A novel locus involved in extracellular polysaccharide production and virulence of *Xanthomonas campestris* pathovar *campestris*

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Xanthomonas campestris pathovar *campestris* (*Xcc*) is the causal agent of black rot disease in cruciferous plants. The extracellular polysaccharide (EPS) produced by *Xcc* is an important pathogenicity factor and also has a range of industrial uses. In preliminary work a number of transposon-mediated insertion mutants in *Xcc* with defects in EPS production were identified. Here, one of these mutated loci was investigated in detail. Six ORFs within the locus (ORFs *XC3811*–*3816*) were disrupted by plasmid integration. Mutation of *XC3813*, *XC3814* or *XC3815* resulted in significantly reduced EPS production and significantly reduced virulence on the host plant Chinese radish (*Raphanus sativus*). The EPS production and virulence of *XC3813*, *XC3814* and *XC3815* mutants could be restored by intact *XC3813*, *XC3814* and *XC3815* genes, respectively, when provided *in trans*. Although bioinformatic analysis suggested a role for *XC3814* and *XC3815* in lipopolysaccharide biosynthesis, the lipopolysaccharides produced by the mutants were indistinguishable from those of the wild-type, as judged by electrophoretic mobility in SDS-polyacrylamide gels. These results reveal that *XC3813*, *XC3814* and *XC3815* comprise a novel gene cluster involved in EPS production and virulence of *Xcc*.

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INTRODUCTION

The Gram-negative bacterium *Xanthomonas campestris* pathovar *campestris* (hereafter *Xcc*) is the causal agent of black rot disease of cruciferous crops worldwide (Onsando, 1992). The pathogen produces extracellular polysaccharide (EPS), also called xanthan gum, and a series of extracellular enzymes including amylase, endoglucanase, polygalacturonate lyase and protease that collectively contribute to pathogenesis (Dow & Daniels, 1994). EPS plays an important role during bacterial infection. It can enhance the susceptibility of host plants by suppressing defence responses such as callus formation (Yun *et al.*, 2006). EPS may also mask the bacterium to prevent host recognition and to enable colonization of host tissues (Alvarez, 2000). In addition to a role in bacterial virulence, xanthan has a wide variety of applications in agriculture, petroleum production and the food industry as a stabilizing, viscosifying, emulsifying, thickening and suspending agent (Kennedy & Bradshaw, 1984).

The commercial value and role in pathogenesis of xanthan has prompted a number of studies on the genetics and biochemistry of biosynthesis of this EPS in recent decades. Two loci within the *Xcc* genome, the *gum* cluster and a 35.3 kb gene cluster, have been demonstrated to be involved in the biosynthesis of EPS (Capage *et al.*, 1987; Hotte *et al.*, 1990; Vanderslice *et al.*, 1990). The *gum* cluster, which is composed of 12 genes (*gumB* to *gumM*), is responsible for the sequential assembly and polymerization of pentasaccharide repeating units, and also the release of polymers into the growth medium (Ielpi *et al.*, 1993). Certain genes such as *xanA* and *xanB*, which are located within the 35.3 kb gene cluster, are responsible for the biosynthesis of the sugar nucleotide precursors, and are involved in both EPS and lipopolysaccharide (LPS) biosynthesis (Köplin *et al.*, 1992). In addition, the products of *rpf*, *clp* and *pigB* were found to be implicated in either the positive or negative regulation of EPS production (Tang *et al.*, 1990, 1991; De Crècy-Lagard *et al.*, 1990; Poplawsky & Chun, 1998).

The recent rapid development of genomics has brought about a paradigm shift in gene function research. The genomes of two *Xcc* strains, ATCC 33913 (da Silva *et al.*, 2002) and 8004 (Qian *et al.*, 2005), have recently been

Abbreviations: EPS, extracellular polysaccharide; FT-IR, Fourier transform infrared.

