



RESEARCH LETTER

Twitching motility and biofilm formation are associated with *tonB1* in *Xylella fastidiosa*

Luciana Cursino, Yaxin Li, Paulo A. Zaini, Leonardo De La Fuente, Harvey C. Hoch & Thomas J. Burr

Department of Plant Pathology and Plant-Microbe Biology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY, USA

Correspondence: Thomas J. Burr,
Department of Plant Pathology and Plant-
Microbe Biology, New York State Agricultural
Experiment Station, Cornell University,
Geneva, NY 14456, USA. Tel.: +1 315 787
2211; fax: +1 315 787 2276; e-mail:
tjb1@cornell.edu

Present address: Yaxin Li, Pioneer Hi-Bred
International Inc., A DuPont Company, 7300
NW 62nd Avenue, Johnston, IA 50131, USA.

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Introduction

TonB is widely known as a cytoplasmic membrane proton-motive force for the active transport of iron-siderophore complexes and vitamin B12 across the outer membrane of gram-negative bacteria (Postle & Kadner, 2003).

Two integral proteins, ExbB and ExbD, and a TonB protein form the TonB complex in *Escherichia coli* K12. TonB is anchored by its N-terminal region to the cytoplasmic membrane and extends into the periplasm. It directly contacts and transduces energy to active transporters by an allosteric mechanism, whereby TonB first assumes an energized conformation that interacts with the outer membrane receptor and leads to conformational changes that open receptor channels for either iron or vitamin B12 transport (Postle, 2007). More recently, Schauer *et al.* (2008) reported that other molecules such as metals, sugars and oligosaccharides requiring energized transport across the outer membrane are also substrates for TonB transport.

Xylella fastidiosa Temecula is a twitching motile *Deltaproteobacteria* that causes Pierce's disease in grapevines (Meng

Abstract

A mutation in the *Xylella fastidiosa tonB1* gene resulted in loss of twitching motility and in significantly less biofilm formation as compared with a wild type. The altered motility and biofilm phenotypes were restored by complementation with a functional copy of the gene. The mutation affected virulence as measured by Pierce's disease symptoms on grapevines. The role of TonB1 in twitching and biofilm formation appears to be independent of the characteristic iron-uptake function of this protein. This is the first report demonstrating a functional role for a *tonB* homolog in *X. fastidiosa*.

et al., 2005), a disease responsible for significant economical losses to the US wine industry (Siebert, 2001). Following the genome sequencing of *X. fastidiosa* (Van Sluys *et al.*, 2003), many virulence factors have been identified including diffusible signal factor (Chatterjee *et al.*, 2008), fastidious gum (da Silva *et al.*, 2001), polygalacturonase (Roper *et al.*, 2007) and various adhesins (Feil *et al.*, 2007) including type I and type IV pili (Meng *et al.*, 2005).

Despite the annotation of many *tonB* homologs in the *X. fastidiosa* genome, their functions have not been elucidated. This study reports a functional role for a *tonB* homolog, *tonB1*, in *X. fastidiosa* in twitching motility, biofilm formation and virulence that is distinct from the well-known function of *tonB* in iron transport.

Materials and methods**Bacteria, plasmids and growth conditions**

Xylella fastidiosa strain Temecula (ATCC 700964) was cultured at 28 °C on modified PW agar or PD2 broth (Davis

et al., 1981) with 3.5 g L⁻¹ of bovine serum albumin (Sigma, St. Louis, MO) according to Li *et al.* (2007). We previously generated a library of twitching mutants through transposon (*Tn5*) mutagenesis of *X. fastidiosa* strain Temecula (Li *et al.*, 2007). Mutants were cultured on modified PW containing 50 mg L⁻¹ kanamycin (Sigma). Bacterial stocks were maintained at -80 °C on modified PW broth containing 7% dimethyl sulfoxide (Sigma) final concentration. *Escherichia coli* was cultured on Luria-Bertani media amended with the appropriate antibiotics. PW-Chrome Azurol S (PW-CAS) agar was prepared as described previously by Pacheco *et al.* (2006).

