Disruption of *Xylella fastidiosa* CVC *gumB* and *gumF* genes affects biofilm formation without a detectable influence on exopolysaccharide production

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Abstract

Xylella fastidiosa causes citrus variegated chlorosis (CVC), a destructive disease of citrus. *Xylella fastidiosa* forms a biofilm inside plants and insect vectors. Biofilms are complex structures involving *X. fastidiosa* cells and an extracellular matrix which blocks water and nutrient transport in diseased plants. It is hypothesized that the matrix might be composed of an extracellular polysaccharide (EPS), coded by a cluster of nine genes closely related to the xanthan *gum* operon of *Xanthomonas campestris* pv. *campestris*. To understand the role of *X. fastidiosa gum* genes on biofilm formation and EPS biosynthesis, we produced *gumB* and *gumF* mutants. *Xylella fastidiosa* mutants were obtained by insertional duplication mutagenesis and recovered after triply cloning the cells. *Xylella fastidiosa gumB* and *gumF* mutants exhibited normal cell characteristics; typical colony morphology and EPS biosynthesis were not altered. It was of note that *X. fastidiosa* mutants showed a reduced capacity to form biofilm when BCYE was used as the sustaining medium, a difference not observed with PW medium. Unlike *X. campestris* pv. *campestris*, the expression of the *X. fastidiosa gumB* or *gumF* genes was not regulated by glucose.

Introduction

Xylella fastidiosa is a bacterium that colonizes the xylem of various plant hosts, causing diseases such as citrus variegated chlorosis (CVC) in citrus (Chang *et al.*, 1993), and Pierce's disease on grapes (Hopkins, 1989). It is transmitted via grafting and xylem-feeding insects. Inside plant vessels and the insects' foregut, *X. fastidiosa* cells organize themselves into complex structures and the result is a community of cells embedded in an extracellular matrix (Tyson *et al.*, 1985), called a 'biofilm'. Scanning electron microscopy (SEM) analysis shows vessel occlusion by the cluster of *X. fastidiosa* cells in several hosts (Tyson *et al.*, 1985; Alves *et al.*, 2003). The cells adhere to glass slides, the glass walls of culture tubes and plastic flasks during *in vitro* cultivation in a liquid medium, and produce a biofilm (Leite *et al.*, 2002; Marques *et al.*, 2002). It has been hypothesized that exopolysaccharides (EPS) could be involved in the formation of *X. fastidiosa* biofilm (Hopkins, 1989; Simpson *et al.*, 2000).

Xylella fastidiosa has a cluster of genes closely related to the *Xanthomonas campestris* pv. *campestris* gum operon, which is responsible for the synthesis of xanthan gum (Simpson *et al.*, 2000). Xanthan is a polymer of repeating pentasaccharide units with the structure mannose-(β-1,4)-glucuronic acid-(β-1,2)-mannose-(α-1,3)-cellobiose. The gum operon is composed of 12 predicted open reading frames, from *gumB* to *gumM* (Ielpi *et al.*, 1993; Katzen *et al.*, 1998; Vojnov *et al.*, 1998). Nine genes were found in *X. fastidiosa*: *gumBCDEFHJKM* (XF2370–XF2360) (Simpson

et al., 2000; Silva *et al.*, 2001). Comparing *X. fastidiosa* and *X. campestris* pv. *campestris gum* gene clusters, there are three missing genes in *X. fastidiosa*: *gumI*, *gumL* and *gumG* encoding, respectively, a β-1,4-mannosyl GDP-transferase, a pyruvate transferase and an acetyl transferase (Silva *et al.*, 2001). That analysis suggest that *X. fastidiosa* synthesizes a modified polysaccharide consisting of tetrasaccharide repeating units, which differs from the polypentamer structure of xanthan gum. The main difference is the lack of a pyruvilated terminal mannose residue (Silva *et al.*, 2001), which may result in a less viscous EPS in *X. fastidiosa* compared to *X. campestris* pv. *campestris* xanthan, as was suggested elsewhere (Silva *et al.*, 2001). This hypothesis is supported by the fact that *X. fastidiosa* colonies are non-mucoid (Weels *et al.*, 1987), unlike *X. campestris* pv. *campestris* mucoid colonies.

