A high-molecular-weight outer membrane protein of *Xanthomonas oryzae* pv. *oryzae* exhibits similarity to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence

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Summary

Transposon insertions in a novel 3.798 kb open reading frame (ORF) of the rice pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) cause virulence deficiency and altered colony/lawn morphology. This ORF encodes a protein, XadA, of 1265 amino acids that exhibits significant similarity to non-fimbrial adhesins of animal pathogenic bacteria such as *Yersinia* YadA and *Moraxella* UspA1. An interesting feature is that the YadA similarity region is repeated six times within the XadA sequence and encompasses almost the entire length of the protein. Anti-XadA antibodies identified a 110 kDa outer membrane protein that was sensitive to protease treatment of whole cells. XadA expression is induced in minimal medium. Homology modelling suggests that XadA adopts a β-helix conformation-like pertactin, a non-fimbrial adhesin of *Bordetella pertussis*. This work is the first characterization of a non-fimbrial adhesin-like molecule in a plant pathogenic bacterium. It extends our knowledge about the repertoire of homologous virulence factors that are deployed by animal and plant pathogenic bacteria to include functions potentially involved in adhesion.

Introduction

Attachment to host tissues is essential for successful infection by microbial pathogens. Certain microbial surface proteins play important roles during this process and are commonly known as adhesins. Based on their size and structure, Gram-negative bacterial adhesins have been classified as two types: fimbrial and non-fimbrial (Hultgren *et al*., 1993). Recently, there has been a lot of interest in non-fimbrial adhesins because of their wide occurrence, crucial role in host cell attachment and interesting mode of transportation to the cell surface. Examples of non-fimbrial adhesins are the Hia (*Haemophilus influenzae* adhesin) and Hsf (*Haemophilus* surface fibrils) of *Haemophilus influenzae*, YadA (*Yersinia* adhesin A) of *Yersinia* sp., pertactin of *Bordetella pertussis* and the UspA1 (ubiquitous surface protein) of *Moraxella catarrhalis* (Skurnik *et al*., 1994; Everest *et al*., 1996; St Geme and Cutter, 1996; Cope *et al*., 1999). Several non-fimbrial adhesins are autotransporters, which are translocated across the bacterial inner membrane by the Sec machinery but cross the outer membrane by self-transportation (Henderson and Nataro, 2001). Genome sequencing projects have revealed the wide occurrence of non-fimbrial adhesins in symbiotic, animal as well as plant pathogenic and free-living bacteria (Hoiczyk *et al*., 2000). However, molecular characterization has only been reported in animal pathogenic bacteria. The genome sequence of *Xyllela fastidiosa*, a causal agent of citrus variegated chlorosis, has revealed the genes for three non-fimbrial adhesin-like molecules in this bacterium (Simpson *et al*., 2000). Dispersal of this pathogen occurs through an insect vector, and it has been suggested that these molecules might be involved in attachment to insect cells (Lambais *et al*., 2000). In the recently sequenced genomes of the plant pathogens, *Ralstonia solanacearum* (affects a wide range of plants), *Xanthomonas campestris* pv. *campestris* (*Xcc*; pathogen of plants such as mustard and *Arabidopsis*) and *Xanthomonas axonopodis* pv. *citri* (*Xac*; pathogen of citrus plants), proteins that are similar to non-fimbrial adhesin-like molecules have been identified, but their role in virulence has yet to be defined (Salanoubat *et al*., 2002; da Silva *et al*., 2002).

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a Gram-negative bacterium that causes bacterial leaf blight, a serious disease in rice. *Xoo* gains entry into rice leaves either through wounds or natural openings called hydathodes that are concentrated at the edges of the leaves. The

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hydathodes lead into the xylem vessels inside which *Xoo* multiplies. Some previously described genes that are required for virulence of *Xoo* are: *gumG* and *gumM* involved in extracellular polysaccharide (EPS) biosynthesis (Dharmapuri and Sonti, 1999; Rajeshwari and Sonti, 2000); *rpfC*, which is involved in the regulation of EPS biosynthesis (Tang *et al*., 1996); *rpfF* involved in iron metabolism (Chatterjee and Sonti, 2002); and *hrp* and *avr*, which encode components of a type III protein secretion system, its effectors and regulators (Kamdar *et al*., 1993; Bai *et al*., 2000; Yang *et al*., 2000; Zhu *et al*., 2000). Besides these, certain amino acid auxotrophs of *Xoo* have also been shown to be virulence deficient (Yamasaki *et al*., 1964; Goel *et al*., 2001). A virulence-deficient mutant of *Xoo* that is unable to secrete xylanase was reported by Ray *et al*. (2000). A genomic clone, pSR1, restored both xylanase and virulence proficiency to this mutant. Insertional mutagenesis of a 12 kb region of pSR1 revealed that the genes involved were homologues of *xpsF* and *xpsD*, which encode components of the type II protein secretion system (TTPS) in *Xcc*. In this study, we show that pSR1 also contains a novel open reading frame (ORF) of 3.798 kb that encodes XadA (*Xanthomonas* adhesin-like protein A), which shares similarity with several non-fimbrial adhesins of animal pathogenic bacteria. XadA is shown to be an outer membrane protein that is differentially expressed and is required for optimal virulence and normal colony/lawn morphology of *Xoo*. Homology modelling suggests that XadA might adopt a β-helix configuration. This is the first demonstration that a non-fimbrial adhesin-like molecule promotes the virulence of a plant pathogenic bacterium.

Results

XadA sequence features and similarity with non-fimbrial adhesins of animal pathogenic bacteria

A 9.0 kb *Eco*RI fragment from pSR1 was subcloned into the cosmid vector pUFR034 to generate pSR3 (Table 1). A 7609 bp region of pSR3 was sequenced, and four potential ORFs (Fig. 1) were identified in this region. ORF1 is 3798 bp long, extending from 1196 bp to 4993 bp. Three putative promoters were identified upstream of ORF1 (between 907 and 1195 bp), and the most probable one (based on similarity to the consensus promoter sequence) is from 907 to 934 bp. Upstream to the probable promoter, a stretch of 14 pyrimidine bases is present from 845 to 858 bp. There are two inverted repeat regions in this ORF: one is from 1770 to 1791 bp (ACGTGGCGCAGCTGCGCCACGT) and the other is from 2121 to 2139 bp (CCGGCGCTACCAGCGCCGG). The significance, if any, of the stretch of pyrimidine bases and the inverted repeat sequences remains to be established.

