Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2012) **13**(8), 923–934 DOI: 10.1111/J.1364-3703.2012.00800.X

The novel virulence-related gene *nlxA* in the lipopolysaccharide cluster of *Xanthomonas citri* ssp. *citri* is involved in the production of lipopolysaccharide and extracellular polysaccharide, motility, biofilm formation and stress resistance

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SUMMARY

Lipopolysaccharide (LPS) is an important virulence factor of *Xanthomonas citri* ssp. *citri*, the causative agent of citrus canker disease. In this research, a novel gene, designated as *nlxA* (novel LPS cluster gene of *X. citri* ssp. *citri*), in the LPS cluster of *X. citri* ssp. *citri* 306, was characterized. Our results indicate that *nlxA* is required for O-polysaccharide biosynthesis by encoding a putative rhamnosyltransferase. This is supported by several lines of evidence: (i) NlxA shares 40.14% identity with WsaF, which acts as a rhamnosyltransferase; (ii) sodium dodecylsulphate-polyacrylamide gel electrophoresis analysis showed that four bands of the O-antigen part of LPS were missing in the LPS production of the *nlxA* mutant; this is also consistent with a previous report that the O-antigen moiety of LPS of *X. citri* ssp. *citri* is composed of a rhamnose homo-oligosaccharide; (iii) mutation of *nlxA* resulted in a significant reduction in the resistance of *X. citri* ssp. *citri* to different stresses, including sodium dodecylsulphate, polymyxin B, H_2O_2, phenol, $CuSO_4$ and $ZnSO_4$. In addition, our results indicate that *nlxA* plays an important role in extracellular polysaccharide production, biofilm formation, stress resistance, motility on semi-solid plates, virulence and *in planta* growth in the host plant grapefruit.

INTRODUCTION

Xanthomonas citri ssp. *citri* (syn. *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas campestris* pv. *citri* or *Xanthomonas citri*) is the causative agent of citrus canker disease, one of the most serious diseases of citrus worldwide (Civerolo, 1984; Graham *et al*., 2004). Under natural conditions, *X. citri* ssp. *citri* can survive as an epiphyte on host plant surfaces and invade the leaf, stem and fruit through natural openings, including stomata and wounds (Brunings and Gabriel, 2003; Vojnov *et al*., 2010). The diseased citrus plant forms canker symptoms that are characterized by distinctive raised, necrotic lesions surrounded by oily, water-soaked margins and yellow chlorotic rings. Severe invasion by the pathogen eventually leads to defoliation, dieback and fruit drop, and the infected fruits are less valuable commercially and/or completely unmarketable (Gottwald *et al*., 2002).

Lipopolysaccharide (LPS) is an important virulence factor that has been shown to be involved in multiple steps of the disease process for many Gram-negative bacterial pathogens, including *X. citri* ssp. *citri* (Li and Wang, 2011; Newman *et al*., 2001). LPS is a key component of the outer membrane of Gram-negative bacteria and protects bacterial cells from the unfavourable conditions of the plant environment (Dow *et al*., 1995). In plant–pathogen interactions, LPS can function as a pathogen-associated molecular pattern (PAMP) by eliciting or potentiating plant defence-related responses (Zeidler *et al*., 2004). It is a tripartite amphipathic molecule composed of a lipid A moiety, a core oligosaccharide and a polysaccharide chain, termed the O-antigen or O-side chain (Raetz and Whitfield, 2002; Vorholter *et al*., 2001). Recently, the LPS structure of *X. citri* ssp. *citri* has been successfully defined (Casabuono *et al*., 2011). It has been shown that *X. citri* ssp. *citri* LPSs are composed mainly of a penta- or tetra-acylated diglucosamine backbone attached to either two pyrophosphorylethanolamine (PP-$EtNH_2$) groups or to one PP-$EtNH_2$ group and one phosphorylethanolamine group. The core region consists of a branched oligosaccharide and two phosphate groups, whereas the O-antigen is composed of a rhamnose homo-oligosaccharide (Casabuono *et al*., 2011).

Despite the tremendous progress made in the investigation of LPS, the understanding of the genetic pathway for the LPS biosynthesis of *X. citri* ssp. *citri* remains limited as the current understanding is mainly based on sequence similarities with related bacteria. Whole genome sequencing has revealed that *X. citri* ssp. *citri* 306 contains a cluster of 16 genes residing between the conserved *etfA* (XAC3587) and *metB* (XAC3602), which have been proposed to be involved in LPS biosynthesis (da Silva *et al*., 2002). However, only a few genes, including *wxacO* (XAC3596), *rfbC* (XAC3598) and *wzt* (XAC3600), have been experimentally characterized in the

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biosynthesis of LPS and the involvement in plant–pathogen interactions (Casabuono *et al*., 2011; Li and Wang, 2011). In addition, it has been reported that *X. citri* ssp. *citri* LPS shows remarkable differences from other bacterial LPS or *Xanthomonas* LPS, including the presence of two PP-$EtNH_2$ groups in the mainly penta-acylated lipid A moiety, the existence of two 3-deoxy-D-manno-octulosonic acid (Kdo) units linking lipid A to the core region and the presence of a 3*N*-acetylfucosamine (Fuc3NAc) unit in the core (Casabuono *et al*., 2011). Sugar analysis has revealed that *X. citri* ssp. *citri* processes a rhamnose homo-oligosaccharide as the O-antigen moiety (Casabuono *et al*., 2011), which is similar to *X. campestris* pv. *campestris* B100 (Braun *et al*., 2005). However, the configuration and types of sugar linkage of the *X. citri* ssp. *citri* O-polysaccharide have not been determined (Casabuono *et al*., 2011). Moreover, only rhamnose was detected in the sugar analysis of the LPS O-antigen of *X. citri* ssp. *citri* (Casabuono *et al*., 2011), which is different from many other *Xanthomonas* species, in which various sugars [rhamnose and fucose (Molinaro *et al*., 1999, 2003), rhamnose and xylose (Shashkov *et al*., 1999), rhamnose and Fuc3NAc (Molinaro *et al*., 2002)] were identified in the LPS O-antigen. Thus, it is necessary to obtain a further understanding of the genetic determinants of LPS biosynthesis of *X. citri* ssp. *citri*.

In our recent study, nine different mutants of LPS cluster genes were identified by screening the EZ-Tn5 mutant library *X. citri* ssp. *citri* (Yan and Wang, 2011b). Among them, two mutants (406G8 and 415H9) were found to have a transposon located in the intergenic region between *rfbC* (XAC3598) and a hypothetical gene XAC3597. The *rfbC* gene putatively encodes a truncated O-antigen biosynthesis protein and is involved in LPS production (Li and Wang, 2011). XAC3597 encodes a hypothetical protein which is specific to strain *X. citri* ssp. *citri* 306 and is absent from other *Xanthomonas* spp., except *X. campestris* pv. *musacearum* NCPPB 4381. Interestingly, a new hypothetical gene (ORF5) was reannotated in the intergenic region between *rfbC* and XAC3597 where the transposon was located in the 406G8 and 415H9 mutants (Patil *et al*., 2007). However, this reannotated hypothetical gene has not been characterized experimentally. In this study, we present evidence that the new hypothetical gene, designated as *nlxA* (novel LPS cluster gene of *X. citri* ssp. *citri*), is involved in the biosynthesis of not only LPS, but also extracellular polysaccharide (EPS), and plays a role in biofilm formation, stress resistance, motility and pathogenesis of the citrus canker pathogen *X. citri* ssp. *citri*.

