RESEARCH LETTER

Subcellular localization of proteins labeled with GFP in *Xanthomonas citri* ssp. *citri*: targeting the division septum

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Abstract

Xanthomonas citri ssp. *citri* (Xac) is the causal agent of citrus canker, an economically important disease that affects citrus worldwide. To initiate the characterization of essential biological processes of Xac, we constructed integrative plasmids for the ectopic expression of green fluorescent protein (GFP)-labeled proteins within this bacterium. Here, we show that the disruption of the α-amylase gene (*amy*), the site of plasmid integration into the bacterial chromosome, does not alter its pathogenesis while abolishing completely the ability of Xac to degrade starch. Furthermore, our GFP expression system was used to characterize ORF XAC3408, a hypothetical protein encoded by Xac that shares significant homology to the FtsZ-stabilizing factor ZapA from *Bacillus subtilis* ($ZapA^{Bsu}$). GFP-XAC3408 expressed in Xac exhibited a septal localization pattern typical of GFP-$ZapA^{Bsu}$, which indicates that XAC3408 is the Xac orthologue of the cell division protein $ZapA^{Bsu}$. The results demonstrate the potential of GFP labeling for protein functional characterizations in Xac, and, in addition, the Xac mutant strain labeled at the septum constitutes a biological model for the exploration of antibacterial compounds able to inhibit cell division in this plant pathogen.

Introduction

Xanthomonas citri ssp. *citri* (Schaad *et al*., 2005, 2006) (also known as *Xanthomonas axonopodis* pv. citri or Xac) is a Gram-negative, plant-pathogenic bacterium that affects most citrus species and is the causal agent of citrus canker, a very economically important disease of citrus plants worldwide. An effective control for this disease is inexistent, and a more detailed understanding of the biology of the etiological agent may contribute substantially toward the development of strategies to prevent and control infection. A major effort to accomplish this task was the elucidation of the genome sequence of Xac (da Silva *et al*., 2002), which has stimulated a number of molecular studies using Xac as model microorganism, and yet, little information is available regarding technical methods that could enhance its proteome exploration (Galvao-Botton *et al*., 2003; Mehta & Rosato, 2003; Alegria *et al*., 2004, 2005; Cernadas *et al*., 2008).

Our main interest focused on the characterization of some essential biological processes of Xac, more specifically those involved with chromosome segregation and cell division. A common feature of such bacterial systems is that they are usually composed of proteins sharing little homology to their functional analogues in more derived eukaryotes; therefore, these proteins constitute ideal targets for antimicrobial drug development and pathogen control (e.g. Gitai *et al*., 2005; Pan *et al*., 2006; Haydon *et al*., 2008; Beuria *et al*., 2009; Kapoor & Panda, 2009). However, to undertake protein functional studies with/in Xac, we were limited by the lack of biological tools developed and/or accessible for this purpose.

Here, we describe a protein expression system dedicated to Xac, which can also be used for the subcellular localization of the green fluorescent protein (GFP)-labeled factors in this pathogen. We used the system to characterize a hypothetical protein of Xac that shares significant homology

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to the FtsZ-stabilizing factor ZapA, originally described in *Bacillus subtilis* (ZapA^{Bsu}) (Gueiros-Filho & Losick, 2002). Furthermore, we show that the disruption of the α-amylase gene, the site of plasmid integration into the Xac chromosome, does not alter its pathogenesis.

Materials and methods

Bacterial strains and media

The *Xanthomonas citri* ssp. *citri* used was the sequenced strain (da Silva *et al*., 2002), formerly designated *X. axonopodis* pv. citri strain 306 (IBSBF 1594). *Escherichia coli* strain DH10B (Invitrogen) was used for cloning. *Escherichia coli* was cultivated at 37 °C in a Luria–Bertani (LB)/LB-agar medium (Sambrook *et al*., 1989); Xac was cultivated at 30 °C in a Nutrient Yeast Glycerol (NYG)/NYG-agar medium (Daniels *et al*., 1984). The antibiotics ampicillin and kanamycin were used, when required, at 20 and 10 μg mL^{-1}, respectively. For the α-amylase tests, NYG-agar medium was supplemented with soluble starch at 0.2%; development of halos was carried out by exposing the medium, after bacterial growth, to vapors delivered by iodine crystals.