Table 1. Plasmid and strain list.

Strain/plasmid	Relevant characteristics	Reference/source
Xoo strains		
BXO1	Laboratory wild type; Indian isolate	Laboratory collection
BXO43	*rif-2*; Rif^r derivative of BXO1	Laboratory collection
BXO836	*xadA1*::Tn*5gusA40 rif-2*	This work
BXO837	*xadA2*::Tn*5gusA40 rif-2*	This work
BXO838	*xadA4*::Tn*5*Tet *rif-2*	This work
BXO839	*xadA5*::Tn*5*Tet *rif-2*	This work
BXO840	*xadA6*::Tn*5*Tet *rif-2*	This work
BXO841	*xadA3*::Tn*10 rif-2*	This work
BXO845	*zxx-110*::Tn*10 rif-2*	This work
BXO846	*zxx-110*::Tn*10 rif-2*; XadA$^+$ derivative of BXO836	This work
BXO847	*zxx-110*::Tn*10 rif-2*; XadA$^+$ derivative of BXO837	This work
BXO848	*xadA1*::Tn*5gusA40 zxx-110*::Tn*10 rif-2*	This work
BXO849	*xadA2*::Tn*5gusA40 zxx-110*::Tn10 *rif-2*	This work
BXO884	*prt1*::Tn*5*Tet *rif-2*	This work
Plasmids		
pUFR034	IncW Nm^r Mob$^+$ *mob* (P) lacZ alpha Par$^+$ *cos* (8.7kb)	DeFeyter *et al*. (1990)
pRK600	pRK2013 *npt*::Tn*9*; Cm^r	Laboratory collection
pBluescript (KS)	Ap^r	Stratagene
pXop1	pBluescript + 777 bp PCR amplified fragment from *xadA* gene	This study
pGEX-2T	Ap^r	Promega; Smith and Johnson (1988)
pXop2	PGEX-2T + ≈777 bp EcoRI and *Bam*HI digest from pXop1	This study
pSR1	pUFR034 + a 30 kb insert from the BXO1 genome	Ray *et al*. (2000)
pSR3	pUFR034 + 9 kb *Eco*RI fragment of the insert from pSR1	This study
pSR10	pSR1::*zxx-110*::Tn*10*	This study

rif indicates a mutation that confers rifampicin resistance. *zxx-110*::Tn*10* insertion is located on *Xoo* DNA cloned in pSR1 outside the region encoding the type two protein secretion system or any other known virulence factor. *prt1*::Tn*5* indicates an insertion in the ORF for the putative serine protease pseudogene. Ap^r indicates resistance to ampicillin.

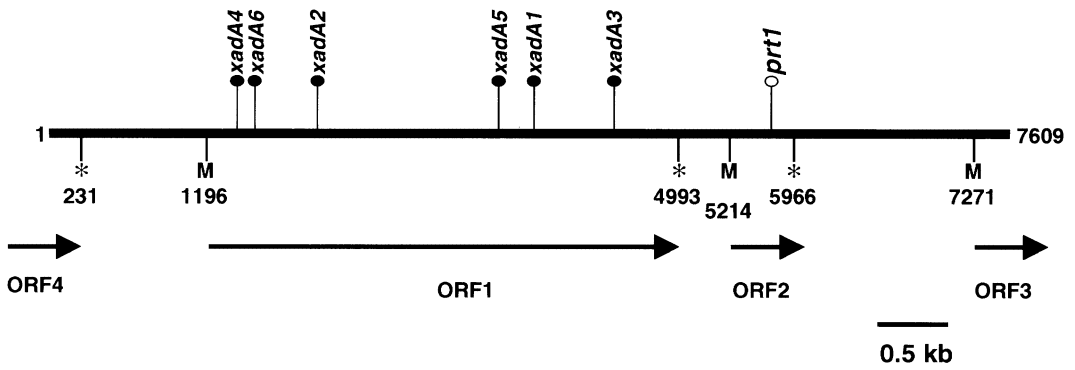

Fig. 1. Schematic of the arrangement of ORFs in a 7.609 kb genomic region of *Xoo*. 'M' and '*' represent the beginning and end of the ORFs, respectively, whereas arrows represent the direction of transcription. *xadA* insertions affect virulence and colony morphology. The *prt1* insertion does not affect either virulence or colony/lawn morphology. ORFs 4 and 3 extend from or into adjacent regions, respectively, and only partial sequences are available.

ORF1 encodes a protein of 1265 amino acids designated as XadA. It is rich in alanine (22.3%), glycine (15.3%), serine (10.1%), valine (9.6%) and threonine (8.1%), whereas it lacks cysteine. Either a TAVG or a similar sequence is present 23 times in this sequence. A nine-amino-acid region (TDAVNVAQL) is repeated four times in XadA, with little variation. There are longer repeat sequences in the protein as shown in Fig. 2A. A novel feature is the presence of repeats of alanine at an interval of seven amino acids almost throughout the sequence of the protein. A cluster of 10 charged amino acids is present towards the C-terminal (1179–1193) region. The N-terminal region of XadA shows the presence of an unusually long putative signal peptide of 66 amino acids. The presence of a long signal peptide has been found in several, but not all, autotransporters (Henderson *et al*., 1998). The C-terminal ends with a tryptophan, which is part of a motif made up of five, alternatively arranged, hydrophobic amino acids found in autotransporters and many other outer membrane proteins such as siderophore receptors, porins, etc. (Struyve *et al*., 1991; Henderson *et al*., 1998).