sequenced and provide a profile of genetic information with which to explore the biological characteristics of *Xcc*. The functions of about one-third of the ORFs are, however, yet to be assigned, and a large repertoire of genes has not been experimentally defined. One aim of the work in our laboratory is to identify further genes that influence EPS biosynthesis in *Xcc*. In preliminary work we isolated a number of transposon insertion mutants in *Xcc* strain 8004 with defects in EPS synthesis. Here we investigate one of these mutated loci in more detail.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium (Miller, 1972) at 37 °C. *Xcc* strains were grown in NYG medium (per litre: 5 g peptone, 3 g yeast extract and 20 g glycerol; Daniels *et al.*, 1984b) or the

minimal medium MMX (per litre: 2.0 g (NH₄)₂SO₄, 4.0 g K₂HPO₄, 6.0 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 1.0 g citric acid, 5.0 g glucose; Daniels *et al.*, 1984a) at 28 °C. Antibiotics were added at the following concentrations as required: kanamycin (Kan) 25 µg ml⁻¹; rifampicin (Rif) 50 µg ml⁻¹; gentamicin (Gm) 5 µg ml⁻¹; spectinomycin (Spc) 50 µg ml⁻¹; and tetracycline (Tet) 5 µg ml⁻¹ for *Xcc* and 15 µg ml⁻¹ for *E. coli*.

DNA manipulations. The methods described by Sambrook *et al.* (1989) were used for preparation of plasmid and chromosomal DNAs, restriction digestion, DNA ligation, agarose gel electrophoresis and DNA transformation of *E. coli*. Conjugation between *Xcc* and *E. coli* strains was performed as described by Turner *et al.* (1984). The restriction endonucleases, T4 DNA ligase and *Pfu* polymerase were provided by Promega (Shanghai).

Construction of insertional mutants. Mutants of ORFs XC3811, XC3812, XC3813, XC3814, XC3815 and XC3816 were constructed using the suicide plasmid pK18mob (Schafer *et al.*, 1994; Windgassen *et al.*, 2000). A 300–500 bp internal fragment of the target ORF was amplified by PCR using *Xcc* chromosomal DNA as the template and

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
JM109	<i>recA1 endA1 gyrA96 thi supE44 relA1</i> Δ(<i>lac-proAB</i>)/F'[<i>traD36 lacI^q lacZΔM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
DH5α	φ80Δ <i>lacZ</i> M15 <i>recA1 endA1 deoR</i>	Gibco-BRL, Life Technologies
HB101	<i>recA13 rps</i>	Boyer & Roulland-Dussoix (1969)
<i>Xcc</i>		
8004	Wild-type, Rif ^r	Daniels <i>et al.</i> (1984b)
151D09	As 8004, but XC3814::Tn5 <i>gusA5</i> , EPS-deficient, Rif ^r Kan ^r Spc ^r Gm ^r	Authors' lab collection
3811nk	As 8004, but XC3811::pK18mob, Rif ^r Kan ^r	This work
3812nk	As 8004, but XC3812::pK18mob, Rif ^r Kan ^r	This work
3813nk	As 8004, but XC3813::pK18mob, Rif ^r Kan ^r	This work
3814nk	As 8004, but XC3814::pK18mob, Rif ^r Kan ^r	This work
3815nk	As 8004, but XC3815::pK18mob, Rif ^r Kan ^r	This work
3816nk	As 8004, but XC3816::pK18mob, Rif ^r Kan ^r	This work
C3813nk	3813nk harbouring pLATC3813, Rif ^r Kan ^r Tet ^r	This work
C3814nk	3814nk harbouring pLATC3814, Rif ^r Kan ^r Tet ^r	This work
C3815nk	3815nk harbouring pLATC3815, Rif ^r Kan ^r Tet ^r	This work
8004/pL6gumGUS	8004 harbouring pL6gumGUS, Rif ^r Kan ^r	This work
3813nk/pL6gumGUS	3813nk harbouring pL6gumGUS, Rif ^r Kan ^r Tet ^r	This work
3814nk/pL6gumGUS	3814nk harbouring pL6gumGUS, Rif ^r Kan ^r Tet ^r	This work
3815nk/pL6gumGUS	3815nk harbouring pL6gumGUS, Rif ^r Kan ^r Tet ^r	This work
Plasmids		
pLAFR3	Broad host range cloning vector, Tet ^r	Staskawicz <i>et al.</i> (1987)
pLAFR6	A promoterless derivative of pLAFR3, Tet ^r	Huynh <i>et al.</i> (1989)
pRK2073	Helper plasmid, Tra ⁺ , Mob ⁺ , ColE1, Spc ^r	Leong <i>et al.</i> (1982)
pK18mob	pUC18 derivative, <i>lacZα</i> Kan ^r , <i>mob</i> site; suicide plasmid in <i>Xcc</i>	Schafer <i>et al.</i> (1994)
pK3813	pK18mob containing a 459 bp internal fragment of XC3813, Kan ^r	This work
pK3814	pK18mob containing a 390 bp internal fragment of XC3814, Kan ^r	This work
pK3815	pK18mob containing a 357 bp internal fragment of XC3815, Kan ^r	This work
pLATC3813	pLAFR3 containing a 1301 bp fragment including the XC3813 gene, Tet ^r	This work
pLATC3814	pLAFR3 containing a 1096 bp fragment including the XC3814 gene, Tet ^r	This work
pLATC3815	pLAFR3 containing a 1478 bp fragment including the XC3815 gene, Tet ^r	This work
pL6gumGUS	pLAFR6 containing the promoter region of the <i>gum</i> operon fused to the coding region for <i>gusA</i>	Vojnov <i>et al.</i> (2001)

