XadM, a Novel Adhesin of *Xanthomonas oryzae* pv. *oryzae*, Exhibits Similarity to Rhs Family Proteins and Is Required for Optimum Attachment, Biofilm Formation, and Virulence

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By screening a transposon-induced mutant library of *Xanthomonas oryzae* pv. *oryzae*, the bacterial blight pathogen of rice, we have identified a novel 5.241-kb open reading frame (ORF) named *xadM* that is required for optimum virulence and colonization. This ORF encodes a protein, XadM, of 1,746 amino acids that exhibits significant similarity to Rhs family proteins. The XadM protein contains several repeat domains similar to a wall-associated surface protein of *Bacillus subtilis*, which has been proposed to be involved in carbohydrate binding. The role of XadM in *X. oryzae* pv. *oryzae* adhesion was demonstrated by the impaired ability of an *xadM* mutant strain to attach and form biofilms. Furthermore, we show that XadM is exposed on the cell surface and its expression is regulated by growth conditions and plays an important role in the early attachment and entry inside rice leaves. Interestingly, XadM homologs are present in several diverse bacteria, including many *Xanthomonas* spp. and animal-pathogenic bacteria belonging to *Burkholderia* spp. This is the first report of a role for XadM, an Rhs family protein, in adhesion and virulence of any pathogenic bacteria.

Adhesion of bacteria to the host surfaces is a prerequisite for the invasion of the host tissue. Bacterial attachment to the host surface is mediated by specific adhesins that are either anchored in the bacterial outer membrane or secreted, and have been classified as nonfimbrial and fimbrial adhesins (Finley and Falkow 1997; Soto and Hultgren 1999). Bacterial adhesins have been studied intensively in animal-pathogenic bacteria and less is known about the virulence functions of adhesins in plant-pathogenic bacteria. In *Xanthomonas* spp. and their close relative, *Xylella fastidiosa*, an insect-transmitted xylem-limited bacterium, it has been shown that nonfimbrial adhesin *Xanthomonas* adhesin A (XadA) and its homolog, XadB, which exhibit similarity to YadA from *Yersinia* spp., are required for virulence and attachment (Das et al. 2009; Feil et al. 2007). Filamentous hemagglutinin (FHA) from animal- and plant-pathogenic bacteria has been shown to be required for virulence and colonization. The best-studied FHA of *Bordetella pertussis*, which causes whooping cough in humans, has been shown to be secreted and a cell-associated adhesin and is responsible for attachment of the bacterium to the host cell

(Locht 2007). In plant-pathogenic bacteria, FHA-like filamentous hemagglutinin from *Erwinia chrysanthemi* (a pathogen that causes soft rot disease in several plants), *X. fastidiosa* (an economically important pathogen of grape, citrus, and several other commercial crops), *Xanthomonas axonopodis* pv. *citri* (a pathogen of citrus), and *X. oryzae* pv. *oryzae* have been shown to be required for virulence and attachment (Das et al. 2009; Gottig et al. 2009; Guilhabert and Kirkpatrick 2005; Ray et al. 2002; Rojas et al. 2002). It has been reported that, in *X. fuscans* subsp. *fuscans*, several adhesins play a role in attachment and fitness in the bean phyllosphere and seed-mediated transmission (Darsonval et al. 2009).

X. oryzae pv. *oryzae*, a member of the genus *Xanthomonas*, is an important pathogen of rice that causes bacterial leaf blight (Shen and Ronald 2002). *X. oryzae* pv. *oryzae* gains entry into rice leaves through either wounds or natural openings called as hydathodes that are concentrated at the edges of the leaves. The hydathodes lead into the xylem vessels inside which *X. oryzae* pv. *oryzae* multiplies. Several virulence traits have been identified in *X. oryzae* pv. *oryzae*, which have been reviewed in detail by Shen and Ronald (2002) and recently by Büttner and Bonas (2010). These include different secretion systems and their effectors (type II and type III), extracellular polysaccharide (EPS), adhesins, and a cell-to-cell signaling system known as diffusible signal factor.

To identify novel virulence functions of *X. oryzae* pv. *oryzae*, we had previously screened 1,600 Tn5gusA40-induced (mTn5) (Wilson et al. 1995) mutants for virulence deficiency (Chatterjee and Sonti 2002; Chatterjee et al. 2003). Several virulence-deficient mutants were identified, and one among these mutants, BXO1701 (henceforth referred as XadM1), was not disrupted in previously recognized virulence genes of *X. oryzae* pv. *oryzae*.

In this study, we demonstrate that the virulence deficiency in XadM1 is due to mutation in a novel open reading frame (ORF) of 5.241 kb that encodes *Xanthomonas* adhesin-like protein M (XadM), which exhibits similarity to recombination hot spot (RHS) family proteins.

The *rhs* genes are a family of enigmatic genes present in diverse bacteria and have been implicated to play a role of a rearrangement hot spot. Despite their ubiquity, *rhs* genes have not been assigned a definitive function (Hill et al. 1994).

We show that XadM is a surface-associated adhesin and is required for *X. oryzae* pv. *oryzae* attachment and biofilm formation. In addition, we also show that XadM plays an important role in the entry and colonization process of *X. oryzae* pv. *oryzae* in rice leaves. Homologs of XadM-like proteins are present in other diverse bacteria, including pathogenic *Xanthomonas* and *Burkholderia* spp. To the best of our knowledge, this is the first demonstration that an RHS family protein

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exhibits adhesin like function and is required for virulence in bacteria.

RESULTS

xadM is required for optimum virulence.

X. oryzae pv. *oryzae* causes bacterial leaf blight (BLB) disease with symptoms of brown, long lesions on the leaf. The lesions caused by the wild-type strain (BXO43; rifampicin-resistant derivative of BXO1) (Table 1) are approximately 4.04 and 11.08 cm long 7 and 14 days after wound inoculation, respectively (Fig. 1A). In contrast, the lesions caused by the XadM1 (BXO1701; mTn5-induced mutant) are 1.36 and 5.25 cm long 7 and 14 days after inoculation, respectively (Fig. 1A). In comparison with BXO43, the XadM1 mutant always exhibited less vigorous lesion symptoms (browning of the rice leaves), which corresponds to typical BLB caused by *X. oryzae* pv. *oryzae* (Fig. 1B). The XadM1 strain also exhibited a virulence deficiency following epiphytic infection (Fig. 1C). In this mode of infection, the bacterial cells are deposited on the rice leaf surface without wounding, and the pathogen gains entry into leaves through the hydathodal openings which are concentrated at the tip of the leaves (Mew et al. 1984; Ray et al. 2002). In this mode of infection, progression of lesion takes place from the tip to the base of the rice blade (Fig. 1D). Infection efficiency was scored by determining the percentage of infected leaves showing disease lesions 16 days after inoculation. Under the conditions of epiphytic infection used in this study, approximately 76% of the leaves infected with BXO43 were found to develop visible lesions. In leaves inoculated with the XadM1 mutant, approximately 26% of the infected leaves developed lesions (Fig. 1C). These data indicate that the XadM1 strain exhibits reduced virulence, as assayed by wound inoculation as well as infection efficiency.