We have produced mutants in a *X. fastidiosa gum* gene cluster to determine whether an inactivation of *gumB* or *gumF* genes would affect biofilm formation, EPS production or cell aggregation patterns. We have chosen these two *gum* genes for mutagenesis in *X. fastidiosa* since the *gumB* mutant in *X. campestris* pv. *campestris* is unable to polymerize the pentasaccharide into mature xanthan, and the production of EPS is highly attenuated (Katzen *et al.*, 1998; Vojnov *et al.*, 1998). Inactivation of the *gumF* gene in *X. campestris* pv. *campestris,* impairing gum acetylation, has not affected gum polymerization and showed no effect on EPS production (Katzen *et al.*, 1998).

Materials and methods

Bacterial strains and growth conditions

The *Xylella fastidiosa* J1a12 strain was isolated from infected sweet orange trees (Monteiro *et al.*, 2001) and cultivated in both solid and liquid media on an orbital shaker (150 r.p.m.) at 28 °C.

Defined and undefined media were used in this work to evaluate the performance of the mutants tested: PW, BCYE, XDM_2 and modifications of them, such as PWG and BCYEG. Their formulations were: PW (0.4% w/v phytone peptone, 0.1% w/v trypticase peptone, 7.35 mM KH_2PO_4, 6.89 mM K_2HPO_4, 1.62 mM $MgSO_4$, 0.001% w/v hemin chloride, 0.002% phenol red, 0.6% w/v BSA, 0.4% w/v glutamine, pH 6.8); BCYE (1% w/w yeast extract, 0.04% w/v L-cysteine-HCl, 0.025% w/v ferric pyrophosphate, 30 mM KOH, and 54.9 mM ACES; pH 6.85); XDM_2 (5.88 mM KH_2PO_4, 12.06 mM K_2HPO_4, 1.62 mM $MgSO_4$, 0.002% ferric pyrophosphate, 0.1% w/v phenol red, 1% glucose, 0.02 mg L^{-1} D-biotin, 1.0 mg L^{-1} nicotinic acid, 0.005 mg L^{-1} vitamin B12, 35.0 mg L^{-1} myo-inositol, 0.4 μg mL^{-1} L-serine, 0.4 μg mL^{-1} L-methionine, 4.0 μg mL^{-1} L-glutamine, and 1.0 μg mL^{-1} L-asparagine, pH 6.8) (Davis *et al.*, 1980; Lemos *et al.*, 2003). Glucose supplemented media (PWG, BCYEG and XDM) were prepared by adding filter-sterilized glucose to 1% (w/v).

Controls used during the assays came from *X. fastidiosa* transformed with pBCK92 and an untransformed culture at the same mutant cultivation stage.

Genetic manipulations

Plasmid constructions were based on pBCK92, a replicative plasmid useful for generating *X. fastidiosa* mutants. The generation mechanism involves an insertion duplication mutagenesis (IDM) containing a 366 bp fragment of the *oriC* chromosomal region of *X. fastidiosa*, the *aacA-aphD* gene coding for kanamycin resistance and a multicloning site into a promoterless copy of the reporter *cat* (chloramphenicol acetyl transferase) gene (Gaurivaud *et al.*, 2002). *gumB* gene specific primers GBF (5′-GAGATCTGTCGAC GATCTGGAG-3′) and GBR (5′-GAGATCTGCAGGATA GATCTC-3′), and *gumF* gene specific primers GFF (5′-GAGATCTCCAGTACGAGGAGGGACATTAGC-3′) and GFR (5′-GAGATCTCATTGTACCAATGACCATTG-3′) were designed based on a *X. fastidiosa* genome sequence for the amplification of the 421 bp (143–564 nt) and 859 bp (28–886 nt) internal regions of the 654 bp *gumB* and 1092 bp *gumF* genes of the *X. fastidiosa* strain, respectively. The *Pst*I site is underlined.

PCR products were digested with *Pst*I and ligated with the plasmid pBCK92. The correct orientation of the fragments was confirmed by restriction analysis. The mutagenic plasmids were named pBCK*gumB* and pBCK*gumF* which contained, respectively, the *gumB* and *gumF* gene fragments (gum_f) transcriptionally fused to the promoterless *cat* reporter gene (Fig. 1a). The plasmid pBCK92 configuration allows – after its integration into the target gene – an indirect evaluation of the expression level of the mutated gene through a chloramphenicol resistance assay.