Identification of *Tn5* insertion

Tn5 insertions were identified by sequencing as described by Li *et al.* (2007). The genomic location of individual insertions was identified by a BLAST search of the *X. fastidiosa* Temecula genome database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and by comparison with the *X. fastidiosa* genome database (<http://www.xylella.lncc.br>). *Tn5* insertions were also confirmed by PCR using primers flanking the insertion.

Colony morphology and twitching motility

Colony morphologies of *X. fastidiosa* wild-type and mutant strains were analyzed after 5–7 days of growth on PW medium at 28 °C. A colony morphology associated with type IV pilus-twitching motility was recognized as a peripheral fringe at the colony edge, clearly visible using a dissecting microscope (SZX12; Olympus, Center Valley, PA). The lack of a peripheral fringe was confirmed with repeated observations of subsequent colonies of mutants. Furthermore, the twitching-impaired phenotype was substantiated microscopically using time-lapse imaging.

Southern blot

Verification of a single *Tn5* insertion in the mutants was confirmed by Southern blots. Analyses utilized the DIG DNA labeling and detection kit (Boehringer Mannheim, Germany) with a *Tn5* probe according to the manufacturer's directions.

Cloning and complementation

All standard cloning procedures were conducted as described by Sambrook & Russell (2001). New England Biolabs (NEB Inc., Beverly, MA) enzymes were used according to the manufacturer's directions. PCR amplifications of *X. fastidiosa* gene *tonB1* – ORF XP0869 (PD0843) – were accomplished with the primers XP0869R (5'-TAAGCTTCTCTAT GCCTGTGCGATTC-3') and XP0869F (5'-TGGATCCGTC GGAATCGACGTGATCG-3'), and the product was analyzed by agarose gel electrophoresis. The resulting 1.7-kb ampli-

con, which contained ORF XP0869 plus 400 bp of flanking regions on both sides, was digested with BamHI and HindIII and cloned into pBBR1 MCS-5 (Kovach *et al.*, 1995), a broad-host-range plasmid that can replicate inside *X. fastidiosa*. pBBR1MCS-5::*tonB* (pLCTONB1) was electroporated into *E. coli* and the presence of the recombinant plasmid was verified by plasmid isolation and double digestion. It was confirmed by DNA sequencing at the Cornell University's CLC facility. Electrocompetent *X. fastidiosa* cells were prepared as described by Guilhabert *et al.* (2001) and electroporation was conducted as described by Li *et al.* (2007). Cells of the *X. fastidiosa tonB* mutant were electroporated with pLCTONB1 and plated on modified PW agar plus 50 mg L⁻¹ of kanamycin (Sigma) and 4 mg L⁻¹ of gentamycin (Sigma).

Biofilm formation

Biofilm formation was analyzed as described by Li *et al.* (2007) with slight modifications. Briefly, wild type, *tonB* mutant and complemented mutant cells from 5-day-old cultures grown on PW agar plates were diluted to an OD_{600nm} of 0.1 and grown in 125-mL Erlenmeyer glass flasks containing 50 mL PD2 broth. The flasks were incubated at 28 °C, with 200 r.p.m. shaking for 10 days, after which biofilm formation at the medium–air interface was assessed.

Growth curves

To assess the influence of the *tonB* mutation on *X. fastidiosa* growth rate, growth curves in PW broth were compared for the wild type, mutant and complemented mutant as described by Galvani *et al.* (2007). Cells were subjected to iron limitation by the addition of 200 µM 2,2-dipyridyl (Sigma) to the basal medium absent of hemin chloride (Zaini *et al.*, 2008).

Microscopy

Temporal and spatial observations of the colony fringes of the wild type, *tonB* mutant and complemented mutant were made using an inverted IMT-2 Olympus microscope using × 20 and × 40 phase-contrast objectives. Time-lapse images were recorded using a CoolSNAP *Cf* digital camera controlled by METAMORPH image software (Universal Imaging Corp., Downingtown, PA). METAMORPH image software was also used to calculate the rate of speed of twitching motility on PW agar plates overlaid with cellophane.