A BLAST search (Altschul *et al*., 1997) with XadA sequence identified homology to several non-fimbrial adhesins of animal pathogenic bacteria, such as YadA of *Yersinia* sp. and UspA1 of *M. catarrhalis*. An interesting feature is that the homology to YadA is repeated six times within the XadA sequence (Fig. 2B). Similarly, the homology to UspA1 is repeated multiple times within the XadA sequence (data not shown). In addition, XadA was found to be homologous to several uncharacterized ORFs present in other animal pathogenic bacteria and the closely related phytopathogenic bacteria, *Xanthomonas campestris* pv. *pelargonii* (causal agent of bacterial blight in geranium) and *X. fastidiosa*, *Xac* and *Xcc* (data not shown). Interestingly, all these related protein sequences contain multiple copies of TDAVNVAQL or a similar motif.

Genes linked to xadA*: a Xoo serine protease gene may be a pseudogene*

Three other ORFs besides *xadA* were identified in the 7609 bp region (Fig. 1). ORF2 extends from 5214 to 5966 bp and is homologous to serine proteases from a number of bacteria, e.g. a serine protease from *Xcc* (32% identity and 49% similarity in a stretch of 205 amino acids). The homology to serine proteases extends beyond ORF2 (from 5214 to 7049 bp) but is interrupted by nonsense and frameshift (–1) mutations immediately after 5963 bp and 6503 bp respectively. The sequence of this region was reconfirmed by polymerase chain reaction (PCR) amplification from genomic DNA and resequencing. It is therefore possible that ORF2 represents a pseudogene. It is pertinent to note that *Xoo* is deficient for extracellular protease activity (Ray *et al*., 2000), whereas *Xcc* is proficient (Tang *et al*., 1987). Only partial sequences are available for ORF3 and ORF4. ORF3 is homologous to XpsE, a component of the type II protein secretion system of *Xcc* (Dums *et al*., 1991), and ORF4 is homologous to phosphoribosylformylglycinamidine synthetase, an enzyme involved in purine biosynthesis (data not shown).

XadA is required for optimal virulence

To study the effect of *xadA* mutations on *Xoo* virulence, several *xadA*::Tn*5* or Tn*10* (Fig. 1) insertions were obtained on the pSR1 clone by transposon mutagenesis and marker exchanged into the wild-type background. The virulence phenotype of all *xadA* mutants (BXO#s 836,

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1    MNQIYRKVWNKSLGVWAVASELSSGDSPGAVASASFIDRRHRLALTAAIALALGGAGFAT
61   PLPANAQSVEVGRGASAPASKATAIGANSHASATGAVATGADSSASGVNSSAIGRPTNAI
121  GENALAIGHNSFVRQSGENGVAPGANAGVSGANSRTYEDDVVSIGSGNGRGGPATRRITN
181  VTAGVNATDAVNVAQLRHVADVAENTAQFFKASPGEESVGAYVEGDSALAAGEGANAVGT
241  ATTALGTGANAVAENATAVGTNALASGQNSAAFGHNAQANGPASVAVGGAAVNEDGEPLI
301  TNGGVPVTTGATSAGVGGTAVGASAKADGFAASSFGVGAYAAGAQASAFGAVANAAGDYA
361  TAVGTQTRASGTSSTAVGGPVDLIPGLGLFVQTQASGEASTALGAGAIASGTYATAVGTL
421  SEASGTEATAVGYFAYAPGEGATAVGPQSLASGELSTALGYFSTARGANSVALGANSVAT
481  RANTVSVGAAGNERQITNVAAGTQGTDAVNLNQLNAVAETAQTTGKYFKASGSAKKDVGA
541  YVEGENALAAGEGAKAVGTGTTALGAGAHAVVRKATAVGVGAVADGIGAAALGNTARALG
601  DNSSAVGSNAVASDIGATANGAGAQALSAYTIALGSKAVASDLQAIAEGFNSTASNVGST
661  ALGGFSQSSGRLSSALGYSAVASSVDSTAVGVAAQATGVSAVAIGEISKATGEESVAVGG
721  GAFSGWIPTQASGKGAAAFGAGAWATADYTTAIGRDSYADGVNATAVGQSADALADNTLA
781  LGGGSRAKAVGASVIGVDASATGINSTGVGRQVNVIGENAVSVGYNSYVRQSAVNGVALG
841  ANAGATGADSVALGSGSSTYDADTVSVGSGNGRGGPATRRIVNVGAGAVASASTDAINGG
901  QLFESLSNAASFLGGGAAIGAQGVFVAPTYLIQGASYNNVGAALTALDSKVTELDARSGG
961  TPANTAARTASLRTATVPAMAATAVSAVSSNVASTAIDATAGVQGTPTAAVVGSITPAAI
1021 STVVGTAAVANNVTGTAIGGSAYAHGANDTAIGSNARVNADGSTAVGANTQIAAVATNAV
1081 AMGDGAQVTAASGTAIGQGARATAQGAVALGQGSVADRANTVSVGSVGGERQVANVAAGT
1141 LATDAVNKGQLDNGVAAANSYTDSRYNAMADSFESYQGDIEDRLRRQNRRLDRQGAMSSA
1201 MLNMSASVAGIASPNRIGAGVGFQNGESALSVGYQRAISPRATVTVGGALSSGDSSIGVG
1261 AGFGW
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B

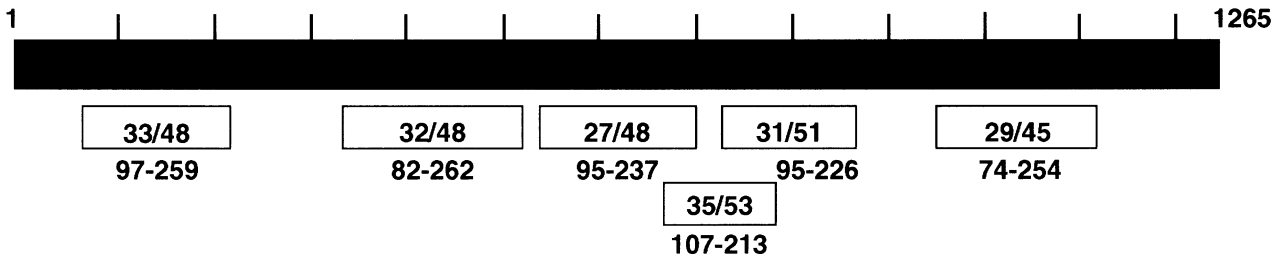

Fig. 2. A. XadA sequence features. Amino acid regions exhibiting similarity to each other have been shown in the same colour. Towards the C-terminal end, amino acids forming a charge cluster have been indicated in bold letters. Alternate hydrophobic amino acids at the C-terminal end of the protein are indicated in bold, underlined letters. A indicates alanine repeated at an interval of seven amino acids.
B. Regions of homology between *Xoo* XadA and *Yersinia* YadA. The filled box represents XadA (1–1265 amino acids), and the vertical lines on it represent every 100 amino acids within this sequence. The open boxes represent regions of XadA that exhibit homology to YadA. Numbers inside the open boxes represent the percentage identity and similarity respectively. Numbers below these boxes indicate homologous regions of YadA.