Transformation of *X. fastidiosa* in the 5th passage in axenic culture was done (Monteiro *et al.*, 2001; Gaurivaud *et al.*, 2002), and for each transformation, five isolated kanamycin resistant colonies were transferred to a PW-broth medium containing 5 μg mL^{-1} kanamycin and incubated at 28 °C, with agitation at 150 r.p.m for oneweek. Volumes of these cell suspensions (1/10) were transferred every week to a fresh flask of PW broth. After six propagations, the DNA of these cell suspensions was extracted in order to assess the plasmid integration. Clones showing integration of the mutagenic plasmid, within the targeted genes, yielded triple clones in solid media (Monteiro *et al.*, 2001; Gaurivaud *et al.*, 2002).

Southern blotting analysis of the total DNA of transformed *X. fastidiosa* J1a12 cells was carried out for the detection of *gumB* and *gumF* gene disruption (Gaurivaud

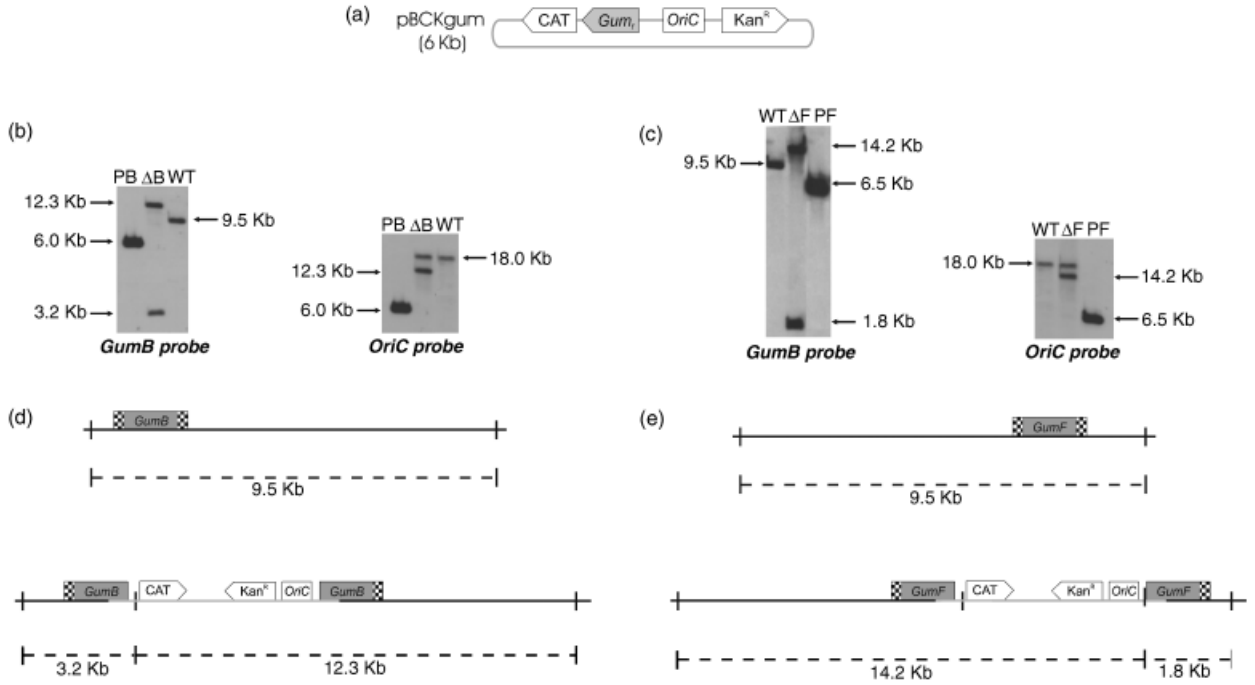

Fig. 1. (a) Representation of pBCK*gum* plasmid used for insertional duplication mutagenesis (IDM) in *Xylella fastidiosa gum* operon. Arrowed boxes represent the direction of the genes in the mutagenic plasmid. (b, c) Southern blotting hybridization of DNA from the untransformed (WT) and pBCK*gumB*-transformed *Xylella fastidiosa* J1a12 clone (ΔB) and pBCK*gumF*-transformed *Xylella fastidiosa* clone (ΔF), with (B) *gumB*, (C) *gumF* and (B, C) *oriC* probes. (d, e) Representation of *Sph*I restriction fragments of the WT *Xylella fastidiosa* genome and (D) *gumB* and (E) *gumF* genes duplicated by IDM. Sizes in kbp. Abbreviations: PB, plasmid pBCK*gumB*; PF, plasmid pBCK*gumF*; *gumf*, *gum* gene truncated fragment; Kan^r, kanamycin resistance.

et al., 2002). All the DNA from the transformed clones was digested with *Sph*I, and integration of the mutagenic plasmids into the target genes was determined using *gumB*, *gumF*, *cat* and *oriC* probes.