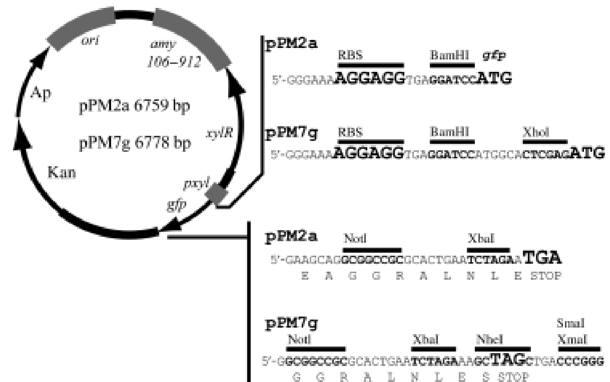

Fig. 1. The GFP expression vectors for Xac. Vectors pPM2a and pPM7g, which differ in the restriction sites flanking *gfp*, were constructed on a pCR2.1-TOPO backbone and carry a pUC-like replication origin (*ori*), and DNA cassettes for ampicillin (Ap) and kanamycin (Kan) resistance. The protein expression system on both vectors is composed of the xylose promoter (*pxyl*), the xylose repressor gene (*xylR*), and the *gfp* gene; promoter and *gfp* cassettes are separated by a short DNA segment containing a RBS. Unique restriction enzymes for DNA insertion are depicted on the right-hand side of the map, together with the ATG and the stop codons of the system. The chromosome integration leading sequence, *amy106–912*, is a fragment of ∼800 bp extracted from the α-amylase gene of Xac (ORF XAC0798).

General methods

Electrotransformation of Xac was performed as described by do Amaral *et al*. (2005). Oligonucleotides are listed in the Supporting Information, Table S1. The integrative GFP expression vectors were constructed by the orderly ligation of several DNA cassettes (Fig. 1). First, we produced a 57 bp double-stranded (ds)DNA by the annealing of two synthetic oligonucleotides: ribosome-binding site (RBS) (top and bottom). This dsDNA carried the RBS and had HindIII compatible ends. The dsDNA was ligated into pUC18/HindIII (NEB), generating pTAS1. pTAS1 was digested with EcoRI/HindIII to receive the xylose promoter and its repressor DNA (*xylR-pxyl*), both extracted from the vector pEA18 (Gueiros-Filho & Losick, 2002), also digested with EcoRI/HindIII, giving rise to pTAS3. The resulting plasmid, pTAS3, was digested with BamHI/XbaI to receive a *gfp* gene (flanked by BamHI/XbaI), thus generating pPM1 (*gfp* was PCR amplified from pEA18 using the primers GFP_WO_STOP/GFP_F_C-ter). Because Xac is naturally resistant to ampicillin, a marker of pPM1, the expression cassette (*xylR-pxyl-gfp*) was moved to pCR2.1-TOPO (Invitrogen), which confers resistance to kanamycin. The strategy used was PCR ligation, exploiting the fact that both pUC18 and pCR2.1-TOPO have identical DNA segments flanking their polylinkers. The expression cassette was removed from pPM1 using the *Pfu* DNA polymerase (Fermentas) and the primers M13F and M13R; the backbone of pCR2.1-TOPO was obtained using the primers M13F-TOPO and M13R-TOPO, both designed to anneal outside of the polylinker, but pointing towards the kanamycin gene. The two amplification products were mixed in equimolar amounts, and ligated in a final PCR, without primers, giving rise to pPM2. Finally, a DNA fragment corresponding to bases 106–912 of the Xac *amy* gene (XAC0798) was PCR amplified using primers XamyFOR5/REV5 and ligated to pPM2/EcoRI, generating pPM2a (GenBank GQ139362). Extra restriction sites were added to pPM2a by reamplifying *gfp* with primers GFP_BHI_XhoI/GFP_NheI. The PCR product was digested with BamHI/XmaI, inserted between pPM2a BamHI/XmaI sites, giving rise to pPM7g (GU988753). Later, the *gfp* gene from pPM7g will be replaced by a mCherry cassette, for future protein colocalization experiments. All plasmids were checked by DNA sequencing. ORF XAC3408 was isolated by PCR using primers 3408F/3408R, digested with XbaI, and ligated to pPM2a/XbaI.

Western blotting was as described by Sambrook *et al*. (1989). The anti-GFP primary antibody used was polyclonal raised in rabbits (F.J. Gueiros-Filho, Departamento de Bioquímica, IQ, USP, SP, Brazil). Subsequent antibody detection and chemiluminescent reaction were according to the Amersham ECL Western Blotting System kit (GE).

Pathogenicity tests

The plant hosts used were Bahia sweet orange [*Citrus sinensis* (L.) Osbeck] and Rangpur lime (*Citrus limonia* Osbeck). Citrus plants were cultivated under greenhouse

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conditions at 25–35 °C. Cells were cultivated in the appropriate medium until $OD_{600\,nm}$~0.6 (10^8 CFU mL^{-1}). Following growth, cell suspensions were used to inoculate leaves on the abaxial surface with the help of hypodermic syringes (1 mL). Symptoms were observed during the course of 3 weeks.