The XadM1 strain carried a single mTn5 insertion, as determined by Southern hybridization using the mTn5 element as a probe (data not shown). Introduction of the mTn5 insertion into a wild-type (BXO43) background by marker exchange led to a virulence deficient phenotype (Supplementary Table S1). Sequence analysis of the chromosomal DNA flanking the mTn5 insertion in XadM1 and partial sequence of the 6,840-bp genomic region of *X. oryzae* pv. *oryzae* strain BXO1 (GenBank accession numbers JQ898330, JQ898328, and JQ898329)

revealed that the transposon insertion is in codon 498 of an ORF (*xadM*), which has potential to encode a protein of 1,746 amino acids (aa) (Fig. 2A). The sequenced 6,840-bp genomic region from the BXO1 strain includes the complete sequence of the *xadM* gene (5,241 bp) and partial sequences (208 bp) of the neighboring ORF encoding glutamate synthase domain 2 (*gsd2*) (Fig. 2A). A BLAST search (Altschul et al. 1997) with *xadM* and its deduced polypeptide sequence indicated substantial similarity with the RHS (locus tag-XOO2631) family protein in the sequenced *X. oryzae* pv. *oryzae* KACC10331 strain (identity 99% in a stretch of 1,746 aa, as well as 99% nucleotide sequence identity) (Lee et al. 2005). It is interesting to note that XadM exhibited a much lower level of similarity to other Rhs family proteins of strain KACC10331, including XOO123 (identity 66%, similarity 73%), XOO2286 (RhsD; identity 24%, similarity 36%), and RhsD precursor protein (XOO1577; identity 22%, similarity 34%).

The ORF *IS1479* and *gsd2* which flank the 5' end of gene *xadM* in strain BXO1 are homologous to similarly located genes in *X. oryzae* pv. *oryzae* KACC10331 (Fig. 2A) (Lee et al. 2005). The ORF *gsd2* (partial sequence of 208 bp) exhibits 100% nucleotide sequence identity to *gsd2* (XOO2632) of *X. oryzae* pv. *oryzae* KACC10331 (Fig. 2A). A BLAST search of a partial sequence of 579 and 201 bp of the BXO1 genomic region flanking the *xadM* gene indicated substantial similarity with upstream flanking sequences of *ISXo8* (99% nucleotide identity; XOO2628) and *IS1479* (100% nucleotide identity; XOO2633) of *X. oryzae* pv. *oryzae* strain KACC10331 (Lee et al. 2005) (Fig. 2A). To further confirm the role of the *xadM* gene in virulence, we made an additional nonpolar mutation in *xadM* designated as *xadM2* (Fig. 2A; Table 1). The XadM2 strain was as virulence deficient as XadM1 in the wound as well as in the epiphytic mode of infection (Fig. 1A and C).

The genomic clone pSC2 containing the wild type allele of the mutated gene was isolated from the genomic library of *X. oryzae* pv. *oryzae* BXO1 strain maintained in DH5 α (Goel et al. 2001; Rajagopal et al. 1995, 1999). Complementation with pSC2 harboring the wild-type *xadM* allele restored normal virulence to the *xadM* mutants (XadM1 and XadM2 as well as the marker exchange mutant XadM3) similar to that of the wild-type strain (Fig. 1C). These results indicate that *xadM* of *X. oryzae* pv. *oryzae* is required for optimum virulence. For the

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics ^a	Reference
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
BXO1	Laboratory wild type; Indian isolate	Laboratory collection
BXO43	<i>rif</i> ^r ; <i>Rif</i> ^r derivative of BXO1	Laboratory collection
XadM1 (BXO 1701)	<i>xadM1::mTn5 rif</i> ^r -2; <i>Vir</i> ⁻ derivative of BXO43	This study
XadM2	<i>xadM2::pK18mobAp</i> ^r <i>rif</i> ^r -2; <i>Ap</i> ^r ; <i>Vir</i> ⁻ derivative of BXO43	This study
XadM3	<i>xadM1::mTn5 rif</i> ^r -2; marker exchange mutant; <i>Vir</i> ⁻ derivative of BXO43	This study
<i>Escherichia coli</i>		
DH5 α	F'/endA1 hsdR17 (rk ⁻ mk ⁺) supE44 thi-1 recA1 gyrA relA1 f80dlacZDM15 (lacZYA-argF) U169	Laboratory collection
S17-1	RP4-2Tc::Mu-Kn::Tn7 pro hsdR recA	Simon et al. 1983
Plasmids		
pBluescript(KS)	<i>Ap</i> ^r	Stratagene, La Jolla, CA, U.S.A.
pUFR034	IncW Nmr Tra ⁻ Mob ⁺ <i>mob</i> (P) lacZ α ⁺ Par ⁺ <i>cos</i>	DeFeyter et al. 1990
pMP2464	pBBR1MCS5 derivative; EGFP ⁺ , Gm ^r	Stuurman et al. 2000
pK18mob	Km ^r , RP4Mob, mobilizable cloning vector.	Schafer et al. 1994
pK18mobAp ^r	A derivative of pK18mob with <i>Ap</i> ^r <i>bla</i> gene cloned in the <i>pvuI</i> site	This study
pSC1	pBluescript + 7.5-kb <i>ClaI</i> fragment of chromosomal DNA, including the <i>xadM1::mTn5</i> insertion in BXO1701 (XadM1)	This study
pSC2	pUFR034 + 31-kb genomic insert of <i>X. oryzae</i> pv. <i>oryzae</i> DNA containing <i>xadM</i>	This study
pSC3	pK18mob + 653-bp fragment of <i>xadM</i> cloned into <i>EcoRI-HindIII</i> site of pK18mobAp ^r	This study

^a *Rif*^r, *Ap*^r, *Gm*^r, and *Km*^r = resistant to rifampicin, ampicillin, gentamycin, and kanamycin, respectively; *Vir*⁻ =, and EGFP = enhanced green fluorescent protein.

rest of our studies, we have used XadM1 (*xadM1*:: mTn5) and *xadM1* (pSC2).

***xadM* sequence features and similarity with Rhs family proteins.**

The *xadM* encodes a protein of 1,746 aa designated as XadM. Sequence analysis of the deduced polypeptide sequence of *xadM* using the signal peptide prediction program SignalP (Nielsen et al. 1997) indicated that it has a potential 35-aa signal peptide, with its most likely cleavage site between amino acids 34 and 35 (AQS-AT) (Fig. 2B). A search through the National Center for Biotechnology Information (NCBI) conserved domain database indicates that the predicted XadM protein exhibited significant similarity to RHS repeat-associated core domain (1.08×10^{-26}), RHS repeat domain (pfam 05593), and RhsA (COG3209; 8.75×10^{-17}), which is also present in the wall-associated surface protein (WASP) from *Bacillus subtilis* 168 (Fig. 2B) (Foster 1993). The WASP from *B. subtilis* contains 31 repeats of the peptide motif Gxxx(Y or F)xYDxxG, which closely resembles the *Rhs* core motif. Sequence analysis of the XadM protein indicated that it contains at least 18 repeats with the consensus gxxvYDxxg (Fig. 2B and C). Among these extensive repeat regions, three repeats with the consensus sequence motif [Gxxx(Y or F)xYDxxG] are similar to the WAPA motif present in a WASP of *B. subtilis* (Fig. 2B and C).

