase (Sumares et al., 2015)), when the inducer arabinose and the repressor glucose were added to the media (Fig. 3A–D, vertical black arrows). After the addition of both sugars, the wild type strain exhibited an almost invariable growth pattern, with only a slight increase in cell mass induced by glucose (Fig. 3A, compare U/black, untreated/control, with A/blue, 0.05% arabinose, and G/orange, 2% glucose). This increase (orange line, glucose, above the blue line, untreated) was due to the abundance of glucose as carbon source. Note that the same behavior was documented for the three *parB*-deleted mutants in the presence of this sugar (Fig. 3, B, C and D). Importantly, this is the condition in which ParB expression is repressed in the mutants (see below), and if glucose repression were to have any effect on cellular growth, the abundance of this carbon source may have masked such an effect (depending on the gene/protein under study), since it boosts the culture.

A modest although conspicuous difference was observed in the curves of the three mutants growing without any sugar (Black lines in B, C and D). Overall, there was a slight retard of growth, seen as a shift of the curves to the right in the exponential phase (compare the black lines of the control in A with the mutants in B, C and D). When arabinose was added to the mutants (blue lines in B, C and D), the phenotypes diverged among strains. For those strains carrying replicative plasmids, *X. citri* Δ*parB*/pLAC1 and *X. citri* Δ*parB*/pLAC2, entry into the exponential phase was retarded, and only *X. citri* Δ*parB*/pLAC1 was actually able to sustain exponential growth (Fig. 3, B and C, blue lines). Cultures of *X. citri* Δ*parB*/pLAC2 exhibited a very soft increment over the following 12 h in which the cells were monitored (until the time point of 15 h). This strain reached only the O.D.600 nm of ~0.5 as opposed to the standard of ~1.2 seen for the wild type *X. citri* (Fig. 3, compare the blue lines in A and C). The behavior displayed by the *X. citri* Δ*parB*/pLAC2 cultures is consistent with a possible toxic effect of ParB-TAP suggested above. Finally, *X. citri* Δ*parB amy*::pLAL6, which expresses ParB-TAP ectopically, had a performance more similar to the control, showing however a very short stationary phase around 10 h, and an anticipation of the death phase beyond ~11 h (Fig. 3, compare the blue lines in A and D). Note here that the toxic effect caused by the accumulation of ParB-TAP in the mutant *X. citri* Δ*parB amy*::pLAL6 happens exactly ~4-5 h after the addition of arabinose, when the blue line splits from the black line (Fig. 3D). So far, our results show that the lack of inducer/ParB led to a delay in culture growth for the three mutants analyzed (untreated condition, black lines, in B, C and D), and overexpression of ParB (arabinose added, blue lines, in B, C and D) had a more deleterious effect in *X. citri* Δ*parB*/pLAC2 than in *X. citri* Δ*parB*/pLAC1 or *X. citri* Δ*parB amy*::pLAL6, because of the rapid/high accumulation of the fusion ParB-TAP.

Concomitantly with the O.D. measurements, we also followed the expression of ParB-TAP in western blots of *X. citri* Δ*parB*/pLAC2 and *X. citri* Δ*parB amy*::pLAL6. At the time of 3 h (before the addition of any sugar), ParB-TAP was not detected (Fig. 3E and F, lane 1). Three hours after the exposure to the sugars (growth-time of 6 h) ParB-TAP could be easily detected for *X. citri* Δ*parB*/pLAC2 (Fig. 3E, lane 3), contrasting sharply with the very faint band seen for *X. citri* Δ*parB amy*::pLAL6 (Fig. 3F, lane 3). Again, the lower expression from pLAL6 reflects the single copy status of this system. The production of ParB-TAP reached a peak at ~6 h of induction for both cultures (Fig. 3E and F, lanes 8), which is consistent with the time in which untreated cultures start the stationary phase (the equivalent of ~10 h of growth). This peak was clearly sustained for approximately 3 h, since the same relative amount of ParB-TAP could be detected at 9 h of induction (12 h culture-growth, lanes 11). Noteworthy, this is the time point in which we observe the most significant difference between the replicative and integrative systems: there is leakage from pLAC2 in the untreated situation (Fig. 3E, lane 10), and although glucose can repress the arabinose promoter, repression is not effective (lane 12). On the contrary, we see a perfect control of ParB-TAP production from pLAL6 without any detectable leakage in all the time points evaluated as untreated or glucose-repressed (Fig. 3F, as example, compare lanes 10–12). Finally, the decline phase, which is clear for *X. citri* Δ*parB amy*::pLAL6, develops beyond 10–12 h of growth, and this is when ParB-TAP starts to disappear (see lanes 14; 15 h of growth). Taken together, data shows that the integrative system was efficient to control the expression of ParB, without leakage in the untreated or repressed (glucose added) situations. Considering that *X. citri* Δ*parB amy*::pLAL6 was able to grow in the absence of ParB (in the form of ParB-TAP) (Fig. 3D), we conclude that this protein is not essential in *X. citri*.