Fluorescence microscopy

Cells were cultivated in the appropriate medium until $OD_{600\,nm}$~0.3. Drops of 20 μL of cell culture were placed on microscope slides covered previously with a thin layer of 1% agarose in 1 × phosphate-buffered saline and covered with a slide cover slip. Visualization of cells was performed using an Olympus BX-60 microscope equipped with a DP-71 refrigerated camera. Images were captured and processed using IMAGEPRO-MC (version 6.0).

Results

Construction of the protein expression vector for Xac

Before we could initiate studies of controlled protein expression into Xac, we had to develop protein expression systems for this bacterium. The expression vectors built (pPM2a and pPM7g) are integrative, and carry the xylose promoter (*pxyl*), the xylose repressor (*xylR*), and a *gfp*-coding sequence (Fig. 1). The xylose promoter is known for its fine-tuned control of protein expression levels, and it has been used extensively in *B. subtilis* (Lewis & Marston, 1999; Gueiros-Filho & Losick, 2002). The xylose promoter and the *gfp* gene are separated by a short synthetic dsDNA that contains a RBS based on a consensus for *B. subtilis* and *E. coli* (Rocha *et al.*, 1999). Unique restriction sites are present at both termini of the *gfp* gene, which allows the ligation of genes and the subsequent production of either N- or C-terminal GFP–protein fusions. Both vectors have a pCR2.1-TOPO backbone, so that they carry a kanamycin cassette, a selectable marker for Xac, and a pUC-like origin of replication. Therefore, these vectors do not replicate in Xac, and can be used for site-directed mutagenesis, a key strategy to study gene function. Finally, pPM2a/pPM7g harbor a fragment of the α-amylase gene of Xac (*amy106–912*), intended to mediate their integration into the chromosome.

The expression system shows stable integration into the chromosome of Xac

The integration of pPM2a/pPM7g into the chromosome is an essential condition for placing the expression cassette into the bacterium. Integration occurs by at least a single homologous recombination event aiming as targets either the ORF to be characterized plus its native chromosomal copy or the *amy106–912* fragment present in the vectors and the chromosomal *amy* gene. Recombination between *amy106–912* and the chromosomal *amy* locus should produce Xac mutants unable to degrade starch on agar medium. To test for this integration, we inserted pPM2a into Xac by electrotransformation and searched for mutant strains on kanamycin-containing NYG-agar plates. Several kan^R Xac putative mutants were selected and subsequently inoculated on NYG-agar supplemented with starch, so that their ability to degrade this substrate could be monitored (Fig. 2a). Note that, apart from the wild-type strain (white arrows), all mutant colonies were deficient in starch degradation, which suggested that pPM2a could be integrated into the *amy* locus of Xac. To establish the site of plasmid integration, we performed a diagnostic Southern blot. Total DNA from two independently generated kan^R mutants was digested with EcoRV and probed with the labeled *amy* gene (Fig. 2b and c). The wild-type strain generated a single signal of ~6051 bp (Fig. 2b, band 1), which corresponds to the EcoRV fragment containing the *amy* gene (Fig. 2c, genome coordinates 946 596 ... 952 647). Conversely, both mutants displayed two signals: ~2249 and ~9686 bp (Fig. 2b, lanes 2 and 3, bands 2 and 3), a result expected in the event of the integration of pPM2a into the bacterial *amy* locus (Fig. 2c). Together, data demonstrate that the expression vector had recombined with the *amy* gene at the chromosome.

Disruption of the *amy* locus does not alter the virulence of Xac

Before addressing the functionality of our protein expression system, it was necessary to check whether the Xac *amy*::pPM2a mutants could still produce disease symptoms *in planta*, i.e., to evaluate whether α-amylase could play a role as a colonization and/or a pathogenicity/virulence factor in this bacterium. We inoculated Xac *amy*::pPM2a mutants in leaves of sweet orange and lime (natural hosts for Xac) alongside the wild-type strain. We observed the appearance of symptoms for a period of 3 weeks, starting from the day of the inoculation, and photos were taken on the 20th day (Fig. 3 shows a representative experiment). As a result, no variation from the wild-type phenotype was detected on our tests, meaning that all the mutants inoculated were as competent as the wild-type strain in producing lesions on leaves. In addition, we did not detect alterations in the pattern of disease development, where lesions were all detected at the same time scale. We also measured the viability of the mutant strains by analyzing their relative doubling times during growth in liquid media along with colony counting on agar plates, and, again, no variations were observed (data not shown). Taking together, these results show that the ability to cause disease is not affected in

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Xac *amy*::pPM2a mutants and strengthen the value of our GFP expression vectors for the characterization of ORFs suspected to be involved in virulence and pathogenicity.