RESULTS

A novel gene involved in the LPS biosynthesis of *X. citri* ssp. *citri*

Similar phenotypes were obtained for the two mutants 406G8 and 415H9; only the data for one mutant (406G8) are presented. The EZ-Tn5 location in the chromosome of *X. citri* ssp. *citri* 306 is indicated in Fig. 1. A growth curve assay was performed by culturing the wild-type and the mutant 406G8 in nutrient broth (NB) medium. The mutant grew in a similar manner to the wild-type (Fig. S1, see Supporting Information). For LPS assay, bacterial cultures were collected at 24 h post-inoculation (hpi). LPS was extracted from the same volume of bacterial culture for both the wild-type and the mutant to avoid growth variation between the tested strains. LPS produced by an *X. citri* ssp. *citri wzt* mutant, with *wzt* encoding an ATP-binding protein of an ABC transporter system for the export of the O-antigen of LPS, was also included. As illustrated in Fig. 2, the four bands of the O-antigen part of LPS were missing in the *nlxA* mutant, which showed a similar LPS pattern to the *wzt* mutant.

For the complementation experiment, the wild-type XAC3597 gene was first used to complement the 406G8 mutant, as we originally reasoned that the phenotype of 406G8 resulted from the transposon insertion in the promoter region of XAC3597 (Fig. 1). This was based on the genomic information for *X. citri* ssp. *citri* from da Silva *et al*. (2002) prior to the publication of the reannotation of the LPS gene cluster (Patil *et al*., 2007). However, attempts to complement the 406G8 mutant using intact XAC3597 plus 155 or 425 bp of upstream fragments did not restore the LPS production and pathogenicity of the mutant (data not shown). Thus, we hypothesized that another gene might exist in the insertion locus of the transposon between XAC3598 and XAC3597.

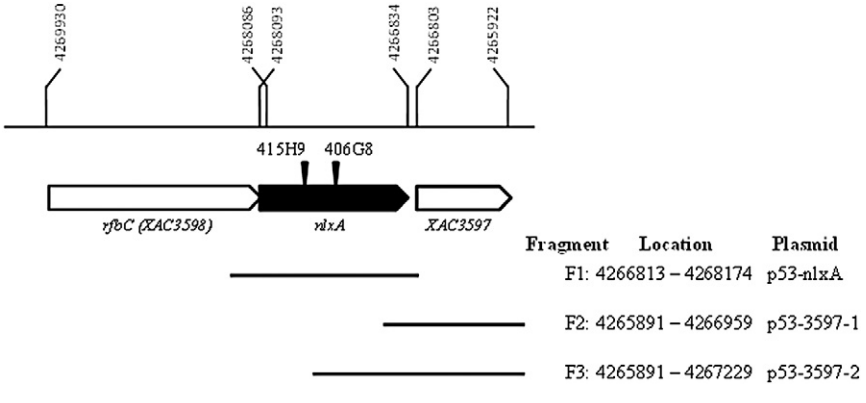

Fig. 1 Schematic diagram of the genetic organization of the *nlxA* and neighbouring genes in the lipopolysaccharide (LPS) gene cluster of *Xanthomonas citri* ssp. *citri* 306. The single-headed arrows represent the locations and orientations of the genes. The *nlxA* gene is indicated by a filled block arrow. The location of each gene in the chromosome is indicated by numbers. The EZ-Tn5 insertions in the mutants are indicated by inverted triangles. F1, F2 and F3 represent the DNA fragments used to construct the complementary plasmids. The locations of the three fragments in the chromosome are also given.

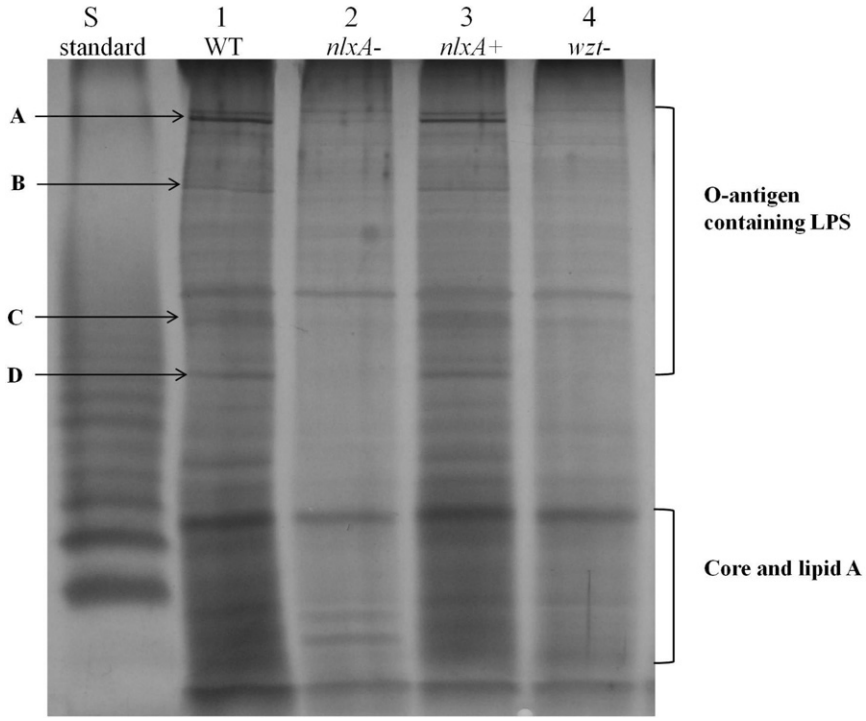

Fig. 2 Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the lipopolysaccharide (LPS) produced by the wild-type *Xanthomonas citri* ssp. *citri* 306 and its derivatives. Lanes: 1, wild-type (WT) containing an empty vector pUFR053; 2, *nlxA* mutant (406G8) containing an empty vector pUFR053; 3, complementary strain, *nlxA* mutant (406G8) containing the vector p53-nlxA; 4, *wzt* mutant (330D3) containing an empty vector pUFR053; S, LPS standard from *Salmonella enterica* serovar Typhimurium (10 μg, Sigma). Four bands that were missing in the O-antigen containing LPS of the *nlxA* mutant were indicated as A, B, C and D. The experiment was repeated three times with similar results, and the result of only one experiment is presented.

Interestingly, a new hypothetical gene (ORF5) was reannotated in the intergenic region between XAC3598 and XAC3597 by Patil *et al*. (2007), which further supports our hypothesis. This hypothetical gene contains 1260 nucleotides and is located between nucleotides 4 266 834 and 4 268 093 of the chromosome of *X. citri* ssp. *citri* 306 (Fig. 1). LPS production of the 406G8 mutant was restored to the wild-type level with a fragment containing the intact hypothetical gene (Fig. 2). This experiment indicates that this reannotated gene is a biologically functional gene and plays a role in the biosynthesis of LPS. This hypothetical gene was designated as *nlxA* (novel LPS cluster gene of *X. citri* ssp. *citri*).