3.3. Lack of ParB retards cell division in X. citri

We showed recently that *X. citri* mutant strains expressing only truncated forms of ParB exhibited pleiotropic phenotypes, among them cell filamentation, chromosome segregation defects, and loss of pathogenicity (Ucci et al., 2014). To determine the direct contribution of ParB to the first two phenotypes we modulated the expression of ParB-TAP in *X. citri* Δ*parB amy*::pLAL6, and compared cell morphology and nucleoid organization of mutant and wild type strains under the microscope. First, the wild type strain had an average cell length of ~1.7 μm, while the *parB* mutant was significantly longer exhibiting cells of ~2.36 μm (Table 2; Fig. 4). The increased cell size displayed by *X. citri* Δ*parB amy*::pLAL6 was observed in arabinose (0.05%), glucose (2%), and even in the untreated culture. Long filaments and chains (which are indicative of cell division and chromosome segregation

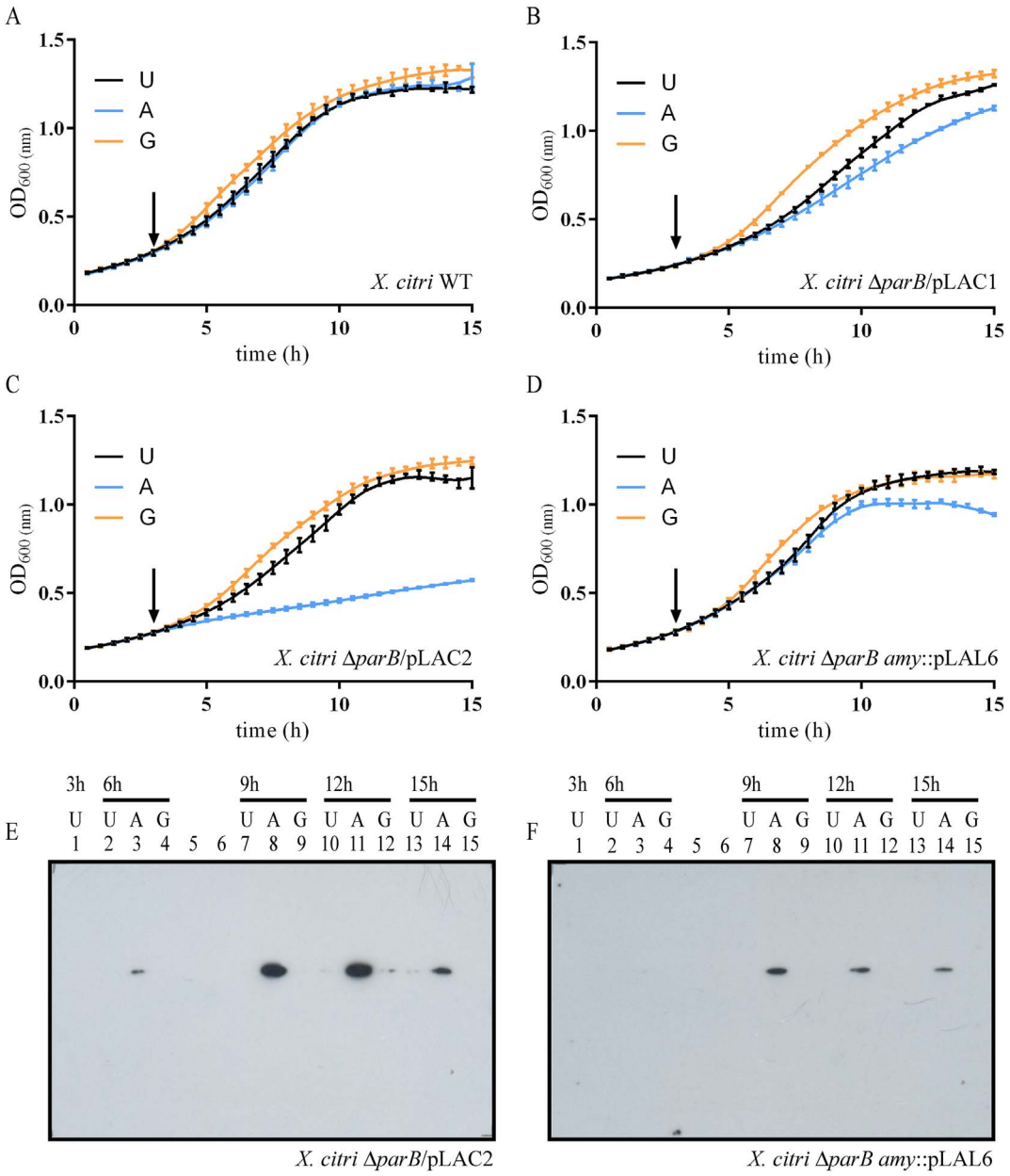

Fig. 3. ParB depletion. Strains of *X. citri*, WT and deleted for *parB*, were cultivated in NYG at 30 °C from the starting O.D.600 nm of ~0.1. After 3 h of growth, arabinose (0.05%), and glucose (2%) were added to the cultures (black arrows in A, B, C and D). O.D.600 nm measurements were taken every 30 min, and each point in the curves corresponds to the average O.D.600 nm calculated from three independent experiments; vertical bars, standard deviation. Curves are color-coded and correspond to U, untreated (control) cells; A, 0.05% arabinose; G, 2% glucose. (E) and (F) immunodetection of ParB-TAP in samples of *X. citri* Δ*parB*/pLAC2 and *X. citri* Δ*parB amy*::pLAL6, respectively. Lanes (U, untreated cells; A, 0.05% arabinose; G, 2% glucose): 1, U3 h; 2–4, U6 h, A6 h, and G6 h; 5–6, molecular weight markers (see Fig. S3 for the correspondent SDS-PAGE); 7–9, U9 h, A9 h, G9 h; 10–12, U12 h, A12 h, G12 h; 13–15, U15 h, A15 h, G15 h. The band shown corresponds to ParB-TAP of ~54 kDa. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)ParB depletion. Strains of *X. citri*, WT and deleted for *parB*, were cultivated in NYG at 30 °C from the starting O.D.600 nm of ~0.1. After 3 h of growth, arabinose (0.05%), and glucose (2%) were added to the cultures (black arrows in A, B, C and D). O.D.600 nm measurements were taken every 30 min, and each point in the curves corresponds to the average O.D.600 nm calculated from three independent experiments; vertical bars, standard deviation. Curves are color-coded and correspond to U, untreated (control) cells; A, 0.05% arabinose; G, 2% glucose. (E) and (F) immunodetection of ParB-TAP in samples of *X. citri* Δ*parB*/pLAC2 and *X. citri* Δ*parB amy*::pLAL6, respectively. Lanes (U, untreated cells; A, 0.05% arabinose; G, 2% glucose): 1, U3 h; 2–4, U6 h, A6 h, and G6 h; 5–6, molecular weight markers (see Fig. S3 for the correspondent SDS-PAGE); 7–9, U9 h, A9 h, G9 h; 10–12, U12 h, A12 h, G12 h; 13–15, U15 h, A15 h, G15 h. The band shown corresponds to ParB-TAP of ~54 kDa. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

defects, respectively) were not observed either for the wild type or the *parB* mutant. The wild type strain exhibited only 0.2% of anucleated rods, which is consistent with our previous reports (Silva et al., 2013; Ucci et al., 2014). On the other hand, *X. citri* Δ*parB amy*::pLAL6 showed on average three times more anucleated cells in comparison with the wild type strain in practically all the treatments (untreated cultures, 0.05% arabinose, and 2% glucose); although, it did not reach an order of magnitude.

When we evaluated the nucleoid organization of both wild type and *parB* mutant, we did not detect clear signs of aberration (Fig. 4; SYTO 9). Apart from the noticeable increase of size of the *parB* mutants, both strains had a proper distribution of the chromosomal mass irrespective of the treatment imposed. Taken together, we conclude that lack of *parB*, which happened in the untreated culture of *X. citri* Δ*parB amy*::pLAL6 and in the presence of glucose, leads to increased cell size (Table 2). In addition, overexpression of ParB by cells of *X. citri* Δ*parB*

Table 2
Cell length of ParB-depleted *X. citri*.