837, 838, 839, 840, 841) was similar to the wild-type strain (BXO43) after wound inoculation on rice leaves at higher concentrations of inoculum (10^7–10^9 cfu ml^{-1}). At ≈10^5 cfu ml^{-1}, all *xadA* mutants exhibited a virulence deficiency (data are shown for BXO#s 836 and 837; Fig. 3A). As complementation of *xadA* mutant strains for virulence was difficult, given the instability of pSR3 *in planta* (data not shown), XadA$^+$ recombinants (BXO#s 846 and 847) were obtained by replacing *xadA*$^-$ alleles in BXO836 and BXO837 with the *xadA*$^+$ allele using the linked *zxx*-Tn*10* insertion (see *Experimental procedures*). These XadA$^+$ recombinants and a *Xoo* strain (BXO845; XadA$^+$) carrying

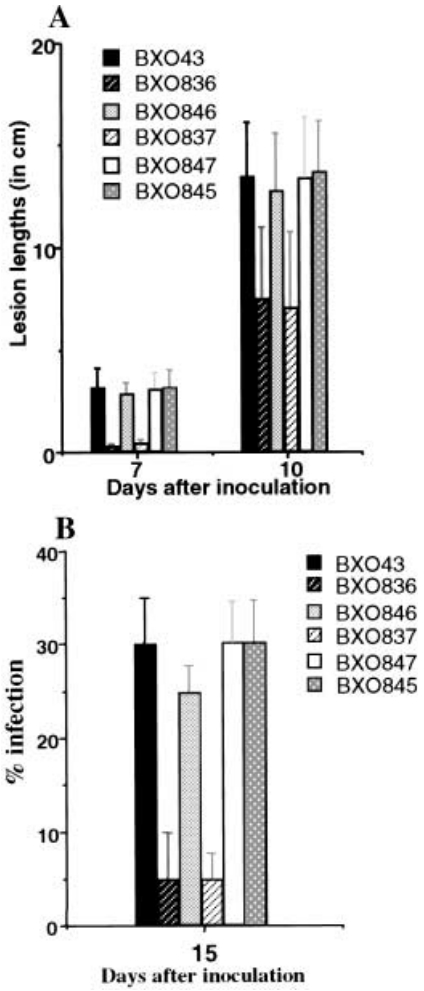

Fig. 3. *xadA* mutations affect *Xoo* virulence. Virulence assays were conducted by inoculating 40- to 50-day-old rice plants of the susceptible cultivar Taichung Native-1 (TN-1) through either wounding (A) or epiphytic (B) infection. Lesion lengths (A) or efficiency of infection (B) (see *Experimental procedures*) were used as a measure of virulence. BXO43, the wild type strain; BXO836 and BXO837 are XadA$^-$; BXO846 and BXO847 are XadA$^+$ recombinants obtained from BXO836 and BXO837 respectively; BXO845, *zxx-110*::Tn*10* (XadA$^+$).

zxx-Tn*10* exhibited a wild-type virulence phenotype. This indicates that mutations in the *xadA* gene cause a virulence deficiency after wound inoculation at low doses of inoculum.

A virulence deficiency of *xadA* mutants was also observed after epiphytic inoculation. In this mode of inoculation, the cells in a bacterial inoculum ($\approx 10^8$ cfu ml^{-1}) are deposited on rice leaf surfaces without wounding, and the pathogen gains entry into rice leaves through the hydathodal openings. Under these conditions of infection, it was observed that lesions develop in ≈30% of the leaves inoculated with BXO43 (Fig. 3B). The infection efficiency for BXO836 and BXO837 was ≈5%, 15 days after infection (Fig. 3B). The XadA$^+$ recombinants (BXO846 and BXO847) and the XadA$^+$ strain (BXO845) carrying the *zxx-110*::Tn*10* were as efficient at epiphytic infection as the wild-type strain. This indicates that the *xadA* gene promotes epiphytic infection of rice leaves. A Tn*5* insertion in ORF2 (*prt*1::Tn*5*; Fig. 1) did not affect the virulence of *Xoo* after either wound inoculation or epiphytic infection (data not shown). This indicates that the virulence deficiency of *xad*A insertion mutants is not the result of a polar effect on downstream genes.

The xadA *mutants exhibit a conditional alteration in colony/lawn morphology*

The *xadA* mutants form extracellular polysaccharide positive (EPS$^+$) colonies/lawns like the wild-type (BXO43) strain upon growing on either complex or minimal medium at 28°C. However, after 7 days of growth in minimal medium, a gradual change occurs in the morphology of the lawns and colonies formed by all the *xadA* mutants (BXO#s 836, 837, 838, 839). The EPS$^+$ regions start becoming EPS$^-$ (Fig. 4; shown only for BXO837). In seven more days, the entire bacterial lawn/colony becomes EPS$^-$. Introduction of the pSR3 clone restores the wild-type pattern of EPS production in these mutants (data not shown). The *xadA* mutants remain EPS$^+$ even after prolonged incubation in rich medium. Upon restreaking, the bacteria from the EPS$^-$ regions form colonies that are EPS$^+$ in rich and minimal medium but slowly become EPS$^-$ only in minimal medium. This suggests that the change in colony morphology does not result from an additional mutation. No such change in bacterial colony/lawn morphology was observed for either BXO43 or other XadA$^+$ strains (BXO#s 846, 847, 843, 844). A Tn*5* inser-