The *nlxA* gene encodes a putative glycosyltransferase (GT)

BLAST analysis revealed that NlxA shares sequence identity (up to 95.02%) with different homologue proteins from more than 10 organisms, including both Gram-positive and Gram-negative bacteria (Table 1). NlxA shares 95.02% and 59.86% identity with only two hypothetical proteins of *X. campestris* pv. *musacearum* and *X. oryzae* pv. *oryzae*, respectively. NlxA shares an identity of 40.14% with WsaF, which was identified as a rhamnosyltransferase in the synthesis of the S-layer glycan of the Gram-positive bacterium *Geobacillus stearothermophilus* NRS 2004/3a (Steiner *et al*., 2010). In addition, NlxA shares an identity of 32.55% with WcrW, which was annotated as a putative GT of *Streptococcus pneumonia* 6803 (Bentley *et al*., 2006). GTs are a ubiquitous group of enzymes that catalyse the transfer of a sugar moiety from an activated sugar donor onto saccharide or nonsaccharide acceptors, and are involved in the biosynthesis of glycoproteins, glycolipids, oligo- and polysaccharides (Coutinho *et al*., 2003). Large numbers of GTs have been found and are currently classified into 94 families in the CAZy database (http://www.cazy.org). Both WsaF of *G. stearothermophilus* and WcrW of *S. pneumonia* belong to the GT4 family, members of which catalyse retaining glycosyl transfer reactions with nucleotide-sugar, phospho-sugar and lipid-phospho-sugar as donors (Coutinho *et al*., 2003; Martinez-Fleites *et al*., 2006). Protein sequence alignment also revealed that the putative protein of NlxA contains a motif 'PHPSYPPLE' which is conserved in all seven proteins of the BLAST result (Fig. 3). The 'PHPSYPPLE' motif has been indicated to be involved in the recognition of deoxythymidinediphosphate (dTDP)-β-L-rhamnose by WsaF to catalyse the glycosyl transfer reaction in *G. stearothermophilus* (Steiner *et al*., 2010).

Mutation of *nlxA* affects EPS production

Both LPS and EPS are polysaccharides and share certain common genetic determinants in polysaccharide biosynthesis. To test whether the mutation of *nlxA* affects EPS production in *X. citri* ssp. *citri*, the EPS assay was performed for the wild-type, mutant and complementary strains after culturing for 24 h in NB containing 2% glucose. No significant difference in cell populations was observed among the wild-type (1.90 $\pm$ 0.03), mutant (1.86 $\pm$ 0.06) and complementary strain (1.91 $\pm$ 0.02) by measuring the optical density at 600 nm (OD_{600}) of the bacterial cultures at the

Table 1 Sequence analysis of NlxA and its homologues.

Protein	Accession number	Source organism	Annotated function	Length (aa)	Identity (%)
NlxA		*Xanthomonas citri* ssp. *citri* 306		419	100
XcampmN_07905	ZP_06489485	*Xanthomonas campestris* pv. *musacearum* NCPPB 4381	Unknown	422	95.02
ORF5	ABI93189	*Xanthomonas oryzae* pv. *oryzae* BXO8	Unknown	417	59.86
WsaF	AAR99609	*Geobacillus stearothermophilus* NRS 2004/3a	Rhamnosyltransferase of GT4	413	40.14
PM8797T_20259	EDL58917	*Planctomyces maris* DSM 8797	Unknown	430	36.70
Roseina2194_02598	ZP_03754177	*Roseburia inulinivorans* DSM 16841	Unknown	448*	34.42
Met49242DRAFT_0030	ZP_08070643	*Methylocystis* sp. ATCC 49242	Rhamnan synthesis F	406†	32.63
WcrW	CAI34499	*Streptococcus pneumonia* 6803	Putative GT4	414	32.55

*The C terminal 448 amino acids (aa) of the complete 1030-aa protein were used for homologue analysis as only the C terminal of the protein showed similarity with NlxA.

†Similarly, the C terminal 406 aa of the complete 812-aa protein were used for homologue analysis with NlxA.

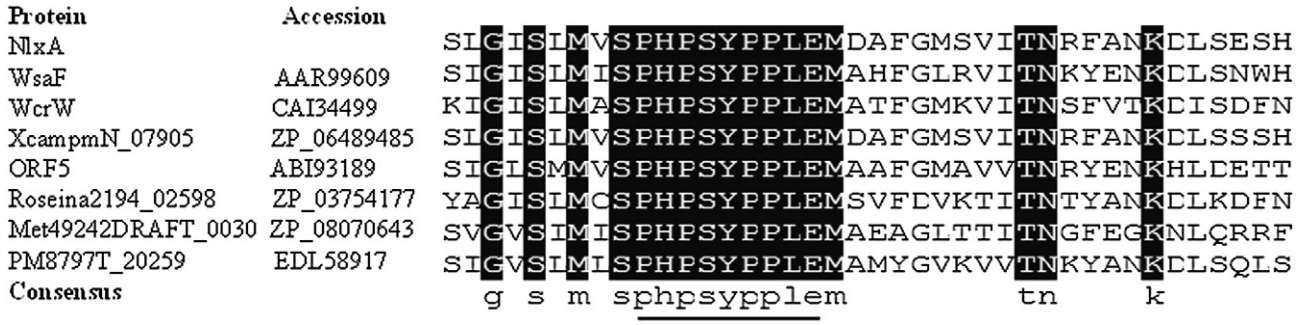

Fig. 3 Alignment analysis of NlxA and its homologues. Multiple alignments of NlxA with its homologues indicate the presence of a conserved motif specific for rhamnosyltransferase in NlxA. Only part of the alignment is shown. The names and GenBank accession numbers of the proteins are given. The nine amino acid residues which were identified by Steiner *et al*. (2010) as a binding signature of the substrate of deoxythymidinediphosphate-β-L-rhamnose of WsaF are underlined.

time point of sampling. The amount of EPS produced by the *nlxA* mutant was 7.13 $\pm$ 0.23 mg/mL, which was significantly lower than that of the wild-type (12.53 $\pm$ 1.93 mg/mL) ($P < 0.05$). EPS biosynthesis by the *nlxA* mutant was restored to the wild-type level (13.93 $\pm$ 1.08 mg/mL) with p53-nlxA, which contains the intact *nlxA* gene.

A capsule assay was conducted to test whether mutation of *nlxA* affects the capsule. No significant difference was observed between the wild-type and the *nlxA* mutant in the capsule staining (data not shown).

nlxA is involved in biofilm formation both *in vitro* and *in vivo*

Biofilm formation is an important bacterial trait associated with virulence and has been related to LPS and/or EPS production in many bacteria. To test whether *nlxA* is involved in biofilm formation of *X. citri* ssp. *citri*, biofilm formation was examined in both a glass tube and on citrus leaves. A significant reduction in biofilm formation was detected in the *nlxA* mutant compared with the wild-type by culturing the bacteria in a glass tube (Fig. 4A). The complementary strain showed similar biofilm formation to the wild-type. The *nlxA* mutant showed earlier biofilm formation than the wild-type and complementary strains at 10 h after inoculation on the citrus leaf surface (Fig. 4B). However, less biofilm formation was observed for the *nlxA* mutant than for the wild-type and complementary strains when the bacteria were incubated for 24 h to fully develop the biofilm on the leaves.

nlxA is involved in the resistance of *X. citri* ssp. *citri* to various environmental and chemical stresses

To investigate whether *nlxA* affects the resistance of *X. citri* ssp. *citri* to environmental and chemical stresses, the bacterial cells were challenged with different stresses. As shown in Table 2, mutation of *nlxA* resulted in a significant reduction in pathogen resistance against various compounds, including hydrogen peroxide, polymyxin B, sodium dodecylsulphate (SDS), phenol, $CuSO_4$ and $ZnSO_4$. The resistance of the *nlxA* mutant to these stresses was restored using the plasmid p53-nlxA containing the intact *nlxA* gene.

nlxA is involved in *X. citri* ssp. *citri* motility on semi-solid plates

The effects of mutation of *nlxA* on the swimming and swarming motility of *X. citri* ssp. *citri* were tested on semi-solid nutrient agar (NA) plates containing 0.3% and 0.7% agar, respectively. The movement of the wild-type on both swimming and swarming plates was very slow in the first 2 days post-inoculation (dpi), but