Colony morphology and aggregation

Xylella fastidiosa colony morphology was evaluated using stereomicroscopy. The formation of cell aggregation in the *X. fastidiosa* culture was inspected both visually, after a gentle homogenization of the culture, and by optical microscopy of methylene blue stained cells. For the methylene blue staining, a drop of exponentially growing culture of *X. fastidiosa* was added to a glass slide. The drop was dried for few minutes and the cells then heat fixed on the glass slide. The fixed cells were stained in a solution of 15 g L^{-1} methylene blue, 50 g L^{-1} phenol, and 10% ethanol for 5 min and rinsed in distilled water. They were then observed under an optical microscope.

EPS production assay

To evaluate the effect of the disruption of *gum* genes on the production of total precipitable EPS in a chemically-defined medium, we utilized a method described for *Xanthomonas campestris* pv. *campestris*. High density cell suspensions of *X. fastidiosa* were obtained in XDM_2 (Lemos *et al.*, 2003). Supernatants were filter-sterilized (0.2 μm) and total EPS was precipitated as described elsewhere (Vojnov *et al.*, 1998). The experiments were replicated three times and the amount of EPS produced was calculated as a average ratio of the weighted precipitated extracellular material over *X. fastidiosa* cell wet mass.

Biofilm formation assay

The biofilm formation assay was conducted following a methodology described for *Pseudomonas fluorescens* and *Salmonella enteritidis* (Kjaergaard *et al.*, 2000). It was conducted in two media for comparison: in PW, a rich undefined medium, and in BCYE, also undefined but less complex, with or without glucose supplementation. Biofilm formation assays were done with cultures from the 24th to 40th passages in the culture medium. We present here the results of assays completed after the 36th passage.

After 8 days of growth in PW broth, *X. fastidiosa* strains were inoculated at same OD_{600} in each media (five tube replicates for each treatment) and cultivated in glass culture tubes at 28 °C, agitated at 100 r.p.m. After 14 days of

cultivation, the cells in suspension were removed and the glass walls of the culture tubes were carefully rinsed with water three times. The formation of biofilm by *X. fastidiosa* cells was inspected by crystal violet staining (Kjaergaard *et al.*, 2000). The biofilm formed on the glass was stained with 0.1% crystal violet for 1 min and the excess was carefully rinsed with water three times. The crystal violet was removed from the biofilm using a 20 : 80 ethanol : acetone solution wash (v/v) and the $OD_{595\,nm}$ was measured. The assay was repeated three times.

Effect of glucose in the expression of the gum operon

Cultures of the *X. fastidiosa gumB* and *gumF* mutants, as well as cultures of either the untransformed wild-type (WT) strain, or J1a12 transformed with plasmid pBCK92 (Gaurivaud *et al.*, 2002), both used as controls, were cultivated in PW or PWG media supplemented with 0, 1, 2, 3, 4, and 5 μg mL^{-1} of chloramphenicol. *X. fastidiosa* cell growth was checked after 6 days of cultivation by $OD_{600\,nm}$ measurement. The results were consistent in the three distinct experiments.

Results and discussion

Xylella fastidiosa gumB and *gumF* mutants

Triple clones from the 6th passage cultures were evaluated in the 14th passage of propagation by Southern blotting (Fig. 1b, c). Using the *gumB* probe, the 9.5 and 6.0 kbp fragments, respectively, corresponding to the wild-type endogenous *gumB* gene and the free pBCK*gumB* plasmid, were not observed in the mutant clone (Fig. 1b). Instead, two different restriction fragments of 12.3 kbp and 3.2 kbp (Fig. 1b), corresponding to the expected *Sph*I restriction pattern of the pBCK*gumB* plasmid integrated into the *gumB* gene (Fig. 1d), were observed. Southern hybridization using an *oriC* probe confirmed that the integration of pBCK*gumB* occurred in the *gumB* gene, since the 18 kbp fragment, corresponding to the endogenous *oriC,* remained intact (Fig. 1b).

As shown in Fig. 1c, the 9.5 kbp fragment containing the *gumF* endogenous gene and the 6.5 kbp plasmid fragment were not detected in the *gumF* mutant, and hybridization with the *oriC* probe showed that integration of the mutagenic plasmid had not occurred in *oriC*. The 14.2 and 1.8 kbp fragments in the *Xylella fastidiosa gumF* clone were in accordance with the expected *Sph*I restriction pattern for the integration of the plasmid in the endogenous *gumF* gene (Fig. 1e). The Southern blotting analysis showing the disruption of both *gumB* and *gumF* genes was confirmed with a *cat* probe hybridization (data not shown).