the corresponding oligonucleotides as primers (Table 2). Primers were designed based on the ORF sequences in the genome of *Xcc* strain 8004 (accession number NC_007086). After being confirmed by sequencing, the amplified DNA fragments were cloned into pK18mob to create the recombinant plasmids. Primers were modified to give *Bam*HI- or *Hind*III-compatible ends to ensure that the internal fragment was cloned in the same orientation as the *lacZ* promoter in pK18mob. The recombinant plasmid was introduced from *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) into *Xcc* strain 8004 by triparental conjugation using pRK2073 (Leong *et al.*, 1982) as the helper plasmid. Mutants were selected on NYG agar plates containing rifampicin and kanamycin. Transconjugants with a mutation in the target ORF were confirmed by PCR. Confirmation PCR was performed using the total DNA of the transconjugants as the template, oligonucleotide P18conF, which is located in pK18mob, and the corresponding oligonucleotide, which is located downstream of the target ORF, as primers. The total DNA of *Xcc* strain 8004 was used as the negative control. The expected PCR products were further confirmed by sequencing analysis.

Complementation of the insertional mutants. For mutant complementation, a 1301 bp DNA fragment containing the *XC3813*

coding region and extending from 226 bp upstream of the 5' end to 16 bp downstream of the 3' end of the ORF, a 1096 bp DNA fragment harbouring the *XC3814* coding region and extending from 259 bp upstream of the 5' end to 12 bp downstream of the 3' end of the ORF, and a 1478 bp DNA fragment including the *XC3815* coding region and extending from 111 bp upstream of the 5' end to 77 bp downstream of the 3' end of the ORF were amplified using the primers C3813F/R, C3814F/R and C3815F/R (Table 2), respectively, and the amplified DNA fragments were cloned into plasmid pLAFR3 (Staskawicz *et al.*, 1987) or pLAFR6 (Huynh *et al.*, 1989) to generate recombinant plasmids. The recombinant plasmids were transferred into the corresponding mutant by triparental conjugation. The transconjugants carrying the recombinant plasmid were screened on NYG agar plates with rifampicin, kanamycin and tetracycline. The resulting strains were named, respectively, C3813nk, C3814nk and C3815nk (Table 1).

Extracellular polysaccharide assay. To estimate EPS production, strains were cultured in 100 ml NYG liquid medium containing 2% (w/v) glucose at 28 °C with shaking at 200 r.p.m. for 3 days. EPS was precipitated from the culture supernatant with ethanol, dried, and weighed as described by Tang *et al.* (1991). EPS structure analysis

Table 2. Primers

The underlined sequences indicate the restriction sites for *Bam*HI and *Hind*III.