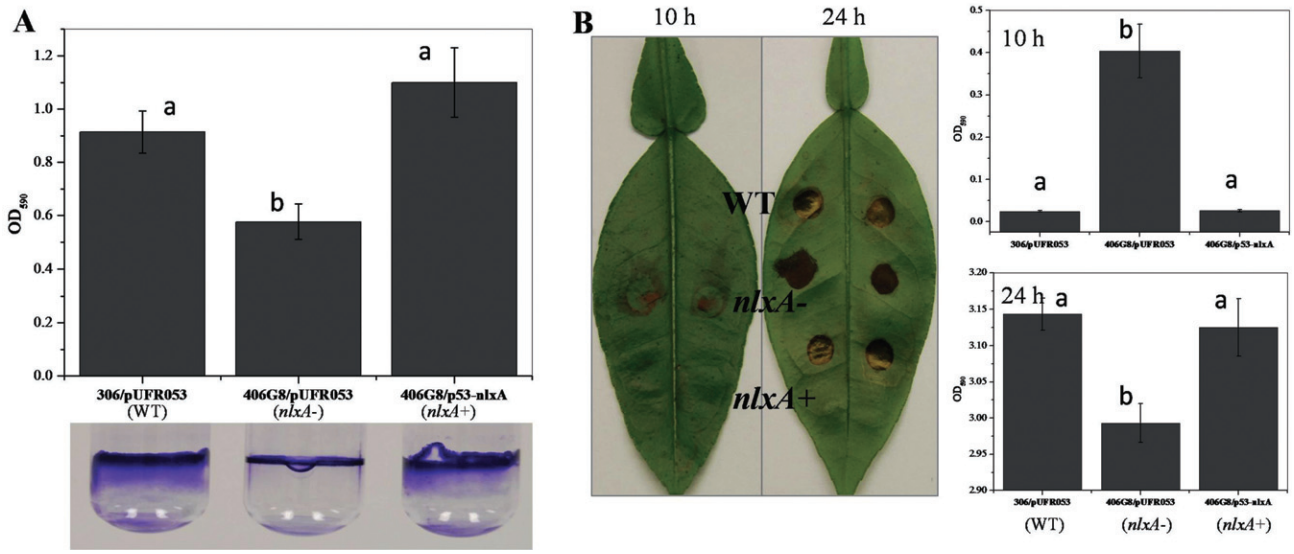

Fig. 4 The *nlxA* gene is involved in biofilm formation. (A) One millilitre of bacterial solution [10^8 colony-forming units (CFU)/mL in nutrient broth (NB)] were incubated at 28 °C for 24 h in a glass tube without shaking. (B) Twenty microlitres of bacterial solution (10^8 CFU/mL in NB) were inoculated onto the citrus abaxial leaf surface and incubated at 28 °C for 10 and 24 h in a humid chamber. Biofilm formation was visualized by crystal violet staining. The stained cells were dissolved in 97% ethanol and quantified by measuring the optical density at 590 nm. Values in the same figure that are significantly different ($P < 0.05$) are indicated by different letters (a, b). The biofilm assays were repeated three times with similar results with four replicates each time. Data from one representative experiment are shown. Vertical bars represent the standard errors of the means.

Table 2 The *nlxA* gene is involved in stress resistance against H_2O_2, polymyxin B, sodium dodecylsulphate (SDS), phenol, $CuSO_4$ and $ZnSO_4$.

Strains		Bacterial concentration before challenge (10^8 CFU/mL)*†	Survival percentage (%) after challenge*					
Name	Genotype		H_2O_2	Polymyxin B	SDS	Phenol	$CuSO_4$	$ZnSO_4$
306/pUFR053	WT	9.33 ± 0.58a	5.76 ± 1.19a	14.81 ± 1.28a	0.57 ± 0.03a	93.51 ± 5.78a	1.02 ± 0.42a	1.42 ± 0.32a
406G8/pUFR053	*nlxA*–	10.33 ± 0.85a	0.85 ± 0.04b	3.84 ± 1.61b	0.07 ± 0.03b	41.76 ± 12.61b	0.18 ± 0.10b	0.37 ± 0.08b
406G8/p53-nlxA	*nlxA*+	10.67 ± 1.53a	10.48 ± 4.62a	14.21 ± 2.42a	0.42 ± 0.13a	95.83 ± 7.22a	1.00 ± 0.57a	1.47 ± 0.19a

All the experiments were repeated three times independently with four replications each time. Similar results were observed and only one representative result is presented.

*For significant difference analysis, the survival percentages of four replications of each strain were calculated independently and then subjected to Student's *t*-test. Data with different letters (a, b) denote significant difference ($P < 0.01$) between strains in the same treatment. Means and standard errors from one representative result are shown.

†All strains were cultured for 20 h in nutrient broth (NB). Initial cell concentrations [colony-forming units (CFU)/mL] were measured by diluting the bacterial cultures appropriately and plating on nutrient agar (NA) plates before challenge with different stresses. For each strain tested, similar cell concentrations were observed in different stress assays. Only one set of the representative data is shown.

was more rapid after the second day (Fig. 5A,C) (which is similar to other *Xanthomonas* bacteria and indicates that the movement is inducible in *X. citri* ssp. *citri*). Although the mutant was slightly motile on the semi-solid plates, the swimming and swarming motilities of the *nlxA* mutant were reduced significantly compared with those of the wild-type (Fig. 5B,D). The deficiency of the mutant in motility could be complemented by the intact *nlxA* gene.

To investigate whether *nlxA* plays a role in the production of flagella, the flagella were observed under transmission electron microscopy (TEM). TEM analysis showed that both the wild-type and the *nlxA* mutant formed a single polar flagellum on the cell surface (data not shown).

nlxA and other genes in the LPS gene cluster were induced in a surface hardness-specific manner

It is known that LPS plays a critical role during bacterial swarming, probably by wetting the surface or acting as a surfactant (Toguchi *et al*., 2000). The expression of genes involved in LPS synthesis of *Salmonella typhimurium* was elevated in a surface hardness-specific manner just before the initiation of swarming, and also maximally induced during late exponential growth coincident with maximum swarming activity (Wang *et al*., 2004). To test whether this also happens in *X. citri* ssp. *citri*, the expression of genes in the LPS gene cluster, including *nlxA*, XAC3588, XAC3590, *wxacO*, *rfbC*