Disruption of *gumB* and *gumF* does not affect colony morphology and growth

A direct observation of *X. fastidiosa* colonies grown in PW solid medium using stereomicroscopy showed these characteristics: circular, discrete, opalescent and nonmucoid *X. fastidiosa* colony morphology, which was not affected by the disruption of either the *gumB* or *gumF* genes (data not shown). Mutations in the exopolysaccharide synthesis genes did not always produce changes in colony morphology (Denny, 1995). Mutations in the *Xanthomonas campestris* pv. *campestris gumD* gene resulted in noticeable changes in the colony mucoid phenotype (Chou *et al.*, 1997).

The growth achieved by the *X. fastidiosa gumB* and *gumF* mutant strains was comparable to the *X. fastidiosa* WT strain in liquid media (Fig. 3). The results consolidate the contention that mutations in *gumB* and *gumF* genes do not interfere with the growth of *X. fastidiosa*. In addition, *X. fastidiosa gumB* and *gumF* mutated cells showed typical aggregation behavior, forming clumps of aggregated cells in liquid media, as is commonly observed in wild-type strain cultures (data not shown).

The disruption of *gumB* and *gumF* does not alter EPS production

The effect of the disruption of the *gumB* and *gumF* genes in EPS biosynthesis was assayed in XDM_2 medium (Lemos *et al.*, 2003). XDM_2, a chemically-defined medium, was chosen because there are no polymers in its composition – in contrast to PW or BCYE media – which would impair EPS quantification. The amount of EPS produced by the *X. fastidiosa* gum mutants is expressed as the relative percentage compared to the WT parental strain (100% $= 1.44 \pm 0.4$ g L^{-1} culture medium), as follows: *gumB* 0.99 ± 0.06 and *gumF* 1.13 ± 0. These data represent the means of three replicates and their standard deviations. No significant difference was observed for the amount of EPS produced by *X. fastidiosa gum* mutant strains in comparison to the wild-type, indicating that the disruption of *gum* genes does not interfere with *in vitro* EPS synthesis by *X. fastidiosa* under the conditions we employed. It should be emphasized that the amount of EPS produced by the *X. fastidiosa* cultures was much lower than that described for *X. campestris* pv. *campestris* (Vojnov *et al.*, 1998).

Disruption of *gumB* and *gumF* genes affects *Xylella fastidiosa* biofilm formation

After 40 passages in an axenic culture, *X. fastidiosa* forms a biofilm at the medium – air interface, reproducing the data obtained after 24 passages (data not shown). We detected no differences in biofilm formation for *X. fastidiosa gum* mutants cultivated in PW-rich medium (irrespective of the

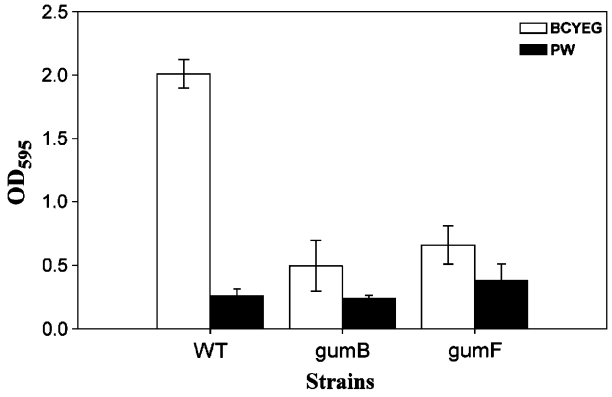

Fig. 2. Comparison of biofilm formation by *Xylella fastidiosa* WT and *gum* mutants after 7 days of incubation, measured as the amount of crystal violet bound to biofilm, as a function of culture medium. Assay conducted in quintuplicates with *Xylella fastidiosa* strains in the 36th passage.