Primer	Nucleotide sequence (5'→3')	Amplified segment or primer location
3811F	ACAGTTGGATCCTGCAGGCCTGGCAATACT	246 bp DNA fragment, spans nucleotides 119 to 364 of the <i>XC3811</i> ORF sequence
3811R	ACAGTTAAGCTT TATGCACCACCAGCGTGG	
C3811R	ATCAATCGCGCTCACAGG	Located downstream of <i>XC3811</i>
3812F	ACAGTTGGATCCGGTGCTGTACCAGATGAT	405 bp DNA fragment, spans nucleotides 591 to 995 of the <i>XC3812</i> ORF sequence
3812R	ACAGTTAAGCTTATCGGCGCCAGTTCCTTC	
C3812R	ATCACCAGCGGAGTTTGC	Located downstream of <i>XC3812</i>
3813F	ACAGTTAAGCTTCATGACCTCTTCAGGCTC	369 bp DNA fragment, spans nucleotides 310 to 678 of the <i>XC3813</i> ORF sequence
3813R	ACAGTTGGATCCTTCGATCTGTATTGCACG	
C3813F	ACAGTTAAGCTTATGGACGCGTTGGTGATT	1301 bp DNA fragment, spans from 226 bp upstream to 16 bp downstream of the <i>XC3813</i> ORF sequence
C3813R	ACAGTTGGATCCAAAATGCCTTCTGGCTAC	
3814F	ACAGTTGGATCCTAGCCAGAAGGCATTTTG	390 bp DNA fragment, spans nucleotides 269 to 658 of the <i>XC3814</i> ORF sequence
3814R	ACAGTTAAGCTTTAGTCGTA CT CGGCCATC	
C3814F	ACAGTTGGATCCTGAAGCCGTGAAACAGCT	1096 bp DNA fragment, spans from 259 bp upstream to 12 bp downstream of the <i>XC3814</i> ORF sequence
C3814R	ACAGTTAAGCTTATCGCCATTGGCTCAGTC	
3815F	ACAGTTAAGCTTAGACCAACACACCCAGGG	357 bp DNA fragment, spans nucleotides 293 to 649 of the <i>XC3815</i> ORF sequence
3815R	ACAGTTGGATCCTACGCGATGTGGA AAAACC	
C3815F	ACAGTTAAGCTTTATGTGCGCCAGAAGACC	1478 bp DNA fragment, spans from 111 bp upstream to 77 bp downstream of the <i>XC3813</i> ORF sequence
C3815R	ACAGTTGGATCCTGATGCGGTCAATATGGT	
3816F	ACAGTTAAGCTTAAAGGCCACGGTCAACAG	373 bp DNA fragment, spans nucleotides 678 to 1050 of the <i>XC3816</i> ORF sequence
3816R	ACAGTTGGATCCCTACCTCAACGACATCCT	
C3816	TCACTCGGTGTCCAGGTT	Located downstream of <i>XC3816</i>
P18conF	GCCGATTCATTAATGCAGCTGGCAC	Located in pK18mob

Fig. 1. Genetic and physical map of the novel gene cluster. The positions and orientations of the three genes are shown; arrows indicate the direction of transcription. The lower element shows the PCR fragments used for mutation and complementation analysis. A, C and E are internal fragments of XC3813, XC3814 and XC3815 respectively. These fragments were cloned into the *Bam*HI/*Hind*III site within suicide plasmid pK18mob, respectively, and the resulting recombinant plasmids were used to mutagenize the corresponding ORF. B, D and F are DNA fragments that contained XC3813, XC3814 and XC3815 respectively. These fragments were cloned into the *Bam*HI/*Hind*III site within pLAFR3 or pLAFR6 for complementation of the corresponding mutants.

was performed with Fourier transform infrared (FT-IR) spectra. FT-IR spectra were recorded on a Nicolet 5DX spectrometer. The dry sample powder was mixed with KBr and pressed into pellets under reduced pressure. The FT-IR spectra were obtained by scanning between 4000 and 450 cm^{-1} .

Lipopolysaccharide analysis. LPS was prepared using an LPS extraction kit (iNtRON Biotechnology). LPS preparations were analysed using Tricine-SDS-PAGE (Lesse *et al.*, 1990) and visualized by silver staining (Kittelberger & Hilbink, 1993).

β -Glucuronidase (GUS) activity assay. *Xcc* strains were cultured overnight and diluted to an OD₆₀₀ of 0.5, and 1.0 ml of each was inoculated into 200 ml NYG medium held in 500 ml flasks. GUS activities were determined at 12 h intervals until 60 h by measurement of the A₄₁₅ using *p*-nitrophenyl β -D-glucuronide as the substrate, as described by Henderson *et al.* (1985).

Virulence assay. The virulence of *Xcc* to Chinese radish (*Raphanus sativus*) was tested by the leaf-clipping method (Dow *et al.*, 2003). Seedlings with four fully expanded leaves were used for inoculation. Bacteria grown overnight in NYG liquid medium were washed and resuspended in water to an OD₆₀₀ of 0.1. Two or three fully expanded leaves per plant were cut with scissors dipped in the bacterial suspensions. Sixty leaves were inoculated in each independent experiment. Each treatment was repeated three times. Lesion length was measured 10 days after inoculation, and data were analysed by *t*-test.