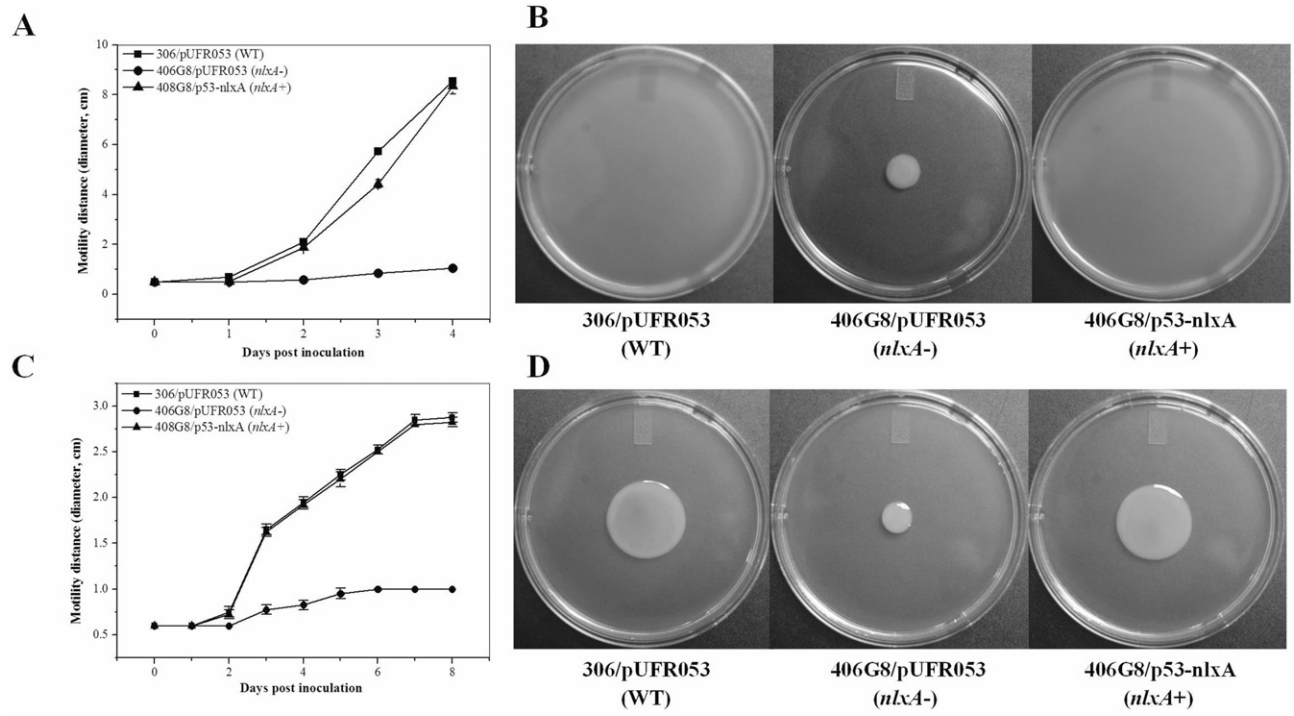

Fig. 5 The *nlxA* gene is involved in swimming and swarming motility. Two microlitres of bacterial solution [10^8 colony-forming units (CFU)/mL in nutrient broth (NB)] were inoculated on the swimming assay (0.3% agar) (A, B) and swarming assay (0.7% agar) (C, D) plates. The bacterial motility was measured at different time points as indicated. The movement of bacterial cells was photographed at 5 and 8 days post-inoculation (dpi) on the swimming and swarming plates, respectively. The experiment was repeated three times with three replicates each time. Means and standard errors of three replicates from one representative result are shown. Vertical bars represent the standard errors of the means.

and *wzm* in NB and on semi-solid NA plates (NB + 0.5% agar), was compared. Bacterial cells were collected at 24 hpi, the time point at which the wild-type starts to show notable movement on motility assay plates (Fig. 5A,C). The expression of *nlxA* was significantly induced ($P < 0.05$) on 0.5% agar plates relative to NB (Table 3). Consistently, the expression of other LPS cluster genes, including XAC3588, XAC3590, *wxacO*, *rfbC* and *wzm*, was also significantly induced on semi-solid NA plates (Table 3).

nlxA is involved in the virulence and bacterial survival *in planta*

As described previously (Yan and Wang, 2011b), the 406G8 mutant was identified by its deficient virulence in grapefruit. In this study, further investigations, including *in planta* bacterial growth assay and complementation experiments, were performed. In addition, a spraying inoculation method, which mimics natural invasion conditions, was also conducted to determine the role of *nlxA* in the virulence of the pathogen. The bacterial population of the *nlxA* mutant *in planta* was significantly lower than that of the wild-type by two orders of magnitude at 6 dpi and by more than one order of magnitude at 8, 10 and 14 dpi. Co-inoculation with the wild-type strain could not rescue the growth of the mutant in the host plant, as indicated by the similar growth curve to that of the *nlxA* mutant alone (Fig. 6D). The growth deficiency of the mutant was successfully complemented by the intact *nlxA* gene. Consistently, the complementary strain caused similar symptoms to the wild-type on citrus leaves (Fig. 6A).

To mimic the natural invasion conditions under which the canker pathogen is spread by rain splash, the bacterial strains were inoculated onto the leaf surfaces by a spraying method. The wild-type caused typical canker symptoms on citrus leaves at 16 dpi (Fig. 6B). However, no obvious symptoms were observed on the leaves inoculated by the *nlxA* mutant. The virulence deficiency of the mutant could be complemented by the intact *nlxA* gene.

DISCUSSION

In this research, we characterized a novel gene *nlxA* in the LPS cluster of *X. citri* ssp. *citri* 306. Our results indicate that *nlxA* is required for O-polysaccharide biosynthesis by encoding a putative rhamnosyltransferase. This is supported by several lines of evidence.

1. NlxA shares 40.14% identity with WsaF (Table 1), which acts as a rhamnosyltransferase (GT4) in the synthesis of S-layer glycan

Table 3 The expression of *nlxA* and other lipopolysaccharide (LPS) biosynthesis genes was induced on semi-solid agar plates.

Target gene†	Locus tag†	Annotated function	Primer sequence (5′ to 3′)	Fold change ± SE*	*P* value (Student's *t*-test)
nlxA	NA		F: AGCCATGCAAACATCGTGTCGTTG R: AAACGTAGGCATCGTCTAAGGCCA	2.611095 ± 0.705173	0.0304023
NA	XAC3588	Integral membrane protein	F: TCGGAATTTCTACTGCAGACGCCA R: CGAATTCTGCAGCAAGCATGGACA	2.694485 ± 0.200239	0.0005489
NA	XAC3590	Oxidoreductase	F: TCCTTCGGGCTTTCTGCAGTATCA R: TGTTGCCGAATTCCTTCAACACGG	3.367230 ± 0.324939	0.001889
wxacO	XAC3596	Putative transmembrane protein	F: TCTTTATGCGCTCTTGGCCGTAGT R: ACATCGGTCATCTGGCGGACATAA	5.199567 ± 0.647125	0.0128718
rfbC	XAC3598	Truncated O-antigen biosynthesis protein	F: ATCCATCACCAGCACCTGTTCGTA R: GAATCCGCCAATGGCATCGAAGTT	2.772365 ± 0.842772	0.0052181
wzm	XAC3601	ABC transporter permease	F: TTGAAATGGTCCCGATGCTGCTTG R: AAAGCAGAACGCTGCTCAGAATGC	2.393728 ± 0.488077	0.0213009

F, forward primer; R, reverse primer.

*The wild-type was cultured in nutrient broth (NB) for 20 h or on a semi-solid plate for 24 h; the fold change was calculated using gene expression of the wild-type after culture in NB for 20 h as calibrator according to the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). SE, standard error.

†Target genes and the corresponding ID are listed. NA denotes a gene name or ID that has not yet been assigned.

and transfers rhamnose sugars from the substrate of dTDP-β-L-rhamnose for the extension of the polysaccharide chain in the Gram-positive bacterium *G. stearothermophilus* (Steiner *et al*., 2010). Structural similarities between S-layer glycan and LPS O-antigen have been observed (Schaffer *et al*., 1996), and the assembly of both types of molecule may follow similar pathways (Steiner *et al*., 2007; Valvano, 2003). Further sequence analysis revealed that NlxA contains a conserved motif 'PHP-SYPPLE' (Fig. 3), which is identical to the signature sequence of WsaF for the binding of the rhamnose substrate (Steiner *et al*., 2010).

2. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that four bands of the O-antigen part of LPS were missing in the LPS production of the *nlxA* mutant (Fig. 2), indicating that *nlxA* is required for LPS O-antigen biosynthesis. This result is also consistent with the recent report that the O-antigen moiety of *X. citri* ssp. *citri* LPS is composed of a rhamnose homo-oligosaccharide (Casabuono *et al*., 2011). In addition, the LPS pattern of the *nlxA* mutant is similar to that of the *wzt* mutant (Fig. 2). The *wzt* gene encodes an ATP-binding protein of an ABC transporter system and is required for O-antigen export.
3. Mutation of *nlxA* resulted in a significant reduction in resistance of *X. citri* ssp. *citri* to different stresses, including SDS, polymyxin B, H_2O_2, phenol, $CuSO_4$ and $ZnSO_4$ (Table 2). LPS is known to be a key component of the outer membrane of Gram-negative bacteria and a protective barrier against harsh environmental conditions (Dow *et al*., 1995). Therefore, NlxA probably functions as a rhamnosyltransferase in the O-antigen biosynthesis of *X. citri* ssp. *citri*.