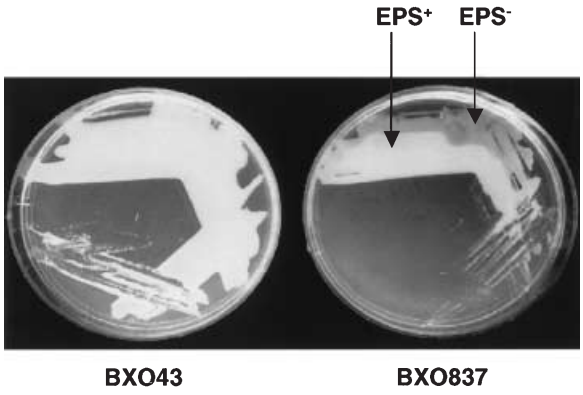

Fig. 4. Extracellular polysaccharide (EPS)-deficient phenotype of *xadA* mutant. BXO43, wild type strain; BXO837, XadA$^-$ mutant. Photographs showing the presence of both EPS-proficient (EPS$^+$) and -deficient (EPS$^-$) regions in a 10-day-old minimal medium-grown culture of BXO837. No EPS$^-$ region is observed in a culture of the BXO43 strain.

tion in ORF2 (*prt*1::Tn*5*; Fig. 1) did not affect the EPS phenotype, suggesting that the conditional EPS deficiency associated with *xad*A insertions is not caused by a polar effect on downstream genes.

XadA is an outer membrane protein, and its expression is regulated by growth conditions

The conditional change in colony phenotype of the *xadA* mutants described above indicated differential expression of XadA. To study XadA expression, *Xoo* strains were grown in either rich or minimal media, and whole-cell lysates were analysed in Western blots using anti-XadA antibodies. Western analysis identified a prominent ≈110 kDa band (expected size of XadA based on sequence) in the BXO43 strain grown in minimal medium (Fig. 5). In BXO#s 836 and 837, this band was replaced by ≈78 kDa and ≈27 kDa bands respectively (the latter band is very faint in Fig. 5 and was visible only upon longer staining). The altered bands in the above mutants were of the expected sizes for the truncated XadA protein, given the Tn*5gusA40* insertions at 782 (after the 2345th bp) and 278 codons (after the 832nd bp) in the *xadA* gene respectively. The *xadA* mutant alleles were converted to *xadA*$^+$ by allelic exchange using the linked *zxx-110*::Tn*10* marker (see *Experimental procedures*). The 110 kDa protein reappeared in the *xadA*$^+$ recombinant strains (BXO#s 846 and 847), and the altered bands were present in recombinants that remained *xadA*$^-$ (BXO# 848 and 849; Table 1; Fig. 5), suggesting that this protein is encoded by the *xadA* gene. Neither the 110 kDa nor the altered bands were present in the *Xoo* strains grown in rich medium, indicating that the XadA protein is expressed only in minimal medium-grown cells. Coomassie brilliant blue (CBB) staining was done on a second gel run under the same conditions to indicate that an equal amount of protein was loaded in all lanes (data not shown). Even in a CBB-stained gel, the ≈110 kDa protein was detected only in lysates from minimal medium-grown wild-type cells but not from rich medium-grown cells. This 110 kDa protein was missing in the *xadA* mutants (data not shown).

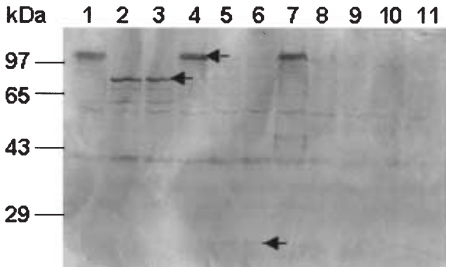

Fig. 5. Differential expression of XadA protein. Western blot analysis of the cell lysates of minimal and rich media-grown *Xoo* strains were done using anti-XadA antibodies. Lanes 1–7, strains grown in minimal medium; lanes 8–11, strains grown in rich medium; lanes 1 and 8, BXO43 (XadA$^+$); lanes 2 and 9, BXO836 (XadA$^-$); lanes 5 and 11, BXO837 (XadA$^-$); lanes 4 and 10, BXO846 (XadA$^+$); lane 3, BXO848 (XadA$^-$); lane 6, BXO849 (XadA$^-$); lane 7, BXO847 (XadA$^+$). In lanes 1, 4 and 7, an ≈110 kDa band is present. In lanes 2 and 3, an ≈78 kDa band is present and, in lanes 5 and 6, an ≈27 kDa band (visible clearly only after prolonged staining) is present.

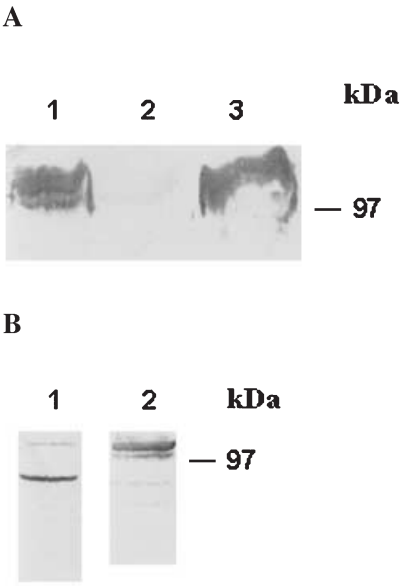

Fig. 6. Localization of XadA to the outer membrane of *Xoo*.
A. Outer membrane preparations of minimal medium-grown *Xoo* cells were separated by SDS-PAGE and subjected to Western blot analysis using anti-XadA antibody as described in *Experimental procedures*. Lane 1 (BXO43; XadA$^+$); lane 2 (BXO836; XadA$^-$) and lane 3 (BXO846; XadA$^+$ recombinant in BXO836 background).
B. Trypsin digestion of physiologically intact BXO43 cells was performed. Whole-cell lysates were separated by SDS-PAGE and subjected to Western analysis using anti-XadA antibodies. Lane 1, trypsin treated; lane 2, trypsin untreated.