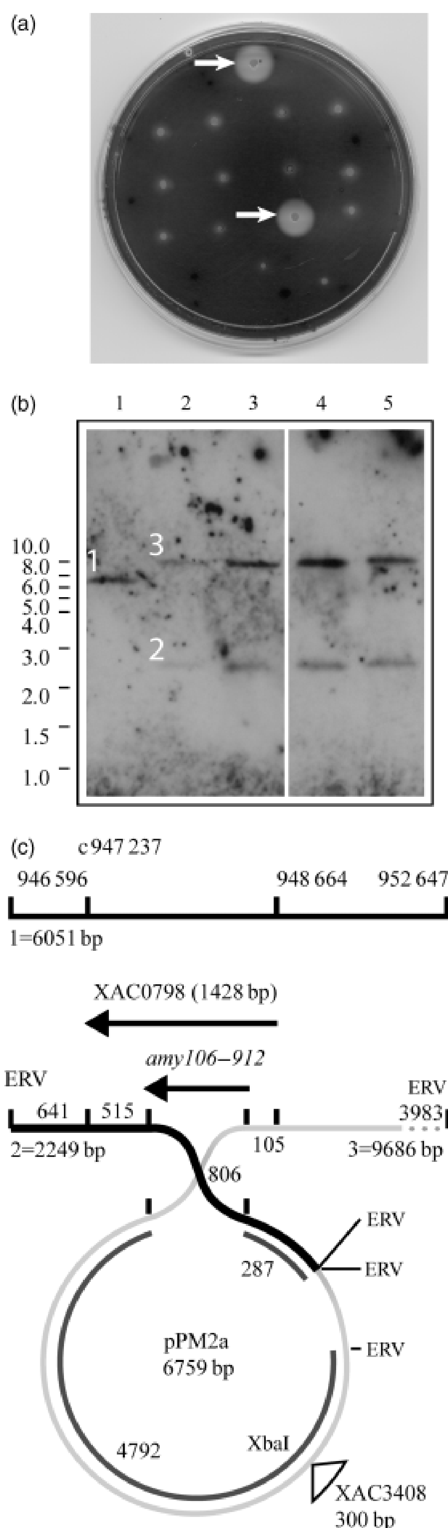

Fig. 2. The integration of the expression vector pPM2a into the chromosome of Xac produces a disruption of the *amy* gene. (a) Integration into the *amy* locus can be detected by plating the kanamycin-resistant candidates on NYG-agar containing soluble starch (0.2%). Xac mutants deficient for α-amylase fail to degrade the starch around the colonies and do not form halos like the wild-type strain (white arrows). Halos were visualized by exposing the medium to iodine vapor. (b, c) Southern blot analysis of a selection of four Xac mutants unable to degrade starch. Total DNA was digested with the enzyme EcoRV (ERV) and hybridized using the *amy* gene as a probe (b). Lanes: 1, Xac 306 wild-type strain; 2 and 3, two independently produced Xac mutants harboring pPM2a integrated into the *amy* locus; 4 and 5, two Xac mutants carrying pPM2a-XAC3408 (see subsequent sections). Bands labeled 1, 2, and 3 on the X-ray film correspond to the DNA fragments estimated using the Xac 306 genome coordinates (c): 1, ERV fragment of 6051 bp containing the *amy* gene (946 596 . . . 952 647); 2 and 3, fragments of 2249 and 9686 bp (for mutants in lanes 4 and 5, add 300 bp corresponding to ORF XAC3408), respectively, are produced by mutant strains carrying pPM2a. The position of the *amy* gene (XAC0798) within the fragment 1 is represented by a black arrow (genome coordinates c947 237 . . . 948 664); the relative position of fragment *amy106–912* (see Fig. 1) is also shown.

Subcellular localization of proteins in Xac

The functionality of our GFP expression vectors was first analyzed by Western blotting. A Xac *amy*::pPM2a mutant strain, harboring a single copy of the expression cassette integrated into the *amy* locus, was cultivated in NYG medium alongside a wild-type strain (negative control) and treated with xylose to induce the GFP production by the mutant. Total protein extracts were prepared, separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane in order to detect the production of GFP by the mutant strain using a polyclonal antibody raised against it. A band of the expected size for GFP (∼27 kDa) was clearly detected for the Xac *amy*::pPM2a mutant (Fig. 4, lane 2), whereas no band of the same size could be visualized for the wild-type strain (lane 1). The bands higher than the GFP mark represent nonspecific interactions, and may be due to the nature of our polyclonal antibody-containing serum. The detection of GFP confirmed the functionality of our expression plasmid.