	Average cell length ± SD (μm)[+]	
	X. citri	*X. citri* Δ*parB amy*::pLAL6
Untreated	1.65 ± 0.45[a]	2.42 ± 0.71[b]
Arabinose	1.71 ± 0.42[a]	2.32 ± 0.66[b]
Glucose	1.85 ± 0.52[a]	2.34 ± 0.69[b]

[+] Average cell length ± standard deviation (n = 100) of *X. citri* and *X. citri* Δ*parB amy*::pLAL6 measured after 6 h of treatment.
[a] Averages differ statistically (one-way ANOVA followed by Tukey post-test; $P < 0.05$).
[b] Averages differ statistically (one-way ANOVA followed by Tukey post-test; $P < 0.05$).

amy::pLAL6 treated with arabinose produced the same effect. Therefore, increased cell size might be attributed to a delay in chromosome segregation/clearance with subsequent delay of cell division that happens in the absence of ParB or when there is an imbalance/overexpression of this protein.

3.4. ParB depletion does not interfere with the ability of X. citri to colonize citrus

The ability to colonize citrus and cause disease was evaluated using the mutant strain *X. citri* Δ*parB amy*::pLAL6. As demonstrated above, *X. citri* Δ*parB amy*::pLAL6 does not express ParB in the absence of arabinose (Fig. 3F, untreated conditions). Bacterial strains were cultivated in NYG without arabinose or glucose, and then spray-inoculated onto leaves of the susceptible host sweet orange Pera. The appearance of citrus canker symptoms were scored over the course of 30 days post-inoculation. The wild type strain *X. citri* induced the typical brownish corky-like symptoms of citrus canker, which became visible ~20 days post-inoculation (Fig. 5B). No lesions were observed on leaves sprayed with the carrier saline (5A). The mutant strain *X. citri* Δ*parB amy*::pLAL6 was as competent as the wild type to colonize citrus leaves and induce the symptoms of citrus canker (5C). The incidence of lesions, and time of appearance were identical. Therefore, we conclude that the lack of ParB does not interfere with pathogenicity of *X. citri*.

4. Discussion

Chromosome segretation is a vital process that coordinates the proper partitioning of the genetic material to the daughter cells before cytokinesis. In *X. citri*, segregation of newly replicated chromosomal origins is asymmetric and depends, at least in part, on the function of the centromere organizing factor ParB (Ucci et al., 2014). Chromosomally encoded ParB is a well conserved bacterial protein (Livny et al., 2007), which is essential in only a few organisms, but its absence may lead to diverse pleiotropic effects (reviewed by Mierzejewska and Jagura-Burdzy (2012)). A preliminary characterization of *X. citri* ParB by our group was conducted using truncated forms of the protein, since we were not able to knockout the *parB* gene in this plant pathogen. Here, we extended our analyses of *X. citri* ParB using protein depletion, which was possible due to the use of newly developed protein expression vectors dedicated to this plant pathogen. ParB supplied *in trans* was decisive to allow a clean deletion of the *X. citri parB* gene, and the subsequent modulation of ParB expression from an integrative complementation vector corroborated its involvement in chromosome segregation.

In the current work we demonstrate that ParB is not essential in *X. citri*, since we documented growth of *X. citri* Δ*parB amy*::pLAL6 in conditions where ParB cannot be detected. However, the *parB* null mutant (*X. citri* Δ*parB amy*::pLAL6) was obtained only after we used the depletion system, which may seem contradictory. A tentative explanation for this is that chromosome segregation is an essential process for all living cells; therefore, cells may have evolved in such a way as not to rely entirely on a single system to accomplish this task. The ParAB system qualifies for a redundant player as it helps segregation in conjunction with forces such as replication (the extrusion-capture model for segregation coupled to replication), transcription (genes are oriented in such a way that concerted transcription may exert force upon them and the chromosome towards the cellular poles), and chromosome compaction (Dworkin & Losick, 2002; Lemon and Grossman, 2001; Wang et al., 2013). However, being redundant does not imply that *parB* can be easily knocked out. It is conceivable that *parB* knockout is an unfavorable event that becomes possible and easier when it has a smoother transition. The smoother transition happens when we put within the cells an extra copy of *parB* (the depletion system). Only after that we succeeded in deleting the native gene, perhaps transforming a rare event in a frequent one.