Outer and inner membrane fractions were purified from minimal medium-grown BXO43, BXO836 and BXO846. Lipopolysaccharide (LPS) and succinate dehydrogenase were taken as markers for outer and inner membranes, respectively, as described by Goel *et al*. (2002). LPS was localized only to the outer membrane fraction, and succinate dehydrogenase activity was detected only in the inner membrane fraction (data not shown), indicating that separation of the membranes was achieved. Western blot analysis using anti-XadA antibodies detected the presence of the ≈110 kDa band only in the outer membrane fractions of BXO43 and BXO846 (Fig. 6A) but not in either inner membrane or extracellular fractions (data not shown). The ≈78 kDa truncated protein that was detected in the whole-cell lysate of BXO836 was present in neither the outer membrane fraction nor the inner membrane fraction (data not shown). This indicates that XadA is an

outer membrane protein and that the C-terminal end is required for outer membrane localization/docking. As expected, the 110 kDa band was not detected in the outer membrane fractions isolated from BXO43 and BXO846 cells grown in rich medium (data not shown). As protease treatment of physiologically intact cells is a suitable tool for testing the surface exposure of bacterial outer membrane proteins (Lattemann *et al*., 2000), minimal medium-grown *Xoo* cells were subjected to trypsin treatment before Western blot analysis. Trypsin treatment of the BXO43 strain resulted in the disappearance of the 110 kDa band and the appearance of a smaller band (≈90 kDa; Fig. 6B), suggesting that only a portion of the XadA protein is sensitive to protease treatment. Trypsin treatment of detergent-solubilized cells and Western blot analysis with XadA antibody yielded a low-molecular-weight band of ≈20 kDa (data not shown), indicating that the 90 kDa band is not simply resistant to trypsin.

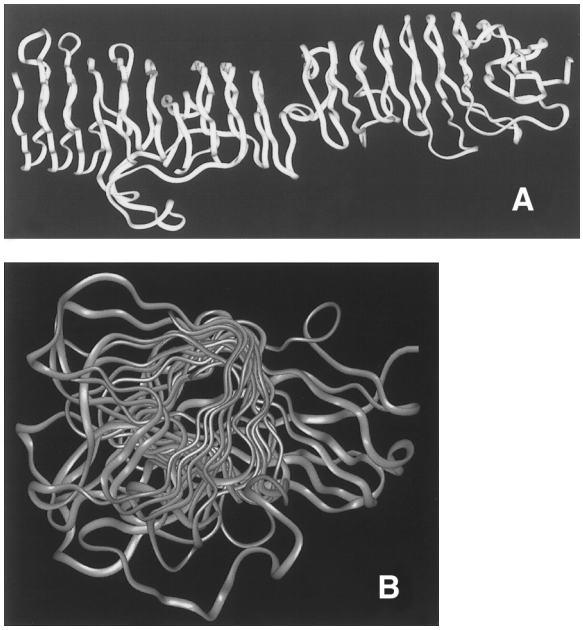

Fig. 7. Three-dimensional structure for the 650–1206 amino acid region of XadA.
A. Ribbon diagram of the simulated three-dimensional structure of XadA indicating parallel β-sheets arranged as a hanger-like β-helical conformation.
B. Ribbon diagram of the see-through structure of the above part of XadA.

Modelling of the three-dimensional structure of XadA

The predicted structure of XadA was generated using the SWISS-HOMOLOGY modeller program. Co-ordinates were obtained for the C-terminal part of XadA (from 650 to 1206 amino acids), and the structure was simulated and optimized on INSIGHTII. The three-dimensional model suggests a parallel β-helix-type structure (Fig. 7A and B), similar to that found in virulence factors such as the non-fimbrial adhesin pertactin P.69 of *B. pertussis* (Emsley *et al*., 1996) and pectate lyase C of *Erwinia chrysanthemi* (Yoder *et al*., 1993). In the modelled structure, a total of 19 rungs are formed, which are made up of both three β-strands and two β-strands comprising 22 and 14 residues per rung respectively. The total length of the β-helix is about 120.20 Å. Like other β-helix proteins, the β-strands are mostly amphipathic in nature with their hydrophilic amino acids facing away from and the hydrophobic amino acids facing towards the core. There are two loop regions protruding out from the helices (894–918 amino acids and 1004–1018 amino acids). Analysis of the XadA sequence using the β-helix predicting program, BETAWRAP (Bradley *et al*., 2001), suggested the presence of β-helix folds throughout the protein. The best wrap region predicted (790–924 amino acids) lies in the modelled region with a raw score of −20.99, substantiating the results of homology modelling. These results are significant because it has been reported that many predicted β-helix-forming proteins are virulence factors of microbial pathogens (Bradley *et al*., 2001). Using the AMPHI algorithm (Jahnig, 1990), four potential amphipathic β-strands, which can form a membrane-spanning region, were detected at the C-terminal end (1198–1265 amino acids) of XadA.

Discussion

In this report, we have described a high-molecular-weight, outer membrane protein of *X. oryzae* pv. *oryzae* that is homologous to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence. It is possible that XadA is involved in attaching to the surfaces lining the hydathodal openings and xylem vessels. As it is difficult to perform adhesion assays on these surfaces, because of their inaccessibility, we have performed assays for attachment to rice leaf surfaces and callus cultures. In preliminary experiments (data not shown), we have not found a difference between wild type and *xadA* mutants in their ability to attach to these surfaces. It is possible that additional functions, besides XadA, might be involved in the attachment of *Xoo* to these particular surfaces. Alternatively, XadA might have another role in promoting *Xoo* virulence. Pectate lyases of *E. chrysanthemi* and other bacteria (Bradley *et al*., 2001) adopt an β-helical structure, and it could be that XadA acts like a pectate lyase or has a similar polysaccharide-degrading activity. We consider this to be less likely because XadA exhibits no sequence similarity with known pectate lyases. Also, unlike the β-helical pectate lyase family members, which are secreted proteins, the outer membrane localization of XadA is more consistent with its role as an adhesin.