To view cells for the presence of pili by transmission electron microscopy (TEM), three-day-old cultures were collected from PW plates that had been overlaid with cellophane, resuspended in water, deposited on Formvar-coated grids, dried and subsequently stained with either

phosphotungstic acid or uranyl acetate, and examined with a JEOL S-100 TEM (JEOL USA Inc., Peabody, MA).

Pathogenicity assay

Vitis vinifera L. cv. Cabernet Sauvignon grapevines grown in the greenhouse for 2 months were inoculated by needle puncture. Ten plants per treatment were inoculated at four basal internodes with 20- μ L drops containing about 1.0×10^8 CFU mL⁻¹ bacterial cells of wild type, *tonB1* mutant or water. Twenty-one weeks after inoculation, symptomatic leaves were scored for Pierce's disease symptoms (Guilhabert & Kirkpatrick, 2005).

Statistical analysis

Plants were arranged in a completely randomized design on greenhouse benches, each individual plant representing an experimental unit. For each treatment, 10 *X. fastidiosa*-inoculated plants (wild type and *tonB1*) and 10 water-inoculated plants were used.

Data were analyzed using one-way ANOVA and a Tukey's HDS test with STATISTIX 9 software (Analytical Software, Tallahassee, FL).

Results

Identification of the *tonB1* mutant

It was determined by Southern blot and sequencing analyses that the twitching-minus mutant had a single transposon

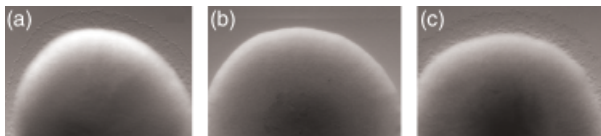


Fig. 1. Colony fringe morphologies. (a) *Xylella fastidiosa* wild-type strain. (b) *tonB1* mutant. (c) Complemented mutant. All cells were grown on PW agar medium.

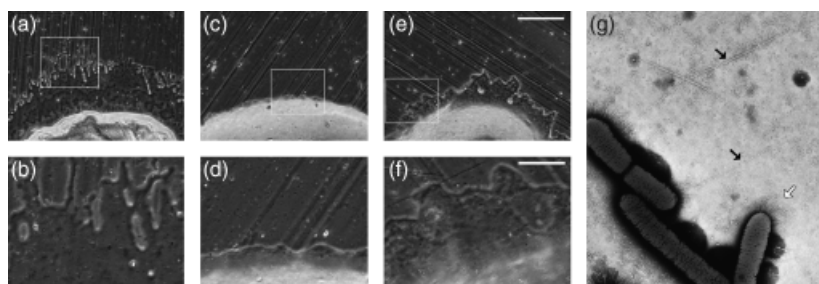


Fig. 2. Light microscopy of twitching zones of *Xylella fastidiosa*. Micrographs of the wild type at $\times 10$ (a) and $\times 40$ (b), *tonB1* mutant at $\times 10$ (c) and $\times 40$ (d) and the *tonB1*-complemented mutant at $\times 10$ (e) and $\times 40$ (f). Transmission electron micrograph of negatively stained cells of the *X. fastidiosa tonB1* mutant showing (white arrow) type I and (black arrow) type IV pili at the cell pole (g). White squares represent the area amplified to $\times 40$. The bar represents 200 μ m for the $\times 20$ panel and 50 μ m for the $\times 40$ panel.

insertion in ORF XP0869 (PD0843), encoding a predicted TonB protein. We designated the gene as *tonB1* because it is the first *tonB* gene characterized in *X. fastidiosa*. *tonB1* is apparently an orphan gene that does not have an associated cognate *exbB* and *exbD*. *tonB1* encodes a putative 299 aa protein (33 kDa) that contains a conserved TonB domain in its C-terminal at 212–246 aa and a proline-rich repetitive region at 267–271 aa (not shown), thus comprising a typical domain structure of TonB proteins.