The growth of bacteria in radish leaf tissue was measured by homogenizing a group of leaves (five leaves for each sample) in 9 ml sterile water. Diluted homogenates were plated on NYG agar plates supplemented with rifampicin (for wild-type) or rifampicin plus kanamycin (for mutants). Bacterial c.f.u. were counted after incubation at 28 °C for 3 days.

RESULTS

Mutation analysis reveals three ORFs involved in EPS production

Our preliminary work identified a number of EPS-deficient mutants by Tn5gusA5 transposon insertional mutagenesis of the *Xcc* wild-type strain 8004. One of the mutants, 151D09, was due to disruption of the ORF XC3814 (Table 1). In the reported genome sequence of strain 8004, the protein encoded by XC3814 (YP_244874) was assigned as a glycosyl-transferase (Qian *et al.*, 2005). The upstream ORFs XC3811, XC3812 and XC3813, and the downstream ORFs XC3815 and XC3816 are poorly characterized genes, and the proteins encoded by these ORFs (YP_244871, YP_244872, YP_244873, YP_244875, YP_244876) were annotated as 'conserved hypothetical proteins' (Qian *et al.*, 2005). These six ORFs are located in the genome sequence between position 4505214 and position 4512185 (Fig. 1).

To assess the function of these genes, mutants carrying insertional disruptions within the ORFs XC3811, XC3812, XC3813, XC3814, XC3815 and XC3816 were constructed and named 3811nk, 3812nk, 3813nk, 3814nk, 3815nk and 3816nk, respectively (Table 1; see Methods). To test the EPS production, these insertional mutants were grown on NYG agar plates supplemented with 2% glucose for 5 days. Mutants 3813nk, 3814nk and 3815nk displayed smaller colonies than the wild-type strain (Fig. 2a), while the other mutants formed normal wild-type colonies. The growth rates of the mutants did not differ from that of the wild-type in NYG liquid medium (Fig. 2b) or in MMX minimal

Fig. 2. Growth of *Xcc* strains in glucose-containing medium. (a) Strains grown on NYG agar plates supplemented with 2% glucose for 5 days. Mutants of *XC3813*, *XC3814* and *XC3815* displayed smaller colonies than the wild-type strain 8004, but the complemented strains formed colonies of normal size. (b) Growth curves of the mutants. Strains were inoculated into 100 ml NYG liquid medium containing 2% glucose. Samples were taken in triplicate at intervals of 4 h, diluted, and plated on NYG agar. Bacterial c.f.u. were counted after incubation at 28 °C for 3 days. The mutants displayed normal growth rates.

medium (data not shown). These findings suggested that mutants 3813nk, 3814nk and 3815nk might produce less EPS than the wild-type. To quantitatively estimate the EPS production of the mutants, strains were grown in NYG liquid medium containing 2% glucose for 3 days and EPS was extracted from the cultures. The results showed that the mutants 3813nk, 3814nk and 3815nk produced significantly less EPS than the wild-type strain 8004 (Table 3). For complementation, plasmids pLATC3813, pLATC3814 and pLATC3815 (Table 1) were constructed by cloning a 1301 bp fragment, a 1096 bp fragment and a 1478 bp fragment, each of which included the corresponding ORF (Fig. 1), into the vector pLAFR3. These plasmids were introduced into mutants 3813nk, 3814nk and 3815nk to form the complemented mutants C3813nk, C3814nk and C3815nk (Table 1; see Methods for details). The EPS yield of the complemented mutants showed no significant difference

Table 3. EPS production by *Xcc* strains

Data are the mean $\pm$ SD of triplicate measurements; different letters in each data column indicate significant differences ($P=0.05$; t -test). Data presented are from a representative experiment and similar results were obtained in two other independent experiments.

Strain	EPS yield (g l ⁻¹)
8004 (wild-type)	9.75 $\pm$ 0.25 ^a
3813nk (<i>XC3813</i> mutant)	4.03 $\pm$ 0.35 ^b
C3813nk (complemented mutant)	9.65 $\pm$ 0.58 ^a
3814nk (<i>XC3814</i> mutant)	4.84 $\pm$ 0.33 ^b
C3814nk (complemented mutant)	9.53 $\pm$ 0.60 ^a
3815nk (<i>XC3815</i> mutant)	3.12 $\pm$ 0.23 ^b
C3815nk (complemented mutant)	9.39 $\pm$ 0.93 ^a
3811nk (<i>XC3811</i> mutant)	9.48 $\pm$ 0.47 ^a
3812nk (<i>XC3812</i> mutant)	9.55 $\pm$ 0.22 ^a
3816nk (<i>XC3816</i> mutant)	9.81 $\pm$ 0.37 ^a

from that of the wild-type strain (Table 3, Fig. 2a), indicating that the mutations in *XC3813*, *XC3814* and *XC3815* could be complemented by the corresponding ORF *in trans*. The promoterless plasmid pLAFR6 harbouring the 1301 bp fragment, the 1096 bp fragment or the 1478 bp fragment could also complement the corresponding mutant (data not shown), suggesting that *XC3813*, *XC3814* and *XC3815* have their own promoter.