The protein expression vectors described here use the arabinose

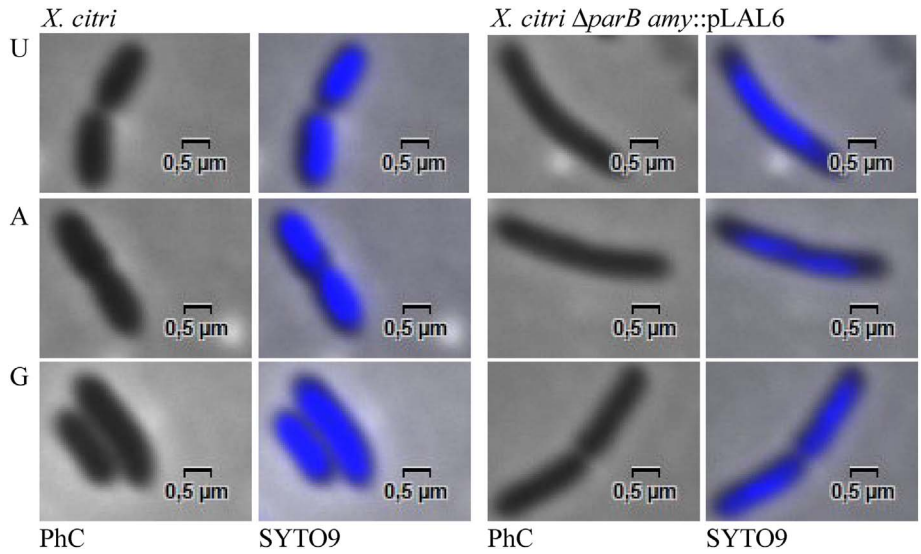

Fig. 4. Cell morphology. Wild type *X. citri* and *X. citri* Δ*parB amy*::pLAL6 were cultivated from the starting O.D.600 nm of ~0.1 in NYG medium, supplemented or not with arabinose (0.05%) or glucose (2%). When cultures reached the O.D.600 nm of ~0.4 (~6 h of growth), cells were stained with SYTO 9 and visualized by phase contrast (PhC) and fluorescence (SYTO 9) microscopy. U, untreated; A, 0.05% arabinose; G, 2% glucose. Magnification of 100 ×.

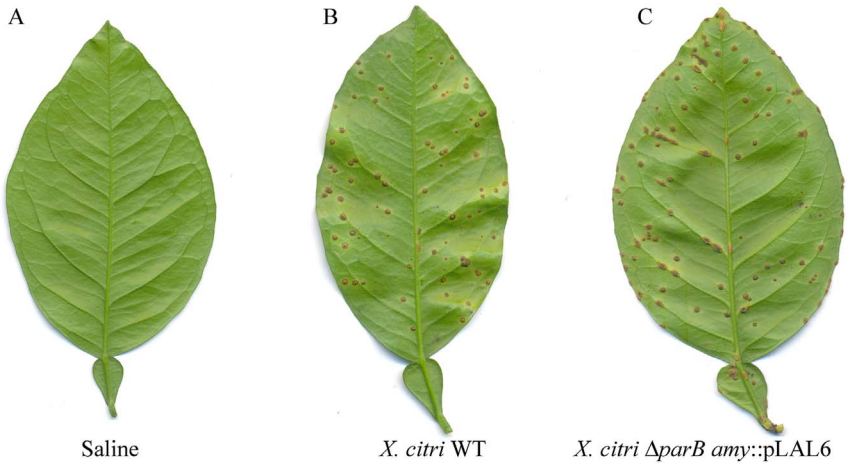

Fig. 5. Lack of ParB does not interfere with the pathogenicity of *X. citri*. Bacterial strains were cultivated in NYG for 12 h, and subsequently diluted in saline to reach the O.D.600 nm of ~0.4. Cell suspensions were spray-inoculated on leaves of sweet orange cultivar Pera. Pictures were taken 30 days post-inoculation. Saline, 0,9% NaCl.