The *tonB1* null mutant has an insertion in the 245th codon of the gene.

tonB1 is required for twitching motility

The *tonB1* mutant produces a colony with a smooth periphery (Fig. 1b), indicating reduced or lack of twitching motility. Time-lapse video microscopy showed that single cells of the *tonB1* mutant at the outer colony periphery do not move (twitch) on PW agar (Fig. 1a), while wild-type cells exhibited twitching motility at rates of 4 μ m min⁻¹ and formed a wide peripheral fringe (Fig. 2a and b).

Although the mutant is clearly twitching negative, its growth rate in liquid medium is similar to that of the wild type (Fig. 3a).

TEM revealed the presence of polar type I (short) and type IV (long) pili on wild type, *tonB1* mutant and complemented cells. *Xylella fastidiosa tonB1* pili types are indicated by arrows in Fig. 2g.

Complementation of the *tonB1* mutant

To verify the association of *tonB1* with twitching motility and biofilm formation, we complemented the mutant via transformation with a complete copy of the gene. Complementation restored the colony phenotype and twitching motility to that of the wild-type strain (Fig. 1a). The complemented mutant cells expressed twitching rates of 3.5 μ m min⁻¹ on agar, resulting in a wide peripheral fringe (Fig. 1c).

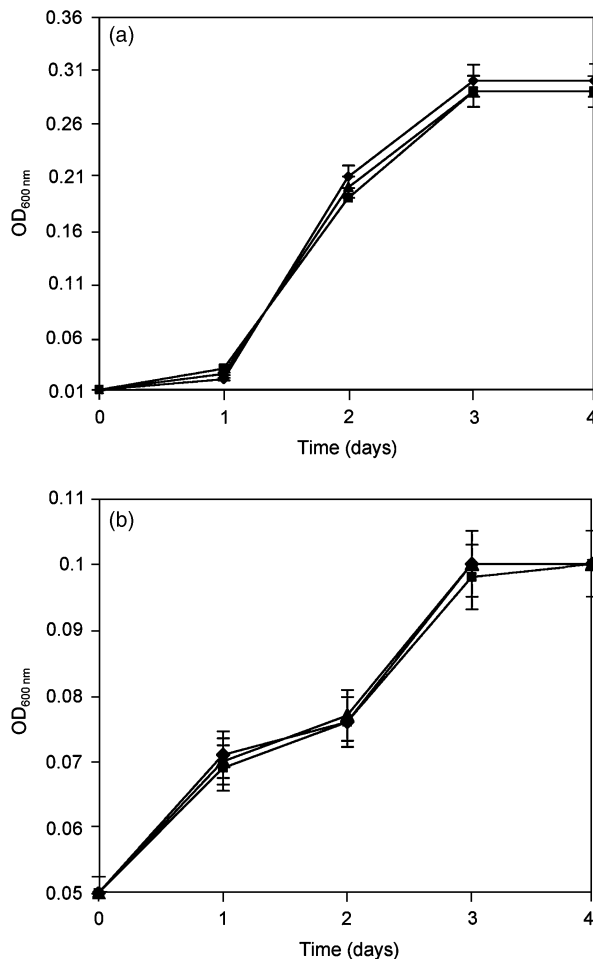


Fig. 3. Bacterial growth of the wild type, *tonB1* mutant and complemented mutant in PW medium. (a) Bacteria growing on PW. (b) Effects of iron limitation on the growth rate. Square, *Xylella fastidiosa* wild type; circle, *X. fastidiosa tonB1* mutant; and triangle, complemented mutant (filled symbols). Data are the average of five replications.

Effect of *tonB1* on growth under iron-limited conditions

To investigate whether the mutation in *tonB1* influenced iron uptake, we grew *X. fastidiosa* wild type, *tonB1* mutant and complemented mutant in liquid media under iron-limited conditions. The wild type, mutant and complemented mutant grew at similar rates and exhibited twitching motility under iron-limited conditions (Fig. 3b).