Our expression system was subsequently tested in protein localization studies by expressing the product of ORF XAC3408 as a GFP fusion within Xac. XAC3408 encodes for a hypothetical protein annotated as the Xac candidate for the cell division factor ZapA, firstly characterized in *B. subtilis* ($ZapA^{Bsu}$) (da Silva *et al*., 2002; Gueiros-Filho & Losick, 2002). If the product of XAC3408 were really the Xac orthologue of $ZapA^{Bsu}$, GFP-XAC3408 would be expected to localize to the division septum, because $ZapA^{Bsu}$ is known to associate with the Z-ring. XAC3408 was cloned into pPM2a for Xac transformation, and the subsequent selection of Xac *amy*::pPM2a-XAC3408 mutants was performed on an

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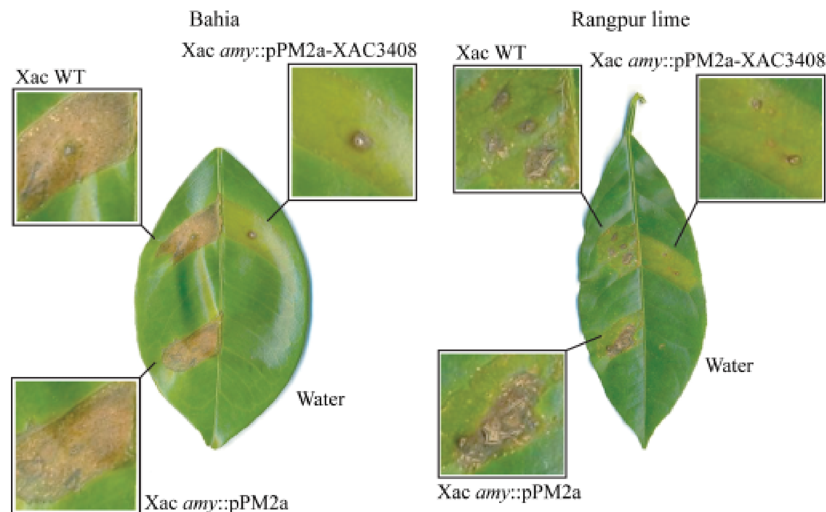

Fig. 3. The disruption of the *amy* gene of Xac does not alter its pathogenicity or virulence. Mutant strains of Xac carrying the expression vector pPM2a (Xac *amy*::pPM2a) or pPM2a-XAC3408 (Xac *amy*::pPM2a-XAC3408) were inoculated in leaves of sweet orange and lime plants and the development of disease symptoms was monitored during a period of 3 weeks. The position of the mutant relative to the control wild-type strain (WT) is labeled on the zoom in panels. Above, a representative test is displayed. Tests were carried out in triplicate.

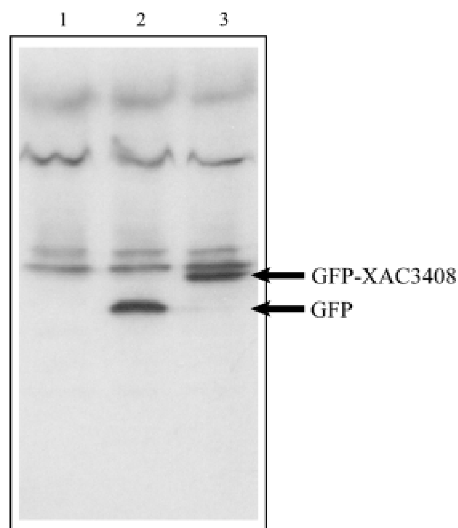

Fig. 4. Western blot to detect the production of GFP and GFP-XAC3408 by Xac mutant strains. The functionality of pPM2a was verified by the expression of GFP and GFP-XAC3408 proteins by Xac *amy*::pPM2a and Xac *amy*::pPM2a-XAC3408 mutant strains, respectively. Lanes: 1, Xac WT; 2, Xac *amy*::pPM2a; and 3, Xac *amy*::pPM2a-XAC3408. Protein expression was induced by growing the mutant strains in the presence of 0.5% xylose, added at $OD_{600\ nm}$~0.5, for 2 h before protein extract preparation. The relative positions of the expressed proteins are marked with black arrows.