The EPS of *Xcc* consists of pentasaccharide repeating units with the structure mannose-1,4- β -glucuronic acid-1,2- β -mannose-1,3- α -cellobiose (Jansson *et al.*, 1975). The assembly and polymerization of the repeated units have been attributed to the protein products of the *gum* genes (Ielpi *et al.*, 1993). To estimate the effect of inactivation of *XC3813*, *XC3814* or *XC3815* on the EPS chemical structure, EPS from the mutant strains and the wild-type strain was analysed using FT-IR spectra. These spectra revealed that the EPS patterns of the mutants were similar to that of the wild-type (data not shown). The characteristic vibration peaks appeared in all the EPS samples and no obvious shift of these characteristic peaks was observed. This indicates that mutation in any of the three ORFs does not result in change in the pentasaccharide repeating units.

Characterization of *XC3813*, *XC3814* and *XC3815*

Domain analysis with the SMART program (<http://smart.embl-heidelberg.de/>) showed that the 352 amino acid protein *XC3813* contains a glycerophosphotransferase domain (PF04464, 4.7e-08) (residues 148–317). It has been reported that in *Staphylococcus epidermidis*, glycerophosphotransferase, encoded by the *tagF* gene, is responsible for polymerization of the main chain of teichoic acid (Fitzgerald & Foster, 2000). The deduced *XC3814* protein comprises 274 amino acids and contains a domain characteristic of glycosyltransferase family 2 (PF00535,

2.80e–20) at its N-terminus (residues 14–177). Glycosyl-transferase family 2 is a diverse family that transfers the sugar from UDP-glucose, UDP-*N*-acetylgalactosamine, GDP-mannose or CDP-abequose to a range of substrates including cellulose, dolichol phosphate and teichoic acids (Campbell *et al.*, 1997). The deduced XC3815 protein, of 429 amino acids, harbours a Wzy conserved domain (PF04932, 1.7e–19), residues 270–341. The protein Wzy, an O-antigen polymerase encoded by the gene *wzy* (Reeves *et al.*, 1996), is responsible for polymerization of the repeated O-antigen unit of LPS (Bengoechea *et al.*, 2002). One of the features of the Wzy protein is that it has multiple putative membrane-spanning domains (Collins & Hackett, 1991; De Kievit *et al.*, 1995; Bengoechea *et al.*, 2002). The deduced protein XC3815 is predicted to have 11 transmembrane segments, suggesting an integral membrane location.

These bioinformatic analyses suggested that XC3814 and XC3815 might affect LPS synthesis. To address this issue, LPS from the mutant and wild-type strains was prepared and analysed using SDS-PAGE. Under these conditions, the banding patterns of LPS from the mutant strains 3814nk and 3815nk were indistinguishable from the wild-type strain 8004 (data not shown). This result indicated that the XC3814 and XC3815 may not be involved in LPS synthesis.

To determine whether the inactivation of XC3813, XC3814 or XC3815 reduces the expression level of the *gum* genes, the reporter plasmid pL6gumGUS, which carries the promoter region of the *gum* operon fused to the coding region for *gusA* (Vojnov *et al.*, 2001), was transferred to the wild-type and mutant strains by triparental mating with the *E. coli* DH5 α donor strain and helper strain HB101/pRK2073. In the wild-type background the *GusA* level, determined enzymically, mirrors *gum* gene expression and is correlated closely with the level of EPS production (Vojnov *et al.*, 2001). The transconjugant strains 8004/pL6gumGUS, 3813nk/pL6gumGUS, 3814nk/pL6gumGUS and 3815nk/pL6gumGUS (Table 1) were cultured in NYG liquid medium containing 2% (w/v) glucose with shaking at 200 r.p.m. GUS activities were assayed as described by Henderson *et al.* (1985) at 12 h intervals. The results showed that the GUS activities in the mutant backgrounds

were not significantly different ($P=0.05$; *t*-test) from each other and from the wild-type background at each of the time points tested (Table 4). This indicates that inactivation of any of the ORFs XC3813, XC3814 or XC3815 has no effect on the transcription level of *gum* genes.