promoter of the pBAD series, which is largely used in *E. coli* and considered to be tightly regulated in many bacteria (Guzman et al., 1995). Sukchawalit et al. (1999) reported the construction and characterization of a series of replicative vectors based on the fusion of the arabinose promoter and the broad host range replicon/backbone of the pBBR1 series of cloning vectors (Kovach et al., 1995). Their system was evaluated for optimal expression conditions using *Xanthomonas campestris* pv. *phaseoli* as a host, and subsequently, Dunger et al. (2007) used one of these vectors to complement a *X. citri gumD* mutant strain. However, nobody had ever characterized the responsiveness of the arabinose promoter in *X. citri*. Apart from a replicative arabinose protein expression system, we report here on an integrative version of it (pLAL6). The integrative system enabled us to fine-tune protein expression in *X. citri*, here demonstrated by the possibility of carrying out the depletion of ParB-TAP in the mutant strain *X. citri* Δ*parB amy*::pLAL6 (Fig. 3). Although useful, the arabinose promoter in a replicative vector exhibited considerable leakage (Fig. 3), which alone might be enough to complement a mutant without the need for induction. Control of the arabinose promoter in *X. citri* was better acchieved when we used the integrative vector pLAL6. When pLAL6 was placed as a single copy into the bacterial chromosome, it allowed a finer modulation of ParB-TAP expression with no detectable leakage, even in the absence of the repressor glucose. Comparatively to promoters like *tac*, *lac*, vanillate, and xylose (Amann et al., 1988; Lewis and Marston, 1999; Thanbichler et al., 2007) that were already tested in *X. citri* (A. P. Ucci, unpublished results; Martins et al. (2010)), the arabinose promoter was superior in responsiveness and capability to shutdown protein expression to undetectable levels.

The clean deletion of the *X. citri parB* gene not only enabled us to extend the characterization of its function, but it also clarified the occurrence of some phenotypic traits observed in the previous report of Ucci et al. (2014). Here we showed that lack (*X. citri* Δ*parB amy*::pLAL6; untreated or glucose-repressed) or over-expression (*X. citri* Δ*parB amy*::pLAL6 exposed to arabinose) of ParB (in the form of ParB-TAP) in *X. citri* led to a retard in cell growth/division, and altered cell size, and a slight increase in the number of anucleated rods. These results are in line with the phenotypes observed for several model bacteria (reviewed by Mierzejewska and Jagura-Burdzy (2012)). However, the filamentation phenotype observed in the *parB* disruption mutant *X. citri parB*::pAPU2 (5% of the cells; Ucci et al. (2014)) was not detected here for *X. citri* Δ*parB amy*::pLAL6. Depletion of ParB caused a mild morphological effect in *X. citri* Δ*parB amy*::pLAL6, which was manifested by a slight, although significant, increase in cell length when compared to the wild type strain. Noteworthy, increased cell size accompanied by a slowdown on cell growth have been a trend whenever the chromosome segregation function is affected in *X. citri*. This can be acchieved by thermal shock (Sumares et al., 2015), treatment with alkyl gallates, which are potent FtsZ inhibitors (Krol et al., 2015; Silva et al., 2013), disruption of *parB* (Ucci et al., 2014), and now, *parB* deletion/ParB depletion. Therefore, we conclude that the filamentation phenotype of *X. citri parB*::pAPU2 (Ucci et al., 2014) was directly associated with the expression of truncated forms of ParB within *X. citri*.

Some reports on the literature offer support to a link among ParB, motility and pathogenicity. Lack of ParB, and also of its partner protein ParA, has been implicated with altered motility in *P. aeruginosa* (Bartosik et al., 2009). In *X. citri*, an intact flagellum, and consequently motility, is necessary for mature biofilm formation on citrus leaves, which in turn is required for host plant colonization and disease development (Li & Wang, 2011; Malamud et al., 2011; Rigano et al., 2007). Here, depletion of ParB in *X. citri* Δ*parB amy*::pLAL6 did not affect its ability to colonize citrus plants and to induce citrus canker symptoms. So far, we have not detected alterations in motility in our mutants *X. citri* Δ*parB*/pLAC1, *X. citri* Δ*parB*/pLAC2 and *X. citri* Δ*parB amy*::pLAL6 (unpublished observation). Finally, the arabinose protein expression vectors described in the present work are valuable tools for protein expression, gene complementation, protein depletion, and especially, to assist in the deletion of essential genes in *X. citri*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plasmid.2017.03.005.

Acknowledgments

LAL and LBC received scholarships from CAPES- Brazil. This work was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP Grants 2013/14013-7 and 2013/50367-8). We thank Centro de Citricultura Sylvio Moreira for supplying the citrus plants used in the pathogenicity tests.

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