Effect of *tonB1* in the assimilation of iron-siderophore complexes

The biosynthesis of siderophores by *X. fastidiosa* Temecula was reported previously (Pacheco *et al.*, 2006). Following the same procedure, the ability of the *tonB1* mutant of iron uptake via siderophores was assessed. On the PW-CAS assay

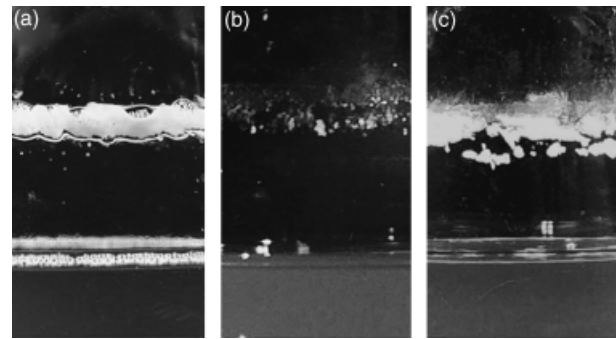


Fig. 4. Biofilm formation of *Xylella fastidiosa* following 10 days of growth in culture with agitation. (a) Wild-type Temecula, (b) *tonB1* mutant and (c) complemented mutant.

plate, the *tonB1* mutant showed siderophore production comparable with that of the wild-type strain. The same was observed for the complemented mutant (not shown).

tonB1 mutation affected biofilm formation

Wild-type Temecula formed a homogeneous biofilm layer (Fig. 4a) whereas the mutant produced a thinner biofilm layer (Fig. 4b). The complemented mutant expressing *tonB1* restored the biofilm phenotype similar to that of the wild type (Fig. 4c).

tonB1 is essential for full virulence in *X. fastidiosa*

The ability of the *tonB1* mutant to cause Pierce's disease symptoms after inoculation into the stems of grapevines was compared with the wild-type strain. At 21 weeks postinoculation, the severity of disease symptoms in plants inoculated with the *tonB1* mutant was significantly less than the wild-type strain ($P < 0.03$) (Fig. 5).

Discussion

Genes encoding TonB are present in all the gram-negative genomes sequenced so far and are conserved within bacterial families (Larsen *et al.*, 1996; Benevides-Matos *et al.*, 2008). For this reason, the *tonB* genes have been used to highlight genetic variability in different species of bacteria including *X. fastidiosa* (Bextine *et al.*, 2008; Stahlhut *et al.*, 2009). The TonB system promotes transport of nonpermeable molecules across the outer membrane and was initially thought to be restricted to iron complexes and vitamin B12 (Nikaido, 2003). Recent results have shown that other molecules including metals and sugars may also be transported via the TonB system (Schauer *et al.*, 2008). In addition, the role of TonB in providing energy to transport molecules involved in the process of bacterial movement has been reported (Huang *et al.*, 2004; Abbas *et al.*, 2007).

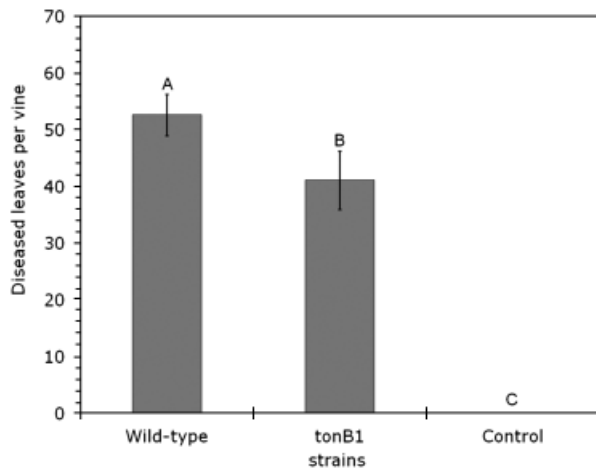


Fig. 5. Severity of Pierce's disease of grape incited by the wild type and *tonB1* mutant of *Xylella fastidiosa*. The vertical lines represent the SE of the mean of the number of infected leaves per vine determined from 10 replicate plants for each treatment. Means with different letters differ significantly ($P < 0.05$).