presence of glucose) in comparison to the *X. fastidiosa* wild-type strain (Fig. 2). However, a striking difference was observed when the strains were cultivated in BCYE medium supplemented with glucose, in which the *gum* mutants showed a reduced capacity to produce biofilm compared to the wild-type (Fig. 2). This difference must be due to the inability of the *X. fastidiosa gumB* and *gumF* mutants to form or maintain the biofilm architecture on a less complex medium (BCYE). It was observed that after periods of growth longer than 2 weeks, a thin ring of adhered *X. fastidiosa* cells of *gum* mutant clones was formed on the air/BCYE medium interface, which was easily disturbed and detachable from the glass through culture aging (data not shown). We evaluated the formation of biofilm by *X. fastidiosa* in PW and BCYE media with or without glucose supplementation, since this sugar stimulates the growth of *X. fastidiosa* (Lemos *et al.*, 2003) and positively regulates the *X. campestris* pv. *campestris gum* operon (Vojnov *et al.*, 2001). We avoided a possible quorum-sensing effect among different strains by inoculating the same amount of cells, as measured by their OD_{600}. This is important because like *X. campestris* pv. *campestris*, *X. fastidiosa* has genes for quorum sensing (Silva *et al.*, 2001; Vojnov *et al.*, 2001).

Adhesion and biofilm formation is a well-known strategy used by bacteria as virulence factors and to gain easier access to nutrients. The formation of *X. fastidiosa* biofilm inside plant vessels and under *in vitro* conditions has already been characterized (Leite *et al.*, 2002; Marques *et al.*, 2002), and it has been noted as an important factor in the pathogenic mechanism developed by this bacterium (Hopkins, 1989; Simpson *et al.*, 2000). Leite and colleagues (Leite *et al.*, 2002) demonstrated that microcolonies of *X. fastidiosa* can form on artificial surfaces. These early stage colonies were not found to be associated with visible EPS, although cell adhesion was observed, and that report supports the results obtained in the present work. Our findings are also supported by recent results obtained with Pierce's disease on strains of *X. fastidiosa* growing in distinct media formulations. The media had a profound effect on the behavior of cells by affecting: (a) the number of cells in suspension, (b) protein concentration, (c) biofilm formation, and (d) aggregation (Leite *et al.*, 2004).

Glucose has no effect on the expression of the *Xylella fastidiosa* gum operon promoter

It is known that *X. campestris* pv. *campestris* metabolizes 28% of glucose into xantham gum (Pielken *et al.*, 1988), which is primarily produced in the late logarithmic and stationary growth phases (Harding *et al.*, 1987). The main promoter located upstream of the *X. campestris* pv. *campestris gumB* gene, which drives the expression of the *gum* operon as a transcriptional unit, is strongly induced by glucose and other sugars (Vojnov *et al.*, 2001). In the *X. fastidiosa gumB* mutant, the *cat* reporter gene is under control of the *gumB* endogenous gene promoter, allowing the use of chloramphenicol resistance to detect expression of the main promoter of the *X. fastidiosa gum* operon. As observed, the wild-type strain is highly susceptible to chloramphenicol (Fig. 3), unlike the *X. fastidiosa gumB* and *gumF* mutants and *X. fastidiosa* transformed with pBCK92 (control plasmid). *Xylella fastidiosa gumB* and *gumF* mutants grew on media containing chloramphenicol, irrespective on the presence of glucose, and with different amounts of antibiotic, indicating that glucose does not regulate expression of the *gum* operon as was observed for *X. campestris* pv. *campestris* (Fig. 3). The *X. fastidiosa gumB* and *gumF* mutant strains, as well as the *X. fastidiosa* wild-type, have the same growth capabilities when cultivated in liquid medium without antibiotic selection (Fig. 3), and they also have the same growth pattern in either PW or BCYE solid media, despite the presence of glucose (data not shown).

Even though there is homology at the DNA level between *X. fastidiosa* and *X. campestris* pv. *campestris*, the characterization of *X. fastidiosa gum* mutants indicates remarkable behavior differences between these two bacteria, which certainly reflect the differences between the environments they inhabit, and, consequently, the distinct regulatory mechanisms for their *gum* operons. *Xylella fastidiosa* is restricted to the xylem vessels and insect mouths, being transmitted to plant hosts by xylem-feeding insects. Conversely, *X. campestris* pv. *campestris* mostly colonizes leaf plant mesophyl, infecting plants through the hydathodes (Hugouvieux *et al.*, 1998), even though it also colonizes xylem vessels and is able to survive outside a plants' live tissue.