Interestingly, the C-terminal end of the XadM protein also exhibited similarity to a domain of unknown function (NLPC_P60; Fig. 2B), which is found in several lipoproteins (Anantharaman and Aravind 2003). Although the N-terminal of XadM (600 aa) did not exhibit similarity in the conserved domain database, analysis using PROSITE search revealed the presence of the RGD cell-attachment motif (Arg-Gly-Asp) (Fig. 2B) at amino acids 211 to 213. The tripeptide RGD has been found in numerous proteins, including multiple FHA from *Bordetella pertussis* and *X. fastidiosa* (Relman et al. 1990; Voegel et al. 2010), and it has been implicated in mediating cell adhesion through binding to integrin receptors (D'Souza et al. 1991a and b).

A search through the NCBI GenBank indicates that the predicted XadM protein exhibits significant similarity (amino acid identity of 99 to 71%) (Supplementary Table S2) to Rhs family proteins from closely related *X. oryzae* pv. *oryzae* strains and *X. oryzae* pv. *oryzicola* (a bacterial pathogen of rice that infect parenchyma tissue). It is interesting to note that the Rhs family proteins from *X. gardneri*, *X. albilineans* (a xylem-limited sugarcane pathogen), and *Burkholderia pseudomallei* exhibit similarity throughout the entire length of XadM, including the less-conserved N-terminal region (Supplementary Fig. S1). Phylogenetic analysis of XadM and other Rhs family homologs from different bacterial species indicates that it forms a more closely related group (clade) within *X. oryzae* strains and, in particular, with *X. gardneri*, *X. albilineans*, and *Burkholderia* spp. (Supplementary Fig. S2).

***xadM* is required for attachment and biofilm formation in *X. oryzae* pv. *oryzae*.**

Because several sequence features of the XadM proteins indicate that it may play a role in an attachment-like function, we did a bacterial attachment assay using rice leaves. In this assay, detached leaves of 30- to 40-day-old plants of rice were inoculated with different strains of *X. oryzae* pv. *oryzae* by dipping the leaves in bacterial culture. Wild-type (BXO43) *X. oryzae* pv. *oryzae* cells exhibited approximately 16-fold higher attachment than the *xadM* mutants (Fig. 3A; data shown for XadM1). Complementation of the XadM1 mutant with the wild-type *xadM* allele restored the adhesion deficiency phenotype (Fig. 3A).

To examine the role of *xadM* in attachment and biofilm formation of *X. oryzae* pv. *oryzae*, we did quantitative cell attachment and static biofilm assays using 12-well polystyrene culture plates as described previously (Chatterjee et al. 2010). It is important to note that the wild type (BXO43), *xadM* mutants, and *xadM* mutants harboring the complementing plasmid exhibited similar growth characteristics (growth rates) in liquid medium (data not shown). Specifically, we incubated the cells in 12-well polystyrene culture plates without shaking and then removed the planktonic bacteria (Fig. 3B and C). Cells strongly attached to the polystyrene culture plates were removed by repeated vortexing (three times, each for 40 s) with Milli-Q water and then plated with appropriate dilution to determine CFU of attached cells per well (discussed below). The XadM1 mutant exhibited approximately eightfold reduced attachment (Fig. 3B), 2 h postinoculation, compared with either BXO43 or XadM1(pSC2) (XadM1 mutant harboring the complement-

Fig. 1. *xadM* mutations affect *Xanthomonas oryzae* pv. *oryzae* virulence. Virulence assays were conducted by inoculating 40- to 50-day-old rice plants of the susceptible 'Taichung Native-1' (Tn-1) through either **A** and **B**, wound or **C**, epiphytic infection. BXO43 indicates the wild-type strain of *X. oryzae* pv. *oryzae*. **A**, Lesion lengths or **C**, efficiency of infection were used as a measure of virulence. **B**, Representative pictures of typical bacterial leaf blight (BLB) symptom caused by different strains of *X. oryzae* pv. *oryzae* by wound inoculation. For wound inoculation experiment, 15 leaves were inoculated, and the values are presented as mean lesion length $\pm$ standard deviation. **D**, Representative picture of typical water soaking symptom of BLB in epiphytic mode of infection by the wild-type (BXO43) strain. Arrow indicates lesion progression from the tip to the base of the rice blade. In epiphytic inoculation (surface inoculation), 20 leaves were inoculated, and the mean $\pm$ standard deviation of data from three independent experiments are presented. BXO43, the wild-type strain; XadM1 (BXO1701) = *xadM1*::mTn5; XadM2 = *xadM2*::pK18mobAp^r; XadM1(pSC2) and XadM2(pSC2) (XadM1 and M2 mutants harboring the complementing plasmid). Asterisk (*) indicates significant difference ($P < 0.001$) in comparison with the BXO43 (wild-type) strain.

ing plasmid). We also did quantification of bacterial cells attached to polystyrene culture plates 24 h postinoculation by Crystal Violet (CV), as described previously (Chatterjee et al. 2010) (Fig. 3D). Quantification of CV bound to the attached cells indicated that the XadM1 mutant exhibited reduced (approximately fourfold) attachment compared with the parental wild-type strain (BXO43) and XadM1 (pSC2) (Fig. 3D). This further corroborated that the *xadM* mutant is deficient in attachment and biofilm formation.

We studied the kinetics of biofilm formation by different strains of *X. oryzae* pv. *oryzae* by measuring the bacteria attached to the polystyrene culture plates in a 24-h time-course experiment (Fig. 3C). The time course of an attachment assay (Fig. 3C) indicated that the XadM1 mutant was consistently decreased in biofilm or attachment compared with either the BXO43 or XadM1(pSC2) (Fig. 3C).

Biofilm formation by different strains of *X. oryzae* pv. *oryzae* was also analyzed by confocal laser-scanning microscopy (CLSM) using bacterial live/dead staining. In this method,

bacterial biofilm was formed on glass slides at the air-media interface (Fig. 4) (discussed below). Analysis of the thickness of the biofilm formed using Z-projection of x-y stacks (optical sections) indicates that the XadM1 mutant formed biofilm with a considerably reduced thickness compared with the wild type (BXO43) and the XadM1(pSC2) strains (Table 2; Fig. 4). The average thickness of the biofilm formed by BXO43 and the XadM1 mutant harboring the complementing plasmid, XadM1 (pSC2), were approximately 14.03 and 14.2 μ m, respectively, after 48 h (Table. 2). In contrast, thickness of the biofilm formed by the XadM1 mutant was approximately 6.9 μ m after 48 h.

The *xadM* mutants exhibits alteration in colony or lawn morphology.

Wild-type (BXO43) *X. oryzae* pv. *oryzae* produces copious amounts of EPS and appears as a dense and opaque lawn or colony growing on rich medium after 5 days of growth at 28°C. Interestingly, all the *xadM* mutants (XadM1, XadM2, and XadM3; data shown for XadM1) exhibit an altered colony