NYG-agar/starch medium, based on their inability to degrade starch. Next, two mutants were evaluated on Southern blot to confirm the specific integration of the plasmid into the *amy* locus (Fig. 2b). Note that both Xac *amy*::pPM2a-XAC3408 candidates exhibited the same band profile as that observed for the Xac *amy*::pPM2a mutants (compare lanes 2–3 with 4–5); the only difference is in the size of the larger fragment (band 3), which now has extra 300 bp corresponding to ORF XAC3408. These results demonstrate the integration of pPM2a-XAC3408 with *amy* disruption in the Xac mutants.

Before the microscope observations, a Western blot was performed to verify whether GFP-XAC3408 could be expressed in Xac (Fig. 4). A band of ~38 kDa was detected (lane 3), which is consistent with the size expected for the fusion GFP-XAC3408, and produced only by the Xac *amy*::pPM2a-XAC3408 mutant strain tested. Next, we observed Xac *amy*::pPM2a-XAC3408 mutant cells under the fluorescent microscope, and as a result, the majority of the cells displayed a bar-like structure at the middle of the rod, oriented perpendicular to its longitudinal axis (Fig. 5), a localization pattern characteristic of $GFP\text{-}ZapA^{Bsu}$ (Gueiros-Filho & Losick, 2002). To confirm that the localization seen was not an artifact, we treated the Xac *amy*::pPM2a-XAC3408 mutant cells with the protein synthesis inhibitor chloramphenicol before microscope inspection. After the antibiotic treatment, the septal bars disappeared, which indicates that the pattern observed was a real localization of GFP-XAC3408.

Finally, we tested the ability of the Xac *amy*::pPM2a-XAC3408 mutant to induce disease symptoms *in planta* and detected a decrease in virulence (Fig. 3). Mutants were unable to produce the typical brown/eruptive lesions observed for the wild type, and this is probably due to the fact that the mutants are expressing extra copies of XAC3408 (in

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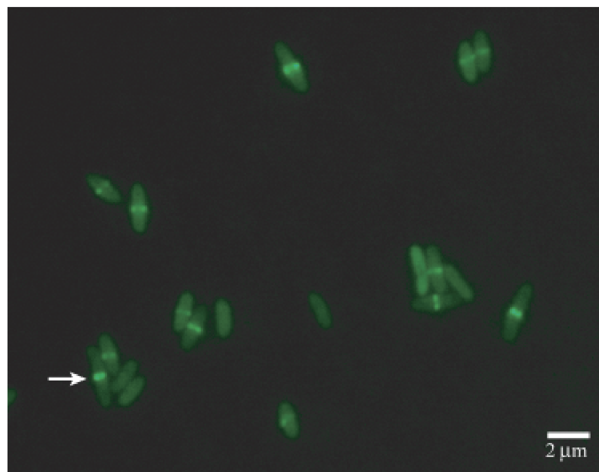

Fig. 5. Subcellular localization of GFP-XAC3408. A Xac *amy*::pPM2a-XAC3408 mutant strain was cultivated in NYG at 30 °C until $OD_{600\ nm}$~0.3. GFP-XAC3408 was examined in living cells immobilized on microscope slides covered with a thin layer of 1% agarose. The site of GFP-XAC3408 localization is pointed out with a white arrow (magnification of × 1000).

the form of GFP-XAC3408), which may perturb normal cell division and hence pathogenesis.

Discussion

Half a decade has gone by since the publication of the genome sequence of Xac (da Silva *et al.*, 2002), and apparently, its large-scale proteome analyses are confined to a few reports using techniques such as two-dimensional protein gels, yeast two-hybrid, and nuclear magnetic resonance scans for folded proteins (Mehta & Rosato, 2001, 2003; Galvao-Botton *et al.*, 2003; Alegria *et al.*, 2004, 2005; Khater *et al.*, 2007). Moreover, the two last methods used heterologous expression of proteins in organisms different from the one under investigation. Most of the limitation to explore the biology of Xac lies in a complete lack of protein expression systems adapted to this bacterium. Here, we showed the construction and test of new protein expression vectors dedicated to Xac, and the subsequent utilization of our system to characterize the hypothetical protein XAC3408. XAC3408 is 30% identical (at the amino acid level) to the *B. subtilis* cell division protein ZapA, and our subcellular localization experiments using GFP-XAC3408 support the hypothesis that XAC3408 is the Xac orthologue of $ZapA^{Bsu}$. ZapA-like proteins are conserved among bacteria, in which they function by promoting the FtsZ bundling and stabilization of FtsZ polymers (Gueiros-Filho & Losick, 2002; Low *et al.*, 2004; Scheffers, 2008). $ZapA^{Xac}$ exhibited a localization pattern similar to that observed for $ZapA^{Bsu}$ (Gueiros-Filho & Losick, 2002), and the availability of Xac mutants labeled at the septum provides a new perspective for antimicrobial drug development trials with Xac aimed to disrupt cell division.