XC3813, XC3814 and XC3815 are required for full virulence

The virulence assay for the *Xcc* strains was performed on Chinese radish by the leaf-clipping method (Dow *et al.*, 2003). *Xcc* is a vascular pathogen and the leaf-clipping inoculation introduces the bacteria directly into the vascular system. Ten days after inoculation XC3813 and XC3815 mutant strains showed markedly reduced virulence compared with the wild-type strain: the mean lesion lengths caused by 3813nk and 3815nk were significantly shorter than that caused by the wild-type strain 8004 at $P=0.01$ (*t*-test). Furthermore the mutant strain 3814nk almost completely lost virulence and hardly induced any symptoms on the inoculated leaves (Fig. 3a,b). In contrast, lesion lengths caused by the complemented strains and the wild-type strain were not significantly different at $P=0.05$ (Fig. 3b), indicating that the virulence of the mutants 3813nk, 3814nk and 3815nk could be fully restored by the recombinant plasmids pLATC3813, pLATC3814 and pLATC3815, respectively.

To determine the role of XC3813, XC3814 and XC3815 in growth of *Xcc* in the host, numbers of bacterial cells of the corresponding mutants in infected radish leaves were calculated. For mutants 3813nk and 3815nk, the number of bacterial cells recovered from the infected leaves was approximately 10-fold lower than that of the wild-type strain 5 days post-inoculation and onward (Fig. 3c). For mutant 3814nk, the number of bacterial cells was approximately 100-fold lower than that of the wild-type strain 5 days post-inoculation and onward (Fig. 3c). The growth of 3813nk, 3814nk and 3815nk *in planta* could be completely restored by pLATC3813, pLATC3814 and pLATC3815, respectively. These results reveal that XC3813, XC3814 and XC3815 affect growth of *Xcc* as well as symptom production *in planta*.

Table 4. GUS activities of *Xcc* strains

Strains were grown in NYG liquid medium with 2% (w/v) glucose. β -Glucuronidase (GUS) activities were measured at 12 h intervals; values are the mean $\pm$ SD of triplicate measurements. Data presented are from a representative experiment and similar results were obtained in two other independent experiments.

Strain	GUS activity (mg <i>p</i> -nitrophenol product min ^{–1} per OD ₆₀₀ unit)				
	12 h	24 h	36 h	48 h	60 h
8004/pL6gumGUS	0.502 $\pm$ 0.089	0.835 $\pm$ 0.066	1.491 $\pm$ 0.072	1.852 $\pm$ 0.095	2.182 $\pm$ 0.062
3813nk/pL6gumGUS	0.452 $\pm$ 0.085	0.829 $\pm$ 0.079	1.537 $\pm$ 0.043	1.830 $\pm$ 0.076	2.232 $\pm$ 0.059
3814nk/pL6gumGUS	0.560 $\pm$ 0.055	0.933 $\pm$ 0.075	1.423 $\pm$ 0.047	1.923 $\pm$ 0.096	2.250 $\pm$ 0.063
3815nk/pL6gumGUS	0.553 $\pm$ 0.059	0.909 $\pm$ 0.124	1.631 $\pm$ 0.088	1.900 $\pm$ 0.099	2.191 $\pm$ 0.142

Fig. 3. XC3813, XC3814 and XC3815 are required for full virulence of *Xcc* to Chinese radish. (a) Symptom production on leaves 10 days after inoculation by clipping with strains and water (mock inoculation). (b) Mean lesion lengths caused by mutants and the wild-type strain. 3814nk has almost completely lost virulence; 3813nk and 3815nk have markedly lost virulence; as analysed by *t*-test, the mean lesion lengths caused by 3813nk and 3815nk were significantly shorter ($P=0.01$) than those caused by the wild-type. Values are the mean $\pm$ SD from 15 measurements. (c) Bacterial populations of strains in host plant leaves. Inoculated leaves for each strain were taken daily and homogenized in sterile water. The homogenates were diluted and then plated on agar plates. Bacterial c.f.u. were counted after incubation for 3 days. Data are the mean $\pm$ SD from three repeats.