Fig. 2. A, Schematic of the arrangement of open reading frames (ORF) based on partial sequence of 6,840-bp genomic region of *Xanthomonas oryzae* pv. *oryzae* BXO1 strain and comparison with the sequenced strain of *X. oryzae* pv. *oryzae* KACC10331. Arrows represent the ORF and direction of transcription in the KACC10331 genome. Open boxes below the predicted ORF indicate sequenced genomic regions from the BXO1 strain; these sequences were highly similar to the KACC10331 sequence. Dashed line indicates genomic region in BXO1 for which sequence was not determined. The predicted ORF *gsd2* and *IS1479*, which are located upstream of *xadM* in KACC10331, encode a glutamate synthase domain 2 and *IS1479* transposase. The predicted ORF downstream of *xadM* gene in KACC10331 exhibit high similarity to *ISXo8A* and *ISXo8B* putative transposases. Mutations in *xadM* affect *X. oryzae* pv. *oryzae* (BXO43) virulence. The open and filled circles indicate the location of mTn5 and pK18mobAp^r insertions, respectively. **B**, Schematic representation of sequence features of XadM. The N and C-terminal ends are indicated as 'N' and 'C'. The 35-amino-acid (aa)-long signal peptide (light gray bar) and the cell attachment motif (Arg-Gly-Asp; represented as RGD) are located at the N-terminal of XadM. Regions exhibiting homology to conserved domains are indicated as 1 and 2 = recombination hot spot (RHS) repeat domain (pfam 05593), 3= RHS repeat-associated core domain, and 4= domain of unknown function (NLPC_P60). Black bars indicates relative position of 18 repeats with the consensus motif (gxxyvYDxxg) within the XadM sequence. Numbers inside the open boxes represents the respective amino acid start and stop positions of these repeats. **C**, Multiple sequence alignment of the 18 repeats within the XadM sequence, each consisting of 12 aa residues. The corresponding amino acid start site of these repeats is indicated. Sequence motif Gxxxx(Y or F)xYDxxG which corresponds to the wall-associated protein of *Bacillus subtilis*, is repeated three times and indicated in gray shading. More conserved motifs (Y or F)xYDxxG and (Y or F)xYD are repeated eight and 14 times, respectively, among these 18 repeats.

or lawn morphology, which appears more translucent compared with the opaque colony or lawn formed by the wild-type (BXO43) strain (Fig. 5A; shown only for XadM1). Introduction of the complementing clone (pSC2) restores the wild-type (BXO43) pattern of colony or lawn morphology in these mutants (data not shown).

It has been reported from several bacteria that mutants altered in cell-wall-associated adhesins and cell-associated EPS (capsular polysaccharide) exhibit altered colony or lawn morphology, and adhesins have been proposed to play a role in attachment of cells with capsular polysaccharide (Enos-Berlage and McCarter 2000; Jobling and Holmes 1997; Swanson 1978).

We isolated secreted and cell-associated EPS from different strains of *X. oryzae* pv. *oryzae*. Specifically, secreted EPS was separated from the cells by centrifugation and the supernatant containing the soluble EPS was precipitated with acetone, as previously described (Dharmapuri and Sonti 1999). Cells harvested by centrifugation were dissolved in phosphate-buffered saline (PBS); and EPS, which is more closely associated with the cells, was removed by repeated vortexing (three times, each for 40 s), as described below. Quantification of both secreted and cell-associated EPS from the wild-type *X. oryzae* pv. *oryzae* strain (BXO43) indicates that, in general, the majority of the EPS produced by *X. oryzae* pv. *oryzae* is in the secreted form, which corresponds to approximately 94%, in contrast to the cell-associated EPS, which is approximately 6% of total EPS (Fig. 5B; Supplementary Fig. S3).

Quantification of secreted EPS from different strains of *X. oryzae* pv. *oryzae* indicated that there were no significant dif-

ferences in the amount of secreted EPS from either BXO43, XadM1 (*xadM1* mutant), or the XadM1(pSC2) (Fig. 5B). In contrast, there was approximately 10-fold less cell-associated EPS in the XadM1 mutant compared with the wild-type (BXO43) or XadM1(pSC2) strains (Fig. 5B).

We did cell-attachment assays with nitrocellulose membrane (NCM) coated with different polysaccharides and also with plant cell wall extract, as described previously (Killiny and Almeida 2009). In this experiment, NCM coated with different polysaccharides such as EPS, xylan, cellulose, and crude rice plant cell-wall extracts were incubated with bacterial cultures for 1 h. Bacterial cells attached to the NCM were isolated by washing the NCM with sterile distilled water and quantified by dilution plating. An attachment assay with different strains of *X. oryzae* pv. *oryzae* indicated that there was not much significant difference in attachment affinities of either BXO43, the XadM1 mutant, or XadM1(pSC2) toward NCM coated with bovine serum albumin (BSA) or with PBS buffer (Fig. 5C). In contrast to the XadM1 mutant, both the wild-type (BXO43) strain and XadM1(pSC2) exhibit significantly higher affinities toward NCM coated with either EPS (threefold) or with crude plant cell-wall extracts (fourfold) (Fig. 5C). Interestingly, both the wild-type (BXO43) strain and XadM1(pSC2) exhibited higher attachment efficiency than the XadM1 mutant toward NCM coated with polysaccharides xylan and cellulose, which are the major constituents of the rice cell wall. These results indicate that mutation in *xadM* reduces the efficiency of *X. oryzae* pv. *oryzae* cells to attach both bacterial EPS and plant xylan or cellulose.

Fig. 3. Cell attachment and static biofilm assay of *Xanthomonas oryzae* pv. *oryzae* strains BXO43 (wild type), XadM1 (*xadM1::mTn5*), and XadM1(pSC2) (XadM1 mutant harboring the complementing plasmid). Quantification of bacterial cells attached to **A**, the leaf surface and **B**, polystyrene culture plates. Fresh rice leaves were dipped in bacterial suspension of 10^9 cells/ml for an hour. Bacterial cells attached on the leaf surface were quantitated by dilution plating to determine CFU of attached cells per centimeter of leaf (y axis) and the values are presented as mean CFU/centimeter of leaf $\pm$ standard deviation from six leaves (three leaves each from two independent experiments). **B**, Bacterial cells attached to polystyrene culture plates were quantified by dilution plating after 2 h postinoculation and the values are presented as mean CFU/well $\pm$ standard deviation from six samples (three samples each from two independent experiments); * indicates significant difference ($P < 0.001$) in comparison with the wild-type (BXO43) strain in both leaf (A) and polystyrene plate (B) attachment assays. **C**, Kinetics of biofilm formation by different strains of *X. oryzae* pv. *oryzae* by measuring the bacteria attached to the polystyrene culture plates in a 24-h time-course experiment. Values on the y axis represent mean CFU/well $\pm$ standard deviation from six samples (three samples each from two independent experiments). **D**, Quantification of bacterial cells attached to polystyrene culture plates 24 h postinoculation by staining with Crystal Violet. Values are presented as mean absorbance (at 570 nm)/well $\pm$ standard deviation from eight replicates; ** indicates significant difference ($P < 0.01$) in comparison with the BXO43 strain.

XadM is required for early attachment and entry into rice leaves.

We did an assay for monitoring leaf attachment and entry by *X. oryzae* pv. *oryzae* by using CLSM and enhanced green fluorescent protein (EGFP)-tagged *X. oryzae* pv. *oryzae* strains, as described previously (Das et al. 2009). Briefly, rice leaves were inoculated with either BXO43, XadM1, or XadM1(pSC2) strains harboring the EGFP-expressing plasmid. The EGFP-expressing plasmid pMP2464 (Table 1) (Stuurman et al. 2000), which is derived from pBRR1MCS5, is compatible with pSC2 (IncW; pUFR03 plasmid harboring the wild-type *xadM* allele) (Table 1). Inoculations were performed by dipping the leaves in bacterial cultures for 1 h. Leaves were then washed to remove loosely adhering bacteria and examined in a confocal microscope. Beginning at the extreme tip of the leaf, optical sections parallel to the leaf dorsal surface were taken to detect EGFP fluorescence in three successive regions (each 200 by 200 by 90 μm^3 along the *x*, *y*, and *z* axes). For each strain, total counts of EGFP spots from this portion of the leaf were considered to be representative of the total number of cells of this strain that were associated with the leaf. An *X. oryzae* pv. *oryzae* cell was considered to be inside the leaf when the EGFP signal was found to colocalize with leaf red autofluorescence in the same *x*, *y*, and *z* axes (Fig. 6A; Supplementary Fig. S4). The data (Fig. 6A and B) indicate that the XadM1 mutant exhibits approximately 70% deficiency (Fig. 6A and B) in both leaf attachment and entry compared with either BXO43 or XadM1(pSC2).