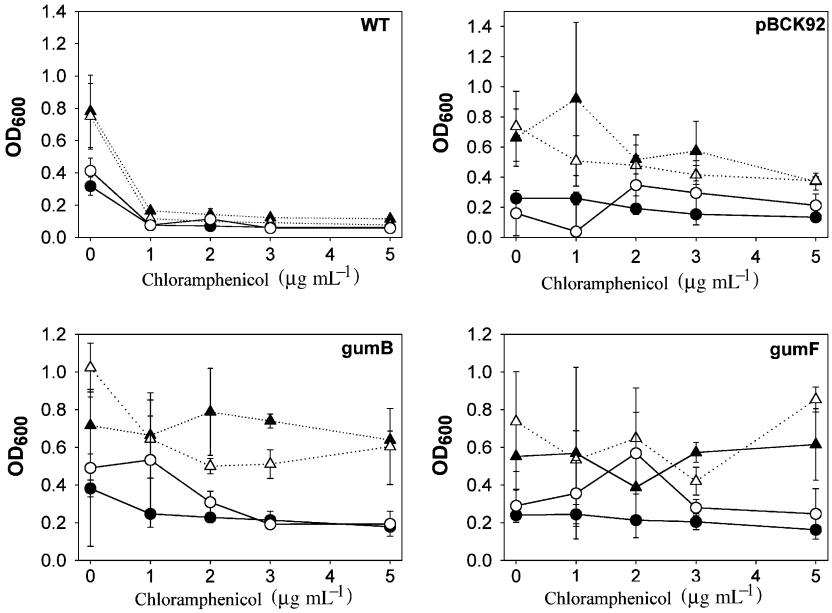

Fig. 3. Chloramphenicol resistance of *Xylella fastidiosa* WT and *gum* mutants as a function of glucose. The same amount of cells were inoculated into PW (. . .▲. . .), PWG (. . .△. . .), BCYE (− ● −) and BCYEG (− ○ −) and *Xylella fastidiosa* culture growth at different concentrations of chloramphenicol was measured spectrophotometricaly ($A_{600\,nm}$). Glucose was added at 1% (w/v).

Although we were not able to detect significant differences in EPS production between the *X. fastidiosa gum* mutants and the wild-type strain, *X. fastidiosa gumB* and *gumF* mutants showed a reduced biofilm formation in BCYE medium and then behaved like the wild-type in PW media. These results indicate that the predicted 'fastidian gum' might be poorly expressed and might not be the major component of the matrix observed in *X. fastidiosa* cell clumps by SEM. Partial evidence that *X. fastidiosa* produces low amounts of fastidian gum has recently been published (Osiro *et al.*, 2004), supporting the contention that *X. fastidiosa* does not need high amounts of EPS for adhesion and biofilm formation (Leite *et al.*, 2002) under low nutrient conditions, as was experienced by *X. fastidiosa* inside xylem vessels.

The role of the fastidian *gum* in *X. fastidiosa* biofilms remains uncertain and additional data are still needed for a better understanding of its function and its involvement in the pathogenicity of this fastidious bacterium. However, up to now this is the first evidence on the effect of gum mutation on biofilm formation dependent on media composition for *X. fastidiosa*.

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References

Alves E, Kitajima EW & Leite B (2003) Interaction of *Xylella fastidiosa* with different cultivars of *Nicotiana tabacum*: a comparison of colonization patterns. *J Phytopathology* **151**: 500–506.

Chang CJ, Garnier M, Zreik L, Rossetti V & Bové JM (1993) Culture and serological detection of the xylem limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Curr Microbiol* **27**: 137–142.

Chou FL, Chou HC, Liu YS, Yang BY, Lin NT, Weng SF & Tseng YH (1997) The *Xanthomonas campestris gumD* gene required for synthesis of xanthan gum is involved in normal pigmentation and virulence in causing black rot. *Biochem Biophys Res Commun* **233**: 265–269.

Davis MJ, Purcell AH & Thomson SV (1980) Isolation media for the Pierce's disease bacterium. *Phytopathology* **70**: 425–429.

Denny TP (1995) Involvement of bacterial polysaccharides in plant pathogenesis. *Annu Rev Phytopathol* **33**: 173–197.

Gaurivaud P, Souza LCA, Virgílio ACD, Mariano AG, Palma RR & Monteiro PB (2002) Gene disruption by homologous recombination in *Xylella fastidiosa* citrus variegated chlorosis strain. *Appl Environ Microbiol* **68**: 4658–4665.

Harding NE, Cleary JM, Cabanãs DK, Rosen IG & Kang KS (1987) Genetic and physical analysis of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*. *J Bacteriol* **169**: 2854–2861.