In this paper, we demonstrated that *tonB1* in *X. fastidiosa* is essential for twitching motility and is also associated with biofilm formation and virulence in grapevines. In *Pseudomonas aeruginosa* ORF PA0406, (*tonB3*) was found to be associated with twitching motility. In this case, *tonB3* was not required for iron transport, but affected twitching motility and extracellular type IV pili assembly (Huang *et al.*, 2004). In contrast, the *P. aeruginosa tonB2* gene was shown to be required for iron transport (Zhao & Poole, 2000). More recently, Abbas *et al.* (2007) reported another gene from *P. aeruginosa*, *tonB1*, which is required for biofilm formation and also affects twitching. It should be noted that the *P. aeruginosa* genome has three *tonB* genes, whereas the annotated genome of *X. fastidiosa* strain Temecula has four *tonB* homologs: PD0843 (*tonB1*), PD0009, PD1319 and PD1359. All appear to encode complete TonB proteins, but only PD0009 has closely linked cognate *exbB*, *exbD* genes. The *X. fastidiosa* TonB1 shows high identity (100–98%) at the amino acid level to corresponding TonB homologs in other *X. fastidiosa* strains (Dixon, 9a5c, M23 and Ann-1), and 36% identity to TonB3 of *P. aeruginosa*, suggesting that their roles in twitching motility may be related.

We previously reported the importance of type I and type IV pili in *X. fastidiosa* in biofilm formation (Li *et al.*, 2007). Although the *tonB1* mutant still possesses both pili types, it did not move via twitching.

We hypothesize that TonB1 is responsible for providing the energy necessary for cellular import of molecules involved in twitching motility other than those involved in type IV pili assembly.

Abbas *et al.* (2007) reported that *tonB1* is responsible for the transport of acyl homoserine lactone signaling

molecules involved in biofilm formation, twitching and swarming motility in *P. aeruginosa*. In *X. fastidiosa*, a number of regulatory systems that use environmental signals have been identified. For example, a putative two-component system involving the *pilR–pilS* gene pair has been identified and a *pilR* knockout was twitching negative (Li *et al.*, 2007). In addition, a gene cluster comprising a putative chemosensory regulatory system located in the vicinity of *tonB1* is also required for twitching movement (Cursino *et al.*, 2008; Hoch *et al.*, 2008). It is possible that TonB1 is involved in the transport of molecules into bacterial cells that are essential to one or more of these regulatory systems.

The *tonB1* mutant and the wild type were able to grow in an iron-depleted PW medium, but at slower rates than that in nonamended PW, similar to the results obtained by Silva-Stenico *et al.* (2005). The ability to grow in media with limited iron indicates that *tonB1* may not be involved in iron uptake or in facilitating the assimilation of iron-siderophores.

tonB1 is also associated with virulence as the mutant caused reduced disease as compared with the wild type. Other bacterial mutations in *tonB* genes have also affected virulence. For example, in *Xanthomonas campestris* pv. *campestris* B100, *tonB* was shown to be required for pathogenicity, iron uptake and a hypersensitive response in nonhost plants (Hung *et al.*, 2003).

Similar results were also reported by Enard & Expert (2000) with the *tonB_{Ech}* mutant of *Erwinia chrysanthemi* 3937. In this case, the mutation led to an increased production of pathogenicity factors such as pectinolytic enzymes and to a slight impairment symptom spread.

Similarly, the *tonB1* mutant in *X. fastidiosa* has reduced biofilm and twitching motility, and causes a reduced disease phenotype. Pathogenicity tests were performed by making inoculations at four basal nodes of the grapevines. Although the disease was reduced by about 30%, inoculated vines still showed clear Pierce's disease symptoms. This could be explained by our previous research (Meng *et al.*, 2005), which demonstrated that even type IV pili mutants are spread passively in grape xylem vessels. Therefore, following multiple inoculations with high inoculum doses, the bacterium may spread passively in the xylem, sufficient to cause disease. Clearly, additional research is needed to identify how the *tonB1* mutant behaves *in vivo* and also to elucidate the specific roles of twitching, biofilm formation and other virulence factors in causing Pierce's disease.

This work describes functions for one out of the four *tonB* genes predicted *in silico* in the *X. fastidiosa* Temecula genome. Further studies of virulence-associated gene functions will provide biological targets for the development of new controls for Pierce's disease.

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