XadM is exposed on the cell surface and its expression is regulated by growth conditions.

To study *xadM* expression, RNA was isolated from bacterial cells harvested from infected rice leaves as well as from *X. oryzae* pv. *oryzae* cells grown to an optical density at 600 nm (OD_{600}) of 1.3 in either rich, minimal (MM9), or plant-growth-mimicking media (XOM2). Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) using primers specific to *xadM* and the 16S rRNA control (Fig. 7A; Supple-

mentary Table S3) revealed that the expression of *xadM* was higher in cells either grown in XOM2 media (plant-growth-mimicking media) or harvested from the infected rice leaves, compared with the cells grown in either rich or minimal media (Fig. 7A). Real-time RT-PCR analysis indicated that the expression of *xadM* was approximately 200- and 20-fold higher in XOM2 media and in planta compared with the cells grown in rich peptone sucrose (PS) media (Fig. 7B). These results indicate that expression of gene *xadM* is induced in planta and is also influenced by growth conditions.

To study the localization of XadM, we used immunofluorescence microscopy, as described below (Buddelmeijer et al. 1998; McNulty et al. 2006). The localization of XadM was studied in the wild type (BXO43), the XadM mutant, and XadM1 (pSC2) (Fig. 8). Interestingly, in both the BXO43 and the XadM1(pSC2) strains, XadM could be visualized as several foci, which appeared to be surface associated (Fig. 8A). Although the XadM1 mutant exhibited faint staining under similar conditions, we could not detect foci-like intense staining in the XadM1 mutant, as compared with foci-like staining seen in either the BXO43 strain or the XadM1 mutant harboring the complementing plasmid, XadM1(pSC2) (Fig. 8A). Trypsin digestion of intact BXO43 *X. oryzae* pv. *oryzae* cells prior to immunostaining abolished the foci-like intense staining (Fig. 8B), which indicated that XadM is exposed on the cell surface. Fluorescence could not be detected under conditions in which only secondary antibodies were used (data not shown).

To further confirm the surface localization of XadM, we did fluorescent-activated cell sorting (FACS) analysis using an *xadM*-specific antibody (Fig. 9). Whole intact cells of *X. oryzae* pv. *oryzae* wild-type (BXO43) and XadM1 strains were treated with XadM-specific antibody prior to FACS analysis. A large population of fluorescent cells was detected after treatment of the wild-type cells with the XadM antibody (Fig 9A), indicating that XadM is exposed on the cell surface but such cells were not observed after treatment of the XadM mutant with the antibody (Fig 9B).

DISCUSSION

In this study, we described the characterization of a novel adhesion, XadM, of *X. oryzae* pv. *oryzae* that is homologous to the Rhs family of proteins and is required for optimum attachment, biofilm formation, and virulence. The Rhs family comprises a set of composite elements found in the chromosome of many naturally occurring *Escherichia coli* strains (Hill et al. 1994). The *rhs* elements have a prototype structure consisting of a 3.7-kb GC-rich core which is conserved between different subfamilies of *rhs* elements followed by an AT-rich region called the core extension which is specific to the particular *rhs* element (Hill et al. 1994). Based on sequence divergence, *rhs*

Fig. 4. Representative confocal laser-scanning microscopy images of biofilms formed on glass slides at the air-media interface by different *Xanthomonas oryzae* pv. *oryzae* strains BXO43, XadM1 (*xadM1::mTn5*), and XadM1(pSC2). Each image represents the layer in the Z-stack that has the maximum bacterial coverage. The 24-h biofilms of each strain were stained with BacLight LIVE/DEAD stain. The *x-z* and *z* axis reconstructions of each biofilm are shown on the left and right panels, respectively. Scale bars = 5 μm .

Table 2. Average biofilm thickness of different strains of *Xanthomonas oryzae* pv. *oryzae* formed on the glass slides at media-air interphase^a

Time (h)	Average thickness of biofilm (μm)		
	BXO43 (wild type)	XadM1	XadM1(pSC2)
24	9.6 $\pm$ 0.4	6.3 $\pm$ 0.5*	10.3 $\pm$ 0.6
48	14.03 $\pm$ 0.57	6.9 $\pm$ 0.63*	14.2 $\pm$ 0.63

^a Each biofilm was scanned with CLSM at six randomly selected positions and *x-z* color detection, corresponding to biofilm thickness, was determined throughout the height of the biofilm. Data are representative of three independent experiments. The results are expressed as the means $\pm$ standard deviations; * indicates significant difference ($P < 0.01$) compared with the wild-type (BXO43) strain. *P* values were determined by an unpaired Student's *t* test, two-sample, assuming unequal variances by using the of Microsoft Office Excel data analysis tool. $P < 0.05$ was considered statistically significant.

cores have been divided into three distinct subfamilies: *rhsA-B-C*, *rhsD-E*, and *rhsG* (Hill et al. 1994; Zhao et al. 1993). Analysis of the Rhs core protein indicates the presence of a peptide motif XXGX(1–3)XXRYXYDXXGRL(I/T) repeated 28 times (Zhao et al. 1993). This motif closely resembles the repeat motif of WapA, a wall-associated protein found in *Bacillus subtilis*, in which the repeated sequence is postulated to be a carbohydrate-binding domain (Foster 1993); and the YD repeat motif observed in teneurin-1, which binds heparin and bacterial polysaccharides (Minet et al. 1999). It has been proposed that the *E. coli* *rhs* genes are under strong positive selection, a feature that is also shared by adhesins and other surface proteins (Petersen et al. 2007).

An FHA of plant pathogens belonging to *Erwinia* spp., which causes diseases on wide variety of plants, has been shown to be required for attachment and virulence (Rojas et al. 2002). In the natural mode of infection (epiphytic), *X. oryzae* pv. *oryzae* gains entry into rice leaves by colonizing the rice leaf surface, particularly hydathodal openings which are concentrated at the tip of the leaf (Mew et al. 1984). It has been proposed that adhesins may play an important role in attaching to the surfaces lining the hydathodal openings and xylem vessels (Ray et al. 2002). Re-

cently, it has been shown that a non-fimbrial adhesin of *X. oryzae* pv. *oryzae*, which exhibits similarity to afimbrial adhesins of animal-pathogenic bacteria, is involved in promoting leaf attachment and entry on rice (Das et al. 2009). The *xadM* mutants of *X. oryzae* pv. *oryzae* exhibits virulence deficiency through either wound or epiphytic infection. An assay for early attachment and entry of *X. oryzae* pv. *oryzae* into rice leaves indicated that the *xadM* mutants are deficient in attachment to the rice leaf and entry through hydathodal openings. These results indicate that XadM plays an important role in the early colonization process of *X. oryzae* pv. *oryzae*, which involves attaching to the surfaces lining the hydathodes and xylem vessel. However, the *xadM* mutants did not exhibit complete loss of virulence and attachment properties. This may be due to the presence of several other adhesins, which may provide redundancy for these functions (attachment and biofilm formation). In our leaf attachment assay, we have observed that the number of cells attached to the leaf was low even though we have used highly concentrated inoculum (10^9 CFU/ml). The rice leaf epidermis presents a highly hydrophobic, wax-coated, cuticular surface for attachment and we think that this might be a reason for the low number of cells attached on the leaf surface.