The vectors described here are integrative and allow the ectopic expression of proteins from the *amy* locus of Xac. Such a strategy has been used extensively in the Gram-positive rod *B. subtilis* (Lewis & Marston, 1999; Gueiros-Filho & Losick, 2002), and it is believed to avoid disturbances to genes/chromosomal regions that might produce undesirable effects in cell growth and altered phenotypes. Besides, integration into *amy* has the advantage of allowing the characterization of essential genes, which may not accommodate changes in their coding sequences. Finally, the disruption of *amy* produces a bacterial phenotype easily detectable on a plate and allows the distinction of insertions that had occurred in *amy* from those in the gene being under investigation.

In the present work, we showed that the α-amylase gene was not essential for Xac to grow on a plate, neither was it found to play any key role during infection, an outcome somewhat expected since it has been demonstrated that in bacteria α-amylases may be essential for growth on starch, but dispensable for growth on other carbon sources (Worthington *et al.*, 2003). However, a recent report has implicated the *amy* locus of Xac as being required for pathogenesis (Laia *et al.*, 2009). In their analyses, a collection of 3300 Xac transposon-insertion mutants was screened for their ability to produce disease *in planta*, and among the ORFs disrupted, XAC0798 (*amy*) was found to be the one that resulted in some alteration in bacterial virulence. In contrast, in our experiments, the disruption of XAC0798 by the insertion of pPM2a (Fig. 2) did not produce any alteration in pathogenesis or virulence even using the same host plant, Rangpur lime, used by Laia *et al.* (2009). This discrepancy could be explained tentatively by the selection of a hypothetical Xac *amy*::transposon mutant strain harboring an additional mutation (perhaps spontaneous) on a region essential for pathogenesis in the screenings performed by Laia *et al.* (2009).

Xac has a repertoire of > 1600 hypothetical ORFs, and probably a considerable part of these might be involved in pathogenesis and virulence to its host plants. Therefore, the GFP expression vectors described here constitute not only extra tools for the study of specific proteins but also an auxiliary method for protein functional assignments, similar to what has already been done with *B. subtilis* and *Caulobacter crescentus* (Meile *et al.*, 2006; Werner *et al.*, 2009).

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thank L.F.F. Donin (Olympus Brazil) for technical support. This work was funded by FAPESP grant 2004/09173-6.

References

Alegria MC, Docena C, Khater L, Ramos CH, da Silva AC & Farah CS (2004) New protein–protein interactions identified for the regulatory and structural components and substrates of the type III secretion system of the phytopathogen *Xanthomonas axonopodis* pathovar citri. *J Bacteriol* **186**: 6186–6197.

Alegria MC, Souza DP, Andrade MO *et al.* (2005) Identification of new protein–protein interactions involving the products of the chromosome- and plasmid-encoded type IV secretion loci of the phytopathogen *Xanthomonas axonopodis* pv. citri. *J Bacteriol* **187**: 2315–2325.

Beuria TK, Singh P, Surolia A & Panda D (2009) Promoting assembly and bundling of FtsZ as a strategy to inhibit bacterial cell division: a new approach for developing novel antibacterial drugs. *Biochem J* **423**: 61–69.

Cernadas RA, Camillo LR & Benedetti CE (2008) Transcriptional analysis of the sweet orange interaction with the citrus canker pathogens *Xanthomonas axonopodis* pv. citri and *Xanthomonas axonopodis* pv. aurantifolii. *Mol Plant Pathol* **9**: 609–631.

Daniels MJ, Barber CE, Turner PC, Sawczyc MK, Byrde RJ & Fielding AH (1984) Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. campestris using the broad host range cosmid pLAFR1. *EMBO J* **3**: 3323–3328.

da Silva AC, Ferro JA, Reinach FC *et al.* (2002) Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* **417**: 459–463.

do Amaral AM, Toledo CP, Baptista JC & Machado MA (2005) Transformation of *Xanthomonas axonopodis* pv. citri by eletroporation. *Fitopatol Bras* **30**: 292–294.

Galvao-Botton LM, Katsuyama AM, Guzzo CR, Almeida FC, Farah CS & Valente AP (2003) High-throughput screening of structural proteomics targets using NMR. *FEBS Lett* **552**: 207–213.

Gitai Z, Dye NA, Reisenauer A, Wachi M & Shapiro L (2005) MreB actin-mediated segregation of a specific region of a bacterial chromosome. *Cell* **120**: 329–341.