DISCUSSION

In this work, we have described a novel cluster, comprising three ORFs, *XC3813*, *XC3814* and *XC3815*, involved in the production of EPS and virulence of *Xcc*. An SSDB Gene Cluster Search (<http://www.genome.jp>) revealed that homologues of *XC3813*, *XC3814* and *XC3815* in the same gene order occur in the genomes of related phytopathogenic bacteria: *XOO0589-0591* in *Xanthomonas oryzae* KACC10331, *XCV3920-3922* in *Xanthomonas campestris* pathovar *vesicatoria*, and *XAC3795-3797* in *Xanthomonas*

axonopodis pathovar *citri*. Homologous genes (*XF1993-1995*) also occur in *Xylella fastidiosa* 9a5c, although in this organism the orientation of the three genes differs from that of *Xcc*. The biochemical characteristics and physiological roles of these homologous proteins have not been reported.

The biosynthesis of EPS in *Xcc* occurs in at least two stages. Firstly, the repeating pentasaccharide unit is sequentially assembled while linked to a polyprenol through a diphosphate bridge. Secondly, the repeating units are polymerized and the polymer is exported from the cell. This second stage

is still poorly understood. The protein products of the *gum* gene cluster direct assembly of pentasaccharide repeating units, polymerization and the export of EPS (Vanderslice *et al.*, 1990; Ielpi *et al.*, 1993). Mutations in certain *gum* genes that affect EPS structure also reduce the level of EPS production (Katzen *et al.*, 1998). In this work, we have shown that mutation of *XC3813*, *XC3814* or *XC3815* causes reduced EPS production but neither the structure of the pentasaccharide repeating unit nor the transcription of the *gum* genes is altered. How then do *XC3813*, *XC3814* and *XC3815* influence EPS production?

Bioinformatic analysis suggested that *XC3814* and *XC3815* have a role in LPS synthesis. *XC3814* is annotated as encoding an LPS core biosynthesis glycosyltransferase by reason of the shared limited similarity with the protein KdtX (39 % identity and 54 % similarity) from *Serratia marcescens* (accession no. Q54435; Guasch *et al.*, 1996), the WaaE protein (39 % identity and 51 % similarity) from *Klebsiella pneumoniae* (accession no. Q9XC90; Regue *et al.*, 2001), and the LpsC protein (33 % identity and 48 % similarity) from *Sinorhizobium meliloti* (accession no. Q9R9M9; Lagares *et al.*, 2001). KdtX, WaaE and LpsC have been predicted to be LPS core biosynthesis glycosyltransferases. Consistent with this assertion, mutation of *kdtX*, *waaE* and *lpsC* led to changes in LPS pattern on SDS-polyacrylamide gels such that the core LPS from the mutant strains migrated faster than that of the wild-type strain (Guasch *et al.*, 1996; Regue *et al.*, 2001; Lagares *et al.*, 2001). *XC3815* was predicted bioinformatically to be a cytoplasmic membrane protein containing the Wzy domain. Wzy has been shown to be responsible for polymerization of the repeated O-antigen unit of LPS in some bacteria, where inactivation of the *wzy* gene led to the loss of the O-antigen ladder in the LPS electrophoresis pattern (Collins & Hackett, 1991; Bengoechea *et al.*, 2002; Grozdanov *et al.*, 2002; Tao *et al.*, 2004). In this work, inactivation of *XC3814* or *XC3815* did not alter the LPS pattern, suggesting that *XC3814* and *XC3815* are not involved in LPS synthesis. Bioinformatic analysis suggested that *XC3813* has a glycerol-phosphate transfer domain. However, structural analyses of LPS have not indicated the presence of glycerol-phosphate residues (Corsaro *et al.*, 2001; Raetz & Whitfield, 2002; Molinaro *et al.*, 2003), and our analysis of LPS failed to detect an effect of mutation of *XC3813* on the electrophoretic mobility of LPS bands.

Although *XC3813*, *3814* and *3815* have no apparent role in LPS biosynthesis, it is possible that these proteins direct the synthesis of a different (as yet unidentified) surface polysaccharide, and that the loss of this polymer adversely affects EPS export or polymerization. A second possibility is that *XC3813*, *3814* and *3815* may act as accessory elements to Gum proteins involved in EPS export or polymerization. Further work is required to understand the molecular basis of the influence of this novel gene cluster on EPS production and this will be the subject of future investigations.

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