Fig. 5. A, *XadM* mutant exhibits altered colony or lawn morphology, which appears more translucent (indicated by arrow) compared with opaque colony formed by the wild-type strain of *Xanthomonas oryzae* pv. *oryzae* (BXO43). **B**, Quantification of secreted (striped bars) and cell-associated (dotted bars) extracellular polysaccharide (EPS) from different strains of *X. oryzae* pv. *oryzae* BXO43, XadM1 (*xadM1::mTn5*), and XadM1(pSC2) (XadM1 mutant harboring the complementing plasmid). Values are presented as mean EPS expressed as milligrams of glucose equivalents/ 10^9 cells $\pm$ standard deviation from six samples (three samples each from two independent experiments). Numbers on the top of each dotted bars (cell-associated) indicate the EPS quantity; * indicates $P < 0.01$ for cell-associated EPS. **C**, Attachment affinities of different strains of *X. oryzae* pv. *oryzae* to nitrocellulose membranes coated with polysaccharides (EPS, cellulose, and xylan), plant cell wall extracts, and bovine serum albumin (BSA). Bacterial cells attached to the nitrocellulose membrane were quantitated by dilution plating. Values are presented as average CFU of attached cells per square centimeter of the nitrocellulose membrane $\pm$ standard deviation from six samples (three samples each from two independent experiments). PBS = phosphate-buffered saline.

Biofilm formation is an important trait for pathogenicity of many animal- as well as plant-pathogenic bacteria (Danhorn and Fuqua 2007; Parsek and Singh 2003). Adhesins play an important role in both cell-to-cell adhesion and cell-to-surface attachment along with EPS, which contribute to initial colonization, attachment, and subsequent biofilm formation (Danhorn and Fuqua 2007). It is interesting to note that, in *Xylella fastidiosa*, an insect-transmitted, xylem-limited bacteria, different sets of adhesins have been proposed to be involved in cell-to-cell attachment and adherence to the host cell surface (Feil et al. 2007). The *xadM* mutant exhibited reduced biofilm formation, which corroborates with its reduced attachment efficiency

Fig. 6. XadM is required for early attachment and entry of *Xanthomonas oryzae* pv. *oryzae* into rice leaves. **A**, Inoculation of rice leaves with enhanced green fluorescent protein-tagged *X. oryzae* pv. *oryzae* strains was done and confocal microscopy-based assays were conducted 1 h after inoculation. Panels depict confocal microscope-based projection images (200 by 200 by 60 μm^3 in the *x*, *y*, and *z* axes, respectively, beginning from the dorsal surface) of rice leaves inoculated with the wild type (BXO43), XadM1 (*xadM*1 mutant), and XadM1(pSC2). Scale bar: 20 μm . **B**, Reduction of leaf attachment and entry is associated with *xadM* mutation of *X. oryzae* pv. *oryzae*. The number of enhanced green fluorescent protein-expressing cells of different strains of *X. oryzae* pv. *oryzae* on the surface as well as inside rice leaves after 1 h of infection was determined using confocal microscopy. Data were collected up to approximately 600 μm in length from the tip of the leaf and a distance of approximately 80 μm in depth from the dorsal surface of each leaf. Each bar shows mean and standard deviation of values obtained from three leaves. Values obtained for the XadM1 mutant were found to be significantly different ($P < 0.05$) compared with either the wild-type strain or the XadM1 mutant harboring the complementing plasmid, XadM1(pSC1), in a Student's two-tailed *t* test for independent means. Similar results were obtained in independent experiments.

on leaf, polystyrene, and glass surfaces. This indicates that XadM also contributes to biofilm maturation by probably mediating cell-to-cell or cell-to-surface attachment.

XadM mutants of *X. oryzae* pv. *oryzae* exhibit a translucent colony or lawn morphology compared with the wild-type strain (BXO43). It has been reported that, in *Neisseria gonorrhoeae* (an animal-pathogenic bacteria), mutants deficient in expression of outer membrane adhesin proteins, which are involved in adherence to the host cell surface, exhibit a translucent colony phenotype compared with opaque colonies formed by the wild-type (BXO43) cells (Lambden et al. 1979; Makino et al. 1991; Swanson 1978). It has been proposed that the outer membrane protein adhesins of *N. gonorrhoeae* recognize carbohydrate structures on the surface of human cells, and that some members of the outer membrane adhesin family confer intergonococcal (cell-to-cell) adherence, probably involving the carbohydrate moieties of gonococcal polysaccharide (Blake et al. 1995). It has been reported earlier that an *X. oryzae* pv. *oryzae* mutant in the *xadA* gene, which encodes a nonfimbrial adhesin, exhibits altered colony or lawn morphology, and it has been proposed that this also might be involved in binding to the EPS as well as to the host cell wall component (Ray et al. 2002). The translucent phenotype exhibited by the XadM mutants of *X. oryzae* pv. *oryzae* may be due to weak attachment or association of cells with secreted EPS. The *xadM* mutants exhibited deficiency in attachment affinities to NCM coated with EPS and plant cell wall polysaccharides (cellulose and xylan), as well as with the plant cell wall extracts. This indicates that XadM is required for optimum binding of *X. oryzae* pv. *oryzae* to host cell-wall components. Intriguingly, it has been shown that a member of the *rhs* subfamily, RhsA, in *E. coli* is associated with an outer membrane capsular polysaccharide biosynthesis or export protein complex and an *rhsA E. coli* mutant is altered in K5 capsular polysaccharide produc-

Fig. 7. **A**, Expression analysis of *xadM* by semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Lanes containing RT-PCR products amplified from total RNA isolated from *Xanthomonas oryzae* pv. *oryzae* wild-type (BXO43) cells either grown in 1 = PS (rich media), 2 = XOM2 (plant-growth-mimicking media), or 3 = MM9 (minimal media) or harvested from infected rice leaves. Primer pairs RT ExpF1/RT ExpR1 (for *xadM*) and 16S rRNA F/16S rRNA R (16S rRNA) yielded the expected DNA fragments of 1.2 and 1.5 kb, respectively. Lanes: -RT indicates without reverse transcriptase and lane M = 1-kb DNA molecular weight marker. **B**, Expression analysis by real-time RT-PCR. Amount of RNA relative to that in the wild-type (BXO43) *X. oryzae* pv. *oryzae* cells grown in PS media is equal to 1.0 and is normalized for 16S ribosomal RNA as an endogenous control. Standard error was calculated based on three independent experiments.

tion (McNulty et al. 2006). It has been proposed that RhsA is involved in coupling and stabilizing capsular biosynthesis and export machinery, and probably is also involved in binding to capsular polysaccharide (McNulty et al. 2006). A very recent study reports that the *Pseudomonas aeruginosa* RhsT (a member of the Rhs family proteins) is induced upon contact with, and transported into, phagocytic cells, where it activates the inflammasome (Kung et al. 2012).