Gueiros-Filho FJ & Losick R (2002) A widely conserved bacterial cell division protein that promotes assembly of the tubulin-like protein FtsZ. *Gene Dev* **16**: 2544–2556.

Haydon DJ, Stokes NR, Ure R *et al.* (2008) An inhibitor of FtsZ with potent and selective anti-staphylococcal activity. *Science* **321**: 1673–1675.

Kapoor S & Panda D (2009) Targeting FtsZ for antibacterial therapy: a promising avenue. *Expert Opin Ther Tar* **13**: 1037–1051.

Khater L, Alegria MC, Borin PF *et al.* (2007) Identification of the flagellar chaperone FlgN in the phytopathogen *Xanthomonas axonopodis* pathovar citri by its interaction with hook-associated FlgK. *Arch Microbiol* **188**: 243–250.

Laia ML, Moreira LM, Dezajacomo J *et al.* (2009) New genes of *Xanthomonas citri* subsp. *citri* involved in pathogenesis and adaptation revealed by a transposon-based mutant library. *BMC Microbiol* **9**: 12.

Lewis PJ & Marston AL (1999) GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. *Gene* **227**: 101–110.

Low HH, Moncrieffe MC & Lowe J (2004) The crystal structure of ZapA and its modulation of FtsZ polymerization. *J Mol Biol* **341**: 839–852.

Mehta A & Rosato YB (2001) Differentially expressed proteins in the interaction of *Xanthomonas axonopodis* pv. citri with leaf extract of the host plant. *Proteomics* **1**: 1111–1118.

Mehta A & Rosato YB (2003) A simple method for *in vivo* expression studies of *Xanthomonas axonopodis* pv. citri. *Curr Microbiol* **47**: 400–403.

Meile JC, Wu LJ, Ehrlich SD, Errington J & Noirot P (2006) Systematic localisation of proteins fused to the green fluorescent protein in *Bacillus subtilis*: identification of new proteins at the DNA replication factory. *Proteomics* **6**: 2135–2146.

Pan PS, Curtis FA, Carroll CL, Medina I, Liotta LA, Sharples GJ & McAlpine SR (2006) Novel antibiotics: C-2 symmetrical macrocycles inhibiting Holliday junction DNA binding by *E. coli* RuvC. *Bioorg Med Chem* **14**: 4731–4739.

Rocha EP, Danchin A & Viari A (1999) Translation in *Bacillus subtilis*: roles and trends of initiation and termination, insights from a genome analysis. *Nucleic Acids Res* **27**: 3567–3576.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schaad NW, Postnikova E, Lacy GH *et al.* (2005) Reclassification of *Xanthomonas campestris* pv. *citri* (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as *X. smithii* subsp. *citri* (ex Hasse) sp. nov. nom. rev. comb. nov., *X. fuscans* subsp. *aurantifolii* (ex Gabriel 1989) sp. nov. nom. rev. comb. nov., and *X. alfalfae* subsp. *citrumelo* (ex Riker and Jones) Gabriel et al., 1989 sp. nov. nom. rev. comb. nov.; *X. campestris* pv. *malvacearum* (ex smith 1901) Dye 1978 as *X. smithii* subsp. *smithii* nov. comb. nov. nom. nov.; *X. campestris* pv. *alfalfae* (ex Riker and Jones, 1935) dye 1978 as *X. alfalfae* subsp. *alfalfae* (ex Riker et al., 1935) sp. nov. nom. rev.; and 'var. fuscans' of *X. campestris* pv. *phaseoli* (ex Smith, 1987) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. *Syst Appl Microbiol* **28**: 494–518.

Schaad NW, Postnikova E, Lacy G *et al.* (2006) Emended classification of xanthomonad pathogens on citrus. *Syst Appl Microbiol* **29**: 690–695.

Scheffers DJ (2008) The effect of MinC on FtsZ polymerization is pH dependent and can be counteracted by ZapA. *FEBS Lett* **582**: 2601–2608.

Werner JN, Chen EY, Guberman JM, Zippilli AR, Irgon JJ & Gitai Z (2009) Quantitative genome-scale analysis of protein localization in an asymmetric bacterium. *P Natl Acad Sci USA* **106**: 7858–7863.

Worthington P, Hoang V, Perez-Pomares F & Blum P (2003) Targeted disruption of the alpha-amylase gene in the

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hyperthermophilic archaeon *Sulfolobus solfataricus*. *J Bacteriol* **185**: 482–488.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotides.

Fig. S1. Growth curves of Xac wild type, and the mutant strains Xac *amy*::pPM2a and Xac *amy*::pPM2a-XAC3408.

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