In the sequenced *Xanthomonas* spp., the homologues of *nlxA* were only found in *X. campestris* pv. *musacearum* NCPPB 4381, a causative agent of banana wild disease (Studholme *et al*., 2010), *X. oryzae* pv. *oryzae* BXO8, a causative agent of rice blight disease (Patil *et al*., 2007), and *X. citri* ssp. *citri* 306, a causative agent of citrus canker disease. This indicates that the *nlxA* gene characterized in this work is a unique LPS biosynthesis gene to only a small and diverse group of *Xanthomonas*. Great variations have been observed among LPS biosynthesis gene clusters of both animal pathogenic (Reeves and Wang, 2002) and plant pathogenic (Patil *et al*., 2007) bacteria. These variations have been proposed to reflect the highly immunogenic nature of LPS and a need for the pathogen to vary LPS to evade detection by the host (Patil *et al*., 2007). The presence of *nlxA* in only a few pathogenic bacteria of *Xanthomonas* might suggest a special role played by *nlxA* in the LPS assembly in these strains. However, the LPS structures of *X. campestris* pv. *musacearum* NCPPB 4381 and *X. oryzae* pv. *oryzae* BXO8 have not been characterized, making it difficult to analyse the structural similarities or differences between them and *X. citri* ssp. *citri*. The genetic evidence presented here demonstrates that *nlxA* is important for the LPS biosynthesis and virulence of *X. citri* ssp. *citri* (Figs 2 and 6). It would be interesting to investigate the role of this unique gene in the configuration of the LPS O-antigen, and whether it is involved in host recognition.

The effect of NlxA on the pathogenicity of *X. citri* ssp. *citri* probably results from its function in both LPS and EPS biosynthesis. The EPS composition of *X. citri* ssp. *citri* remains unknown. The EPS structure of *X. campestris* consists of a β-1,4-linked D-glucose backbone with trisaccharide side chains composed of mannose-(β-1,4)-glucuronic acid-(β-1,2)-mannose attached to alternate glucose residues in the backbone by α-1,3 linkages, and does not contain rhamnose (Jansson *et al*., 1975). However, a recent study has indicated that the EPSs produced by different *Xanthomonas* spp. vary. The EPS synthesized by *X. campestris* pv. *pruni* contains glucose, rhamnose, mannose and glucuronic acid in its chemical

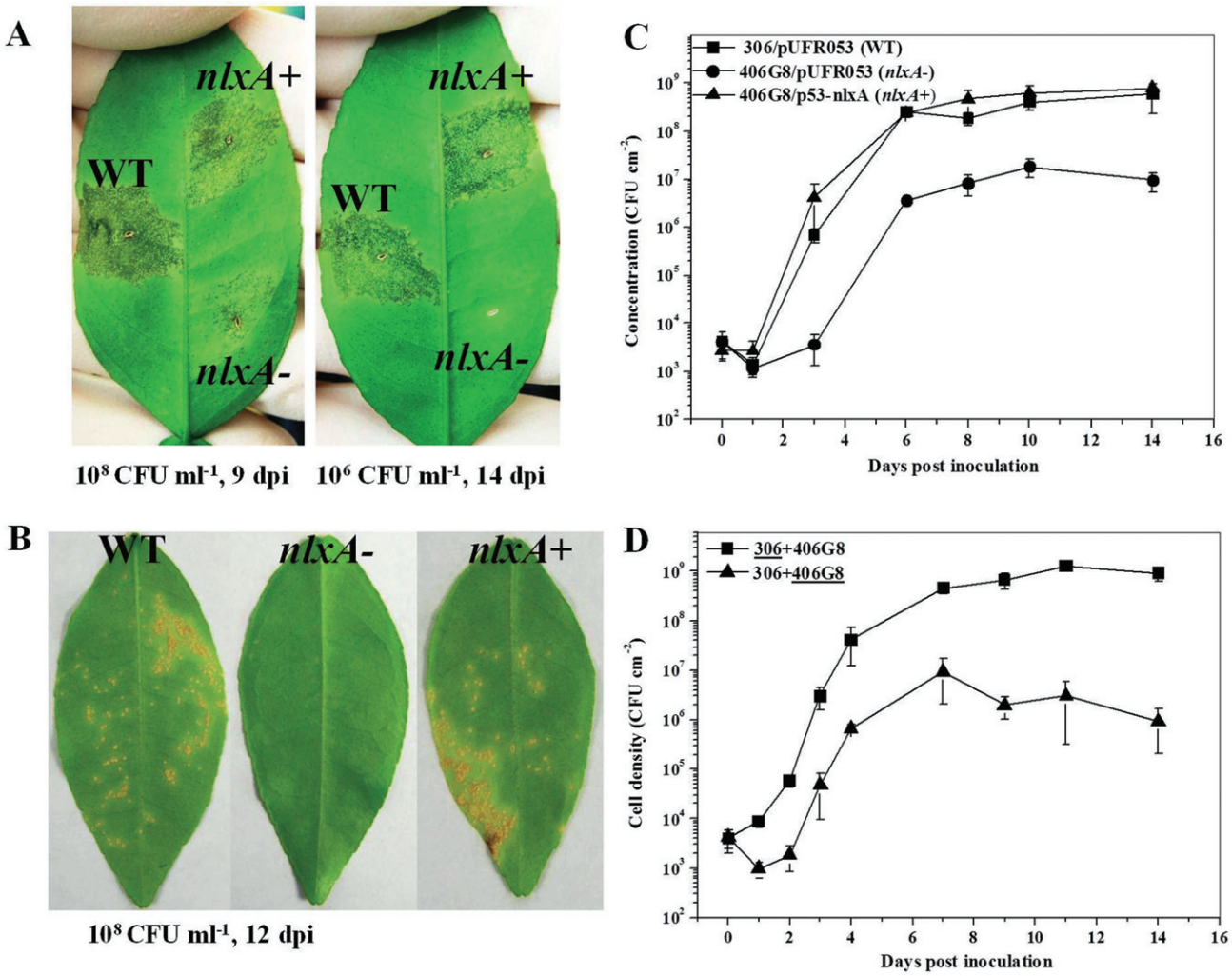

Fig. 6 The *nlxA* gene is important for the virulence and growth *in planta* of *Xanthomonas citri* ssp. *citri*. Grapefruit (*Citrus paradise* Macf. cv. Duncan) was used for virulence and growth assay. For virulence assay, the wild-type, *nlxA* mutant and complementary strain were inoculated into the intercellular spaces (A) or sprayed onto the leaf surfaces (B) of grapefruit. The inoculation concentration of the cell suspensions and the incubation time for photography are shown. The experiments were repeated more than three times with similar results. Only one representative leaf was photographed and presented. For *in planta* growth assay (C), the wild-type, *nlxA* mutant and complementary strain were inoculated into the intercellular spaces of the leaves with a cell suspension of 5×10^5 colony-forming units (CFU)/mL. For co-inoculation growth assay (D), cell suspensions (10^6 CFU/mL) of the wild-type and *nlxA* mutant were mixed equally and inoculated into the leaves following the same method. The growth curves of the wild-type and the *nlxA* mutant in the co-inoculation experiment are indicated by underlining. Bacterial cells from the inoculated leaves were recovered at different time points. The *in planta* growth was measured in quadruplicate and the assays were repeated three times independently. Means and standard errors of four replicates from one representative result are shown. Vertical bars represent the standard errors of the means.