Real-time RT-PCR analysis demonstrated that the expression of *xadM* is induced inside the host (in planta) as well as in plant-growth-mimicking media. We could not identify any consensus plant-inducible promoter box-like sequence in the upstream region of the *xadM* gene by in silico analysis. It is possible that the expression of gene *xadM* is driven by a plant-inducible promoter which has not yet been identified. Future experiments are intended to understand the regulatory mechanism of *xadM* expression.

Our localization studies with anti-XadM antibody by immunofluorescence microscopy and FACS analysis demonstrates that XadM is exposed on the cell surface. Trypsin treatment of physiologically intact wild-type (BXO43) *X. oryzae* pv. *oryzae* cells abolished the surface localization, which corroborates these findings.

In summary, we identified a novel cell surface-localized adhesin of *Xanthomonas* spp., XadM, which exhibits similarity to the Rhs family of protein. XadM-like proteins are widely distributed among many Xanthomonads as well as in other bacteria, including animal-pathogenic *Burkholderia* spp. We show that XadM of *X. oryzae* pv. *oryzae* is required for optimum virulence, attachment to the host cell surface, and biofilm formation, which are all important factors for host colonization and pathogenicity. To the best of our knowledge, this is the first report that demonstrates that a member of the Rhs family of proteins functions as adhesin and is required for attachment and virulence in bacteria.

Fig. 8. A, Immunofluorescent microscopy of *Xanthomonas oryzae* pv. *oryzae* strains BXO43 (wild type), XadM1 (*xadM1::mTn5*), and XadM1(pSC2) bacteria that were fixed but not permeabilized using antisera specific for XadM. Cells were treated. After fixation, the cells were treated with XadM-specific antisera followed by staining with secondary antibody (goat anti-rabbit immunoglobulin G-FITC) and observed in a confocal microscope under a $\times 100/1.4$ oil DIC objective (LSM 510, META; Carl Zeiss, Germany). Argon and infrared lasers were used for detecting FITC, seen as red pseudo color instead of green (excitation = 488 nm and emission = band pass 500 to 550 nm). **B,** Immunofluorescent microscopy of *X. oryzae* pv. *oryzae* BXO43 (wild-type) cells pre-treated with trypsin prior to immune staining with XadM-specific antisera. Bars = 2 μ m. Inset boxes shows a $\times 4$ enlargement of a typical cell from each of the respective confocal images.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

The bacterial strains and plasmids used in this study are described in Table 1. The wild-type *X. oryzae* pv. *oryzae* used in this study (BXO43) is a spontaneous rifampicin-resistant (Rif^r) derivative of BXO1 (Table 2). To aid in the subsequent genetic analysis and to use a selection marker for conjugation experiments, we have used BXO43 (Rif^r derivative of BXO1) as the wild-type strain in this study as well as in several of our earlier works (Chatterjee and Sonti 2002; Chatterjee et al. 2003; Goel et al. 2001; Rajagopal et al. 1995). The virulence properties of the *X. oryzae* pv. *oryzae* (BXO43) Rif^r derivative are similar to those of its respective parent, BXO1 strain (Goel et al. 2001; Rajagopal et al. 1995). *X. oryzae* pv. *oryzae* strains were grown on rich media or peptone sucrose agar (PSA) or in PS broth at 28°C, with shaking at 200 rpm, as described previously (Tsuchiya et al. 1982). For growth of *X. oryzae* pv. *oryzae* in minimal medium (M4) (Kelemu and Leach 1990) and XOM 2 media (minimal media which induces *hrp* genes in *X. oryzae* pv. *oryzae*) (Tsuge et al. 2002), strains were first grown in PS medium to a cell density of 1×10^{10} cells/ml, then concentrated by centrifugation at 7,000 rpm and washed with autoclaved double-distilled water (DDW). Washed cells (1×10^7 cells/ml) were inoculated in M4 and XOM2 medium and grown for 20 h. Antibiotics were used at a concentration of 50 µg/ml for rifampicin, 50 µg/ml for spectinomycin (Sp), 100 µg/ml for ampicillin (Ap), 10 µg/ml for gentamycin, and 50 µg/ml for kanamycin.

Virulence assays on rice plants.

Virulence assays were carried out by two different methods: wound inoculation and surface inoculation. For wound inoculation, greenhouse-grown rice plants (55 days old) of the susceptible rice 'Taichung Native-1' (TN-1) were inoculated by clipping leaf tips with sterile scissors dipped in cultures (10^9 cells/ml resuspended in sterile DDW) of *X. oryzae* pv. *oryzae* (Kauffman et al. 1973). The plants were incubated in a greenhouse with minimum and maximum temperatures of approximately 25 and 30°C, respectively, and a relative humidity of approximately 60%. Lesion lengths were measured at regular intervals. No lesions were observed in control experiments in which the leaves were clipped with scissors dipped in water. In each experiment, 15 leaves were inoculated, and the values are presented as mean lesion length $\pm$ standard deviation. Similar

results were obtained in independent experiments. For surface inoculation (epiphytic infections) (Ray et al. 2002), 40- to 50-day-old TN-1 rice plants were placed inside the greenhouse within a humidity chamber (made 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 1×10^9 CFU/ml for 5 to 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 16 days after inoculation. In each experiment, 20 leaves were inoculated, and the mean $\pm$ standard deviation of data from three independent experiments is presented.

Molecular biology techniques and construction of *X. oryzae* pv. *oryzae* mutants.

All standard molecular biology and genetics techniques were performed as described previously (Sambrook et al. 1989). PCR amplification was done using PCR amplifications performed with Pfu polymerase (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. Restriction digestion and ligation reactions were performed with enzymes obtained from New England Biolabs (NEB; Beverly, MA, U.S.A.), as per the manufacturer's instructions. For cloning of the mTn5 insertion, genomic DNA of BXO1701 (XadM1; *xadM1::mTn5*) was isolated as described by Leach and associates (1990) and digested to completion with *Cla*I (NEB), which does not cut within the mTn5 element (Wilson et al. 1995). pSC1 was obtained after ligation of this DNA into the *Cla*I site of pBSKS+ and selecting *E. coli* DH5 α transformants on Luria-Bertani medium containing Ap and Sp (mTn5 encodes Sp-resistant [Sp^r]). A 7.5-kb *Cla*I fragment is present in pSC1. The XadM3 marker exchange mutant was obtained by electroporating 300 ng of plasmid DNA (pSC1) individually into competent cells of BXO43 (wild type). The transformants were grown in PS for 2 h and then plated on PSA + Sp (for mTn5). Colonies that were Ap-sensitive Sp^r (for mTn5) were analyzed by Southern hybridization to confirm that marker exchange had occurred as expected. The XadM2 mutant (*xadM2::pK18mob*[Ap-resistant]Ap^r) was generated by integration of recombinant (pSC3) pK18mobAp^r plasmid through homologous recombination. An internal fragment of the *xadM* gene that is to be mutated was PCR amplified from strain BXO43 (wild type) genomic DNA using primers pK18Ko F *Eco*RI and pK18Ko R *Eco*RI, cloned in pK18mob

Fig. 9. Fluorescence-activated cell sorting (FACS) analysis of *Xanthomonas oryzae* pv. *oryzae* strains **A**, BXO43 and **B**, XadM1 mutant using antisera specific for XadM. Cells were labeled and then subjected to FACS analysis. The y axis denotes number of cells and the x axis denotes increased fluorescence at 488 nm. α -FITC-only and α -XadM-only controls omit the primary and secondary antibodies, respectively.

plasmid to obtain pSC3. Biparental matings were set up between *E. coli* S17-1 harboring recombinant pK18mobAp^r plasmid and *X. oryzae* pv. *oryzae* strains. Integration events of the recombinant plasmid into the recipient chromosome were isolated by selection for Ap resistance. Gene disruption was confirmed by PCR and sequencing of the PCR products using primers Con F and ConR, which are located outside of the cloned fragment in pSC3. This process resulted in nonpolar mutations when the transcriptional orientation of the gene of interest and the promoter of *lacZ* gene in pK18mob were in the same direction (Wei et al. 2007; Windgassen et al. 2000).