The *xadA* mutants are more affected for virulence after epiphytic infection than after wound inoculation. This indicates that XadA has an important function in *Xoo* virulence somewhere along the path that takes it from the leaf surface, through the hydathodes, into the xylem vessels. The XadA protein also has a moderate, yet significant, effect on virulence after wound infection. One possibility is that other virulence factors might have overlapping functions with XadA in promoting growth within xylem vessels. It is pertinent in this regard to note that many animal pathogenic bacteria have been reported to have multiple adhesins (Henderson and Nataro, 2001). Also, the genome sequences of the plant pathogens *X. fastidiosa*, *R. solanacearum*, *Xcc* and *Xac* have been reported to encode multiple adhesin-like functions (Simpson *et al*., 2000; Salanoubat *et al*., 2002; da Silva *et al*., 2002).

The XadA protein appears to be expressed in minimal medium but not in rich medium. This correlates well with the conditional EPS-deficient phenotype of *xadA* mutants. This pattern of expression is interesting because xylem is considered to be mainly a conduit for transport of water and minerals. Therefore, rice xylem sap is likely to be more akin to minimal medium than to rich medium. Consistent with this, our preliminary observations (data not shown) suggest that XadA is indeed being expressed during *in planta* growth. However, the mechanism by which XadA expression is regulated by the composition of the growth medium has yet to be understood.

Hoiczyk *et al*. (2000) have proposed a 'lollipop'-shaped structure for YadA, based on data from electron microscopy and amino acid sequence analysis. The N-terminal region forms the head domain, which is responsible for host cell contact and contains several degenerate 14-residue repeats. Secondary structure prediction suggests that this repeat region consists primarily of β-strands. This is followed by a neck region, which is highly conserved and is often present as multiple repeats in non-fimbrial adhesins from several proteobacteria. The stalk of the lollipop is postulated to be formed by an elongated coiled-coil domain that is composed of a different class of degenerate repeats. The C-terminal region acts as an outer membrane anchor domain and is composed of four amphipathic transmembrane β-strands. XadA exhibits homology to the N-terminal head region of YadA. The TAVG-like repeats that are present throughout XadA are homologous to consensus sequences in the degenerate 14-residue repeats from the head region of YadA (data not shown). The TDAVNVAQL sequence, which is part of the neck region of YadA, is repeated four times in XadA, but the coiled-coil domain was not detected. As in YadA, the C-terminal region of XadA is composed of four transmembrane amphipathic β-strands that might serve as a membrane anchor. Based on the sequence relationships with YadA and the homology modelling, we postulate that XadA is composed of an elongated β-helix with a C-terminal membrane anchor. This elongated structure might be an adaptation to the presence of EPS, which may otherwise form a barrier for interacting with the host tissue. The multiple repeat motifs in XadA might serve, in an as yet undetermined manner, to elaborate this elongated β-helix structure. This kind of phenomenon has been reported in *H. influenzae* (St Geme *et al*., 1996), in which the Hsf adhesin of typeable strains (EPS^+) is larger than the Hia from non-typeable strains (EPS^-) because of repetition of the Hia homologous region three times in Hsf. Structural analysis with electron microscopy, crystallography, etc. along with site-specific mutagenesis of the *xadA* gene are planned to understand the structure–function relationship of XadA and to test some of the predictions made in this study.

Recent reports have provided the interesting observation that several virulence functions are conserved among Gram-negative bacterial pathogens, irrespective of whether they infect animal or plant cells. These conserved virulence functions are either transport systems that are involved in the secretion of virulence factors across the bacterial cell surface or particular effector molecules (Hardt and Galan, 1997; Staskawicz *et al*., 2001). One particular bacterium, *Pseudomonas aeruginosa*, has been shown to be capable of infecting both animals and plants using the same virulence factors (Rahme *et al*., 2000). This study extends our knowledge about the repertoire of homologous virulence factors that are deployed by animal and plant pathogenic bacteria to include functions potentially involved in adhesion. Future studies will be aimed at understanding the exact role of XadA in *Xoo* virulence.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The strain and plasmid list is shown in Table 1. Media and growth conditions for *Escherichia coli* and *Xoo* have been described previously (Ray *et al*., 2000). Antibiotics used in this study were: cephalexin (Cp) 20 μg ml^{-1}; kanamycin (Km) 50 μg ml^{-1} for *E. coli* and 25 μg ml^{-1} for *Xoo*; spectinomycin (Sp) 50 μg ml^{-1}; tetracycline (Tc) 10 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *Xoo*.

Virulence assays

Wound inoculations were performed on 40- to 50-day-old plants of highly susceptible rice cultivar Taichung Native-1 (TN-1) as described previously (Ray *et al*., 2000). Symptoms were scored by measuring lesion lengths at 7 and 10 days after inoculation (DAI). In each experiment, 15 leaves were inoculated, and the values are presented as mean lesion lengths and standard deviations. Similar results were

obtained in independent experiments. For epiphytic infections, 40- to 50-day-old TN-1 rice plants were kept inside the greenhouse within a humidity chamber (made up of Plexiglas sheets) for 24 h before infection. Inoculations were done by dipping the tips of rice leaves (to a distance of ≈4 cm from the leaf tip) in a bacterial suspension carrying 10^8 cfu ml^{-1} for 5–10 s. Plants were kept in the humidity chamber for an additional week. Subsequently, the plants were transferred to a greenhouse bench, and the frequency of lesion formation was determined 15 DAI. In each experiment, 20 leaves were inoculated, and the mean and standard deviations of data from three experiments are presented.