composition, different from the known xanthan composition (Borges and Vendruscolo, 2007). Thus, NlxA might contribute to EPS biosynthesis by transferring rhamnose. In addition, both LPS and EPS are known virulence factors and protect cells from the harsh *in planta* environment. Mutations in LPS and *gum* genes result in reduced virulence of *Xanthomonas* spp. A mutant of *gumB* is defective in gum production, and forms reduced disease symptoms in lemon (*Citrus limon*) (Rigano *et al*., 2007). Mutation in the LPS biosynthesis genes *wxacO* and *rfbC* result in virulence deficiency in grapefruit (Li and Wang, 2011). The reduced pathogenicity (Fig. 6A,B) and bacterial population (Fig. 6C) of the *nlxA* mutant are consistent with its reduced tolerance to H_2O_2 and phenol (Table 2). H_2O_2 is the primary reactive oxygen species (ROS) generated during the oxidative burst by plants in response to microbial pathogen attack in both compatible and incompatible interactions (Bolwell and Wojtaszek, 1997; Bolwell *et al*., 2002). Peak concentrations of H_2O_2 were observed at 24 h and between 8 and 10 days after inoculation in sweet orange 'Hamlin' infected by *X. citri* ssp. *citri* (Kumar *et al*., 2011). Mutations in LPS- and EPS-related genes resulted in reduced tolerance of *Xanthomonas* spp. against H_2O_2 (Kemp *et al*., 2004; Li and Wang, 2011). In addition, phenolic compounds are another large class of second-

ary metabolites produced by plants to defend themselves against pathogens (Freeman and Beattie, 2008). Large amounts of phenolic compounds were found in the peel of 13 commercial citrus species, with a range of 104.2 to 223.2 mg gallic acid equivalent/g of extract power (Ghasemi *et al*., 2009). The deficiency in phenol tolerance of the *nlxA* mutant (Table 2) is consistent with a previous report indicating that phenol and phenolic compounds cause an injury of membrane permeability of *Escherichia coli* cells (Heipieper *et al*., 1991). Thus, our results further support the involvement of NlxA in LPS and EPS production.

In conclusion, a novel gene, designated as *nlxA*, in the LPS gene cluster of *X. citri* ssp. *citri* was characterized. Our results indicate that the *nlxA* gene might function as a rhamnosyltransferase to transfer rhamnose in the LPS O-antigen biosynthesis. In addition, our results indicate that *nlxA* plays an important role in EPS production, biofilm formation, stress resistance, motility on semi-solid plates, virulence and *in planta* growth in the host plant grapefruit.

EXPERIMENTAL PROCEDURES

Bacterial strains, growth conditions, plasmids

The bacterial strains and plasmids used in this study are listed in Table 4. The wild-type strain of *X. citri* ssp. *citri* 306 and its derivatives were cultured at 28 °C in NB (Difco, Detroit, IL, USA) and on NA (Difco) plates. *Escherichia coli* strain was cultured at 37 °C in Luria–Bertani (LB) medium. When required, the growth media were supplemented with ampicillin (50 μg/mL), chloramphenicol (20 μg/mL), gentamycin (5 μg/mL), kanamycin (50 μg/mL) and rifamycin (50 μg/mL).

Preparation of LPS and SDS-PAGE analysis

For the extraction of LPS, *X. citri* ssp. *citri* strains were cultured for 24 h at 28 °C in NB medium with shaking (200 rpm). The cell populations of the wild-type, mutant and complementary strains were tested by measuring OD_{600} values of the bacterial cultures at the time point of sampling. No significant differences were observed in the OD_{600} values among the wild-type, mutant and complementary strains (Fig. S1). Ten millilitres of the cultures were collected and centrifuged for 40 min at 3202 ***g***. LPS from the harvested bacterial cells was extracted with a 50% phenol–water solution and treated with proteinase K following a previous report (Nesper *et al*., 2000). Ten microlitres of LPS sample were separated by SDS-PAGE and visualized using silver staining following the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Standard LPS from *Salmonella enterica* serovar Typhimurium was obtained from Sigma (St. Louis, MO, USA). The experiment was repeated three times with similar results, and the result of only one experiment is presented.

Construction of complementary vectors

To complement the 406G8 and 415H9 mutants, three different DNA fragments were cloned into the shuttle vector pUFR053. The primers used for the amplification of different fragments are listed in Table 4. As shown in Fig. 1, fragment 1 (F1) was amplified using primers nlxAF1/nlxAR1 and only contains the *nlxA* gene. F2 and F3, containing only XAC3597 with different sizes of the putative promoter region, were amplified using primers 3597F1/3597R1 and 3597F2/3597R1, respectively. The PCR products were then cloned into the shuttle vector pUFR053 using the restriction enzyme sites given in Table 4. The resulting plasmids p53-nlxA, p53-3597-1 and p53-3597-2 (Fig. 1), respectively, were transformed into the mutants.

Table 4 Bacterial strains, plasmids and primers for complementation.

Strains or plasmids	Relevant characteristic*	Reference or source
Escherichia coli strain		
EC100D *pir*$^{+}$	F^{-}*mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80d*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139Δ(*ara-leu*)7697 *galU galK*λ^{-}*rpsL nupG pir*+ DHFR	Epicentre
Xanthomonas citri ssp. *citri* strains		
306	Wild-type; Rif^{r}	DPI
406G8	*nlxA*::EZ-Tn5 derivative of strain 306; Rif^{r} Km^{r}	Yan and Wang (2011b)
415H9	*nlxA*::EZ-Tn5 derivative of strain 306; Rif^{r} Km^{r}	Yan and Wang (2011b)
330D3	*wzt*::EZ-Tn5 derivative of strain 306; Rif^{r} Km^{r}	Yan and Wang (2011b)
Plasmids		
pUFR053	Shuttle vector, IncW Mob^{+}*mob*(P) *lacZ*+Par+ Gm^{r},	El Yacoubi *et al*. (2007)
p53-nlxA	A 1362 bp *Eco*RI-*Hin*dIII fragment containing wild-type *nlxA* gene was ligated into pUFR053. Gm^{r},	This study
p53-3597-1	A 1069 bp *Sac*I-*Hin*dIII fragment containing wild-type XAC3597 gene was ligated into pUFR053. Gm^{r},	This study
p53-3597-2	A 1339 bp *Sac*I-*Hin*dIII fragment containing wild-type XAC3597 was ligated into pUFR053. Gm^{r},	This study
Primers		
nlxAF1	5′-TA*GAATTC*ATCGTGAATCCGGAGATCCGTTC (*Eco*RI)	This study
nlxAR1	5′-AT*AAGCTT*AACAACAAGGACTACAGCTGG (*Hin*dIII)	This study
3597F1	5′-TC*GAGCTC*GAATCAAGGCATATGCTCTCG (*Sac*I)	This study
3507F2	5′-TT*GAGCTC*GATCTCGGGCCGTTCGTCCTG (*Sac*I)	This study
3597R1	5′-TTC*AAGCTT*ACGCGAGCCACTTACCAC (*Hin*dIII)	This study

DPI, Division of Plant Industry of the Florida Department of Agriculture and Consumer Services, Gainesville, FL, USA.

*Gm^{r}, Km^{r} and Rif^{r} indicate resistance to gentamycin, kanamycin and rifamycin, respectively; for primers, specific restriction sites of the primers are shown in italic and the names of the restriction enzymes are given.