Hopkins DL (1989) *Xylella fastidiosa*: xylem-limited bacterial pathogen of plants. *Annu Rev Phytopathol* **27**: 271–290.

Hugouvieux V, Barber CE & Daniels MJ (1998) Entry of *Xanthomonas campestris* pv. *campestris* into hydathodes of *Arabidopsis thaliana* leaves: a system for studying early infection events in bacterial pathogenesis. *Mol Plant Microbe Interact* **11**: 537–543.

Ielpi L, Couso RO & Danjert MA (1993) Sequential assembly and polymerization of the polyprenol-linked pentasaccharide repeating unit of the xanthan polysaccharide in *Xanthomonas campestris*. *J Bacteriol* **175**: 2490–2500.

Katzen F, Ferreiro DU, Oddo CG, Ielmini MV, Becker A, Pühler A & Ielpi L (1998) *Xanthomonas campestris* pv campestris *gum* mutants: effects on xanthan biosynthesis and plant virulence. *J Bacteriol* **180**: 1607–1617.

Kjaergaard K, Schembri MA, Hasman H & Klemm P (2000) Antigen 43 from *Escherichia coli* induces intra and interspecies cell aggregation and changes in colony morphology of *Pseudomonas fluorescens*. *J Bacteriol* **182**: 4789–4796.

Leite B, Andersen PC & Ishida ML (2004) Colony aggregation and biofilm formation in xylem chemistry-based media for *Xylella fastidiosa*. *FEMS Microbiol Lett* **230**: 283–290.

Leite B, Ishida ML, Alves E, Carrer H, Pascholati SF & Kitajima EW (2002) Genomics and X-ray microanalysis indicate that Ca^{2+} and thiols mediate the aggregation and adhesion of *Xylella fastidiosa*. *Braz J Med Biol Res* **35**: 645–650.

Lemos EG, Alves LM & Campanharo JC (2003) Genomics-based design of defined growth media for the plant pathogen *Xylella fastidiosa*. *FEMS Microbiol Lett* **219**: 39–45.

Marques LLR, Ceri H, Manfio GP, Reid DM & Olson ME (2002) Characterization of biofilm formation by *Xylella fastidiosa* in vitro. *Plant Disease* **86**: 633–638.

Monteiro PB, Teixeira DC, Palma RR, Garnier M, Bové JM & Renaudin J (2001) Stable transformation of the *Xylella fastidiosa* citrus variegated chlorosis strain with *oriC* plasmids. *Appl Environ Microbiol* **67**: 2263–2269.

Osiro D, Colnago LA, Otoboni AMMB, Lemos EGM, Souza AA, Coletta-Filho HD & Machado MA (2004) A kinetic model for *Xylella fastidiosa* adhesion, biofilm formation, and virulence. *FEMS Microbiol Lett* **236**: 313–318.

Pielken P, Schimz KL, Eggeling L & Sahm H (1988) Glucose metabolism in *Xanthomonas campestris* and influence of methionine on the carbon flow. *Can J Microbiol* **34**: 1333–1337.

Silva FR da, Vettore AL, Kemper EL, Leite A & Arruda P (2001) Fastidian gum: the *Xylella fastidiosa* exopolysaccharide possibly involved in bacterial pathogenicity. *FEMS Microbiol Lett* **203**: 165–171.

Simpson AJG, Reinach F, Arruda P, *et al.* (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* **406**: 151–157.

Tyson GE, Stojanovic BJ, Kuklinski RF, DiVittorio TJ & Sullivan ML (1985) Scanning electron microscopy of Pierce's disease bacterium in petiolar xylem of grape leaves. *Phytopathology* **75**: 264–269.

Vojnov AA, Slater H, Daniels MJ & Dow JM (2001) Expression of the gum operon directing xanthan biosynthesis in *Xanthomonas campestris* and its regulation in planta. *Mol Plant Microbe Interact* **14**: 768–774.

Vojnov AA, Zorreguieta A, Dow JM, Daniels MJ & Dankert MA (1998) Evidence for a role for the *gum*B and *gum*C gene products in the formation of xanthan from its pentassaccharide repeating unit by *Xanthomonas campestris*. (1998) *Microbiology* **144**: 1487–1493.

Weels JM, Raju BC, Hsueh-Yun H & Weisburg WG (1987) *Xylella fastidiosa* gen. nov., sp. nov: Gram-negative, xylem limited, fastidious plant bacteria related to *Xanthomonas spp. Int J System Bacteriol* **37**: 136–143.