Bacterial conjugations, transposon mutagenesis and marker exchange

Matings between *E. coli* strains and *E. coli* (donor) with *Xoo* (recipient) were carried out as described previously (Ray *et al*., 2000). The pSR1 clone was mutagenized *in vivo* with Tn*5gusA40* (Wilson *et al*., 1995) and *in vitro* using an EZ::TN <TET-1> insertion kit (Epicentre Tech). Transposon insertions obtained in the *xadA* and *prt* (protease) genes were marker exchanged (Ray *et al*., 2000) into the BXO43 background, and the mutants were analysed by Southern hybridization. To obtain $XadA^+$ recombinants, the pSR10 plasmid (Table 1; Ray *et al*., 2000), carrying a *zxx-110*::Tn*10* insertion linked to *xadA*$^+$, was mobilized into BXO836 and BXO837 by conjugation. Tc^r recombinants that were either $XadA^+$ (BXO846 and BXO847; Tc^r Sp^s, Km^s) or $XadA^-$ (BXO848 and BXO849; Tc^r, Sp^r, Km^s) were obtained by growing the transconjugants in rich medium containing Tc for more than 30 generations and screening on medium supplemented with appropriate antibiotics.

Plasmid isolation, DNA sequencing and sequence analysis

Plasmid DNA was isolated by the alkaline lysis method (Sambrook *et al*., 1989) and restriction digested where necessary. Sequencing was performed using transposon-specific primers and primer walking. Sequencing reactions, electrophoresis and sequence data analyses were performed using the ABI Prism 3700 automated DNA sequencer (Perkin-Elmer Biosystems). Homology searches were done using the BLAST algorithm (Altschul *et al*., 1997) through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). SIGNALP (Nielsen *et al*., 1997) and SAP (statistical analysis of proteins; Brendel *et al*., 1992) programs at the http://www.expasy.ch site were used to detect putative signal peptide and repeat regions. Putative promoters were identified using the search launcher at Baylor College of Medicine (http://dot.imgen.bcm.tmc.edu), and ORFs were identified using FRAMEPLOT (Ishikawa and Hotta, 1999; http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl).

Raising of polyclonal antibodies to XadA fusion protein

A 777 bp region of the *xadA* gene was PCR amplified using the following primers (forward primer: GCGGATCCACTGC CGGCGTCAATGCCACCGAT; reverse primer: GCGAATTC TTCGCCCGGCGCATACGCGAAGTA) to yield a protein of 259 amino acids from the N-terminal region of XadA (from 182 to 440 amino acids; accession no. AF288222). The PCR product was cloned in pBSKS vector (Stratagene) using *Eco*RI and *Bam*HI restriction sites to obtain pXop1, and subcloned in pGEX-2T (Amersham Pharmacia Biotech) to obtain pXop2. The fused protein was purified using glutathione-coated agarose beads (Smith and Johnson, 1988) and used for raising polyclonal antibodies in rabbit by standard protocols (Sambrook *et al*., 1989). The antiserum could detect XadA protein in wild-type *Xoo* samples at a dilution of 1:2000.

Outer membrane localization of XadA

The outer and inner membrane fractions were isolated from the saturated cultures of wild-type, *xadA* mutant and *xadA*$^+$ recombinant strains of *Xoo* by the method of Ray *et al.* (1994). Cultures were grown in 100 ml of minimal medium, and cells were pelleted and resuspended in buffer containing 5 mM Tris-Cl (pH 8.0), 0.375 M sucrose, 1 mM EDTA and 30 μg ml^{-1} lysozyme. The cells were lysed by sonication and centrifuged at 5000 r.p.m. for 10 min to pellet unlysed cells and cell debris. The supernatant was centrifuged at 90 000 r.p.m. for 2 h in a table-top ultracentrifuge. The pellets were resuspended in Triton X-100 buffer containing 10 mM Tris-Cl (pH 8.0), 1% Triton X-100 and 5 mM $MgCl_2$ and incubated for 30 min at room temperature. The sample was centrifuged at 70 000 r.p.m. for 30 min. The supernatant was retained as the inner membrane fraction. The pellet was resuspended in buffer containing 50 mM Tris-Cl (pH 8.0), 10 mM EDTA and 1% Triton X-100 and incubated at room temperature for 30 min. The sample was centrifuged at 10 000 r.p.m. for 1 h, and the supernatant was taken as the outer membrane fraction. Both fractions were solubilized in 1× SDS-PAGE sample buffer (Sambrook *et al*., 1989), heated for 5 min at 100°C and separated on 10% acrylamide gels. The samples were transferred to polyvinylidene difluoride (PVDF) membranes by semi-dry method, and Western blot analysis (Sambrook *et al*., 1989) was done using anti-XadA antibodies. The secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase. Detection was performed using chromogenic substrates as described by Sambrook *et al*. (1989).

For the preparation of whole-cell lysates, 1 ml of saturated culture of wild-type, *xadA* mutant and *xadA*$^+$ recombinant strains of PS- or minimal medium-grown *Xoo* cells was pelleted. The pellets were solubilized in 1× SDS-PAGE sample buffer, separated by electrophoresis on 10% acrylamide gels and transferred to PVDF membranes for Western blot analysis using XadA-specific antibodies. Whole-cell trypsin treatment was carried out as described by Lattemann *et al.* (2000). Cells were grown in minimal media for 48 h at 28°C, pelleted and resuspended in phosphate-buffered saline (PBS) to a concentration of ≈1×10^8 cfu ml^{-1}. The cells were incubated at 37°C for 10 min with trypsin (50 μg ml^{-1}) before washing twice in PBS to remove trypsin. The whole-cell lysates were then obtained and subjected to Western blot analysis as described above.

Molecular modelling of XadA

The model of XadA was generated by homology modelling on SWISS-MODEL protein modelling server (SWISS-MODEL version 36.0002; Peitsch, 1996; Guex and Peitsch, 1997). The co-ordinates from 651 to 1204 residues were obtained from the SWISS-MODEL server and used to build the three-dimensional structure. The structure was corrected using INSIGHTII, and minimization was done on this structure using the DISCOVER module on an Onyx workstation at 300 K (Silicon Graphics). After adding hydrogens, charge potentials were assigned, fixed and accepted; hydrogens were uncharged. The 100 steepest iterations and 50 iterations for conjugate were used to minimize the structure. The AMPHI algorithm (Jahnig, 1990) was used to detect amphipathic β-strands in protein sequences. The BETAWRAP program (http://theory.lcs.mit.edu/betawrap; Bradley *et al*., 2001) was used for β-helix prediction.

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