EPS and capsule assays

EPS assay was performed as described previously (Vojnov *et al*., 1998). Briefly, *X. citri* ssp. *citri* strains were grown for 24 h in NB containing 2% glucose at 28 °C with shaking at 200 rpm. The cell populations of the wild-type, mutant and complementary strains were tested by measuring OD_{600} values of the bacterial cultures at the time point of sampling. No significant differences in the cell populations were observed among the wild-type, mutant and complementary strains. The cells were removed from 10-mL portions of the cultures by centrifugation (3202 ***g*** for 40 min). The EPS in the supernatant was precipitated by centrifugation after the addition of three volumes of 99% ethyl alcohol, and the EPS pellet was weighed after drying. The experiments were repeated three times independently with four replicates each time.

A capsule-staining kit (Eng Scientific Inc., Clifton, NJ, USA) was used for capsule assay as described previously (Guo *et al*., 2010).

Biofilm formation assay

Biofilm formation in glass tubes and on leaf surfaces was performed as described previously (Yan and Wang, 2011a). Briefly, fresh bacterial cells were collected from NA plates and re-inoculated into sterilized NB to obtain a final concentration of 10^8 colony-forming units (CFU)/mL. Rifamycin (50 μg/mL) was added to the bacterial suspensions to avoid potential contamination. For biofilm formation assay in glass tubes, 1 mL of the cell suspension was added to sterilized borosilicate glass tubes. The tubes were kept for 24 h in a humidified chamber at 28 °C without shaking. The cultures were then discarded and the tubes were washed three times using tap water. The biofilm formation on the tubes was visualized by staining with 0.1% crystal violet. For biofilm formation assay on leaf surfaces, 20 μL of the cell suspension were dropped onto the citrus leaf surfaces. The leaves were kept for 10 or 24 h in a humidified chamber at 28 °C without shaking. The biofilm formed on the leaf surfaces was stained with 0.1% crystal violet and rinsed three times using tap water. The stain remaining in cells in glass tubes or on leaf surfaces was dissolved in 95% ethanol and quantified by measuring the optical density at 590 nm using an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA). The average of four replicates was used for quantitative measurement. The biofilm assays were repeated three times with similar results with four replicates each time.

Stress tolerance assay

The wild-type and its derivatives were cultured in NB at 28 °C with shaking at 200 rpm. The early stationary stage (20 hpi) cells were collected and challenged with different toxic compounds. Before challenging by different chemicals, the cell populations of the wild-type, mutant and complementary strains were tested by measuring the living cells in the samples. No significant differences in the cell populations were observed among the wild-type, mutant and complementary strains (Table 2). For H_2O_2, the bacteria were incubated for 40 min with a final concentration of H_2O_2 of 20 mM. For polymyxin B, the bacteria were incubated for 90 min with a final concentration of polymyxin B of 15 μg/mL. For SDS, the bacteria were incubated for 10 min with a final concentration of SDS of 0.1% (w/v). For phenol, the bacteria were incubated for 15 min with a final concentration of phenol of 10 mM. For $CuSO_4$ and $ZnSO_4$, the bacteria were incubated for 8 min with a final concentration of 10 mM for both $CuSO_4$ and $ZnSO_4$. The living cells were counted by serial dilution on NA plates containing the corresponding antibiotics. The experiments were repeated three times independently with four replicates each time.

Motility assays

Swimming and swarming motilities were examined as described previously (Li and Wang, 2011) on NA plates containing 0.3% (w/v) agar (Difco, Franklin Lakes, NJ, USA) and 0.7% (w/v) agar, respectively. The experiment was repeated three times with similar results.

TEM analysis of flagella

The wild-type and its derivatives were cultured overnight on semi-solid NA medium (NB + 0.5% agar) in a glass tube at 28 °C. Cells were resuspended by adding 1 mL of sterilized distilled water to the tube and incubating for 15 min without shaking. The bacterial suspension was loaded onto copper grids. The grids were stained with 1% (v/v) uranyl acetate for 1 min and washed three times with water. Samples were examined using a Philips FEI Morgagni 268 transmission electron microscope (FEI Company, Eindhoven, the Netherlands) operating at 60 kV.

Pathogenicity and *in planta* growth assays

The pathogenicity assay was performed using two different methods as described elsewhere (Yan and Wang, 2011a) in a quarantine glasshouse facility at the Citrus Research and Education Center, Lake Alfred, FL, USA. Briefly, fully expanded, immature leaves of grapefruit (*Citrus paradise* Macf. Cv. 'Duncan') were used. The *X. citri* ssp. *citri* strains were cultured for 2 days on NA plates at 28 °C and were resuspended in sterile tap water. For the pathogenicity assays, a bacterial suspension (10^8 CFU/mL) was sprayed onto the leaf surface, and two different bacterial suspensions (10^8 and 10^6 CFU/mL) were injected into the intercellular spaces of leaves with a needleless syringe. All the tests were repeated three times independently.

For the *in planta* growth assay, fresh bacterial cells were collected from NA plates and resuspended in sterile tap water at a concentration of 5×10^5 CFU/mL and infiltrated into the leaves of cv. Duncan grapefruit with a needleless syringe. For co-inoculation experiments, equal volumes of cell suspensions (10^6 CFU/mL) of wild-type and mutant were mixed and infiltrated into the leaves using the same method. Leaf discs (leaf area, 1 cm^2) from inoculated leaves were excised with a cork borer and ground in 1 mL of sterile tap water. The samples were serially diluted and plated onto NA plates with appropriate antibiotics. The bacterial colonies were counted after incubation at 28 °C for 3 days. The *in planta* growth was measured in quadruplicate and the assays were repeated three times independently.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (QRT-PCR)

Bacterial cells for RNA extraction were cultured in liquid or on plates. For experiments in liquid, the wild-type strain was inoculated into sterilized

NB with a start concentration of 10^7 CFU/mL and incubated for 20 h at 28 °C with shaking at 200 rpm. For experiments on plates, 10 mL of bacterial suspension (10^8 CFU/mL) were spread onto the surface of a semi-solid plate (NB with 0.5% agar) and the excess liquid was removed with Kim wipes. The plates were allowed to dry with the lid off for 10 min on the bench top and then shifted to 28 °C for 24 h. The bacterial cells were harvested by centrifugation (3202 ***g*** for 20 min) and treated with RNA Protect Bacterial Reagent (Qiagen, Valencia, CA, USA). Bacterial total RNA was extracted using an RNeasy Mini Kit (Qiagen). Contaminated genomic DNA was removed by treatment with a TURBO DNA-free kit (Ambion, Austin, TX, USA). The concentration of RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to 50 ng/μL.

The RNA obtained was subjected to one-step QRT-PCR assay with a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR green RT-PCR kit (Qiagen). The gene-specific primers listed in Table 3 were designed to generate products of 100–250 bp based on the genome sequence of 306. The 16S rRNA (forward primer, CGCTTTCGTGCCTCAGTGTCAGTGTTGG; reverse primer, GGCGTAAAGCGTGCGTAGGTGGTGGTT) was used as an endogenous control. The relative fold change of target gene expression was calculated using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Three biological replicates were used for each strain. QRT-PCR was repeated once with another three independent biological replicates.

ACKNOWLEDGEMENTS

This work was supported by the Citrus Research and Development Foundation, Lake Alfred, FL, USA.

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SUPPORTING INFORMATION

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

Fig. S1 Growth curve of the 406G8 mutant and the wild-type strain 306 in Nutrient Broth (NB). Fresh bacterial cells were collected from nutrient agar (NA) plates and resuspended in a flask containing 30 mL sterilized NB with a start concentration of OD600 0.03. The flasks were kept at 28°C with shaking at 200 rpm. Bacterial population (OD600) was monitored at different time points as indicated. This experiment was repeated three times with three replicates each time. Similar results were observed at each time and the result of only one experiment was present. Vertical bars represent the standard errors of the mean.

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