Isolation of a genomic clone containing the *xadM* gene.

Individual clones (720, with an average insert size of 30 kb) from a genomic library of *X. oryzae* pv. *oryzae* BXO1 strain (Rajagopal et al. 1995, 1999) maintained in DH5 α were pooled in groups of 12. The pooled cosmid clones were grown and cosmid DNA was isolated by the alkaline lysis method (Sambrook et al. 1989). Pooled plasmid DNA (30 ng) was used for PCR, as described previously (Chatterjee et al. 2003) using *xadM*-specific forward and reverse primers (ConF and ConR). In all, 1 of 60 cosmid pools gave the expected size band (1.06 kb) with the ConF and ConR primers. The 12 individual clones of the positive pool were again screened by PCR, using the same forward and reverse primers. One positive clone (pSC2) with a 31-kb insert was characterized further. Restriction digestion with *EcoRI* and Southern hybridization indicated that the insert contained two *EcoRI* fragments of 25 and 6 kb.

Biofilm and attachment assay.

For attachment assays on the leaf surface, *X. oryzae* pv. *oryzae* cells were inoculated in PS medium (20 ml) supplemented with appropriate antibiotics and grown to early stationary phase, pelleted down, washed, and resuspended in sterile Milli-Q water at a concentration of 10⁹ cells/ml. Fresh leaves (with intact and uninjured tips) from 30- to 40-day-old rice plants (*X. oryzae* pv. *oryzae*-susceptible TN-1) were detached and dipped for an hour at 28°C in bacterial suspension up to a distance of approximately 3 cm from the tip. Leaf pieces extending up to a distance of 2 to 3 cm from the leaf tip were cut with scissors and washed with sterile Milli-Q water to remove loosely attached cells. Leaves (1 cm from the tip) were then taken and vortexed in water for 1 min to remove attached cells and dilution plated to determine CFU of attached cells per centimeter of leaf.

For static biofilm and attachment assay, approximately 1 $\times$ 10⁷ cells/ml were inoculated in 5 ml of PS media in 12-well polystyrene culture plates (Chatterjee et al. 2010). The culture plates were incubated for 24 h at 28°C without shaking. The 12-well polystyrene culture plates were removed, media was discarded, and the wells were washed with DDW to remove planktonic and loosely attached cells at different time points during the 24-h incubation. Cells strongly attached to the polystyrene culture plates were removed by repeated vortexing (three times, each for 40 s) with Milli-Q water and then plated with appropriate dilution to determine CFU of attached cells per well. CV staining for the quantification of attached cells in 12-well polystyrene plates was done as described previously (Chatterjee et al. 2010). Briefly, 1 $\times$ 10⁷ cells/ml were inoculated in 5 ml of PS media in 12-well polystyrene culture plates. The culture plates were incubated for 24 h at 28°C without shaking. After 24 h of incubation, the medium was discarded and the wells were washed with DDW to remove planktonic and loosely attached cells. The adherence was monitored by staining the remaining cells with 1% CV for 10 min at room temperature. Excess CV stain was removed by washing the

wells with DDW. CV bound to the attached cells was solubilized with 1 ml of 90% ethanol and quantified by measuring the absorbance at 570 nm.

For visualization of biofilm formation, *X. oryzae* pv. *oryzae* strains were inoculated at PS media at a concentration of 10⁵ cells/ml in 50-ml falcon tubes containing a sterile glass slide partially dipped in media. Tubes were incubated at 28°C and 200 rpm for 48 h. Biofilms formed at the media–glass slide interface were washed with sterile Milli-Q water and stained with LIVE/DEAD BacLight Bacterial Viability Kits (L7012; Invitrogen, Eugene, OR, U.S.A.) as per the manufacturer's instructions, mounted with 25% glycerol, and analyzed at different time points using a confocal laser-scanning microscope (LSM510 META; Carl Zeiss, Promenade, Jena, Germany). Three-dimensional images were reconstructed using Zeiss LSM Image Browser, version 4.2.0.121. To determine biofilm thickness, a series of horizontal (xz) optical sections at 0.5- μ m intervals were taken through the height of the biofilm for measurement. Each biofilm was scanned at five randomly selected positions. Each sample was observed independently more than three times. Confocal images of green (SYTO 9; excitation and emission = 485 and 530 nm, respectively) and red (propidium iodide, red color; excitation and emission = 485 and 630 nm, respectively) fluorescence were constructed simultaneously using a multitrack mode.

Isolation and quantification of secreted and cell-associated (cell-bound) EPS.

Cells were harvested from the PSA plates and EPS was isolated by precipitation with acetone, as previously described (Dharmapuri and Sonti 1999), and quantitated by the colorimetric method for estimation of pentoses and hexoses by phenol sulfuric acid method, as described previously (Dharmapuri and Sonti 1999). Briefly, *X. oryzae* pv. *oryzae* cells were grown on PSA plates for 3 days at 28°C. Cells along with secreted EPS were harvested from the plates by washing with PBS. Cells were centrifuged at 6,000 rpm for 20 min and the supernatant containing the secreted EPS was precipitated with ice-cold acetone. For isolating cell-associated (cell-bound) EPS, cells harvested by the above-mentioned method was resuspended in PBS, vortexed for 2 to 3 min, and pelleted down by centrifugation, and the supernatant was precipitated separately with acetone. Cells were plated with appropriate dilution to determine CFU.

Attachment assay using NCM coated with different polysaccharides.

Cell-wall extracts from fresh rice leaves were isolated as described previously (Moore et al. 2006). NCM were coated with different polysaccharides as described previously (Killiny and Almeida 2009). Pieces of NCM (1 cm²) were incubated with 1 ml of PBS containing 1% (wt/vol) of different polysaccharides, BSA (Sigma Aldrich, St. Louis), and lyophilized plant cell wall extract (1% wt/vol) under slow shaking conditions for 1 h at room temperature. Polysaccharides tested included cellulose, xylan (Sigma-Aldrich), and EPS (secreted EPS of *X. oryzae* pv. *oryzae*). After 1 h of incubation, the NCM were washed three times (each 15 min) with 0.3% Tween-20 in PBS (PBS-Tween). *X. oryzae* pv. *oryzae* cells were grown to early stationary phase in PS medium supplemented with appropriate antibiotics and pelleted down, washed, resuspended in PBS at a concentration of 1 $\times$ 10⁹ cells/ml, and incubated with the NCM for 1 h. Bacterial cells strongly attached to the NCM were harvested by repeated washing and vortexing (three times, each for 40 s) with Milli-Q water, then plated with appropriate dilution to determine CFU of attached cells per square centimeter of NCM.

Assay for early attachment and entry of *X. oryzae* pv. *oryzae* into rice leaves.

Leaf attachment and entry by *X. oryzae* pv. *oryzae* by using CLSM and EGFP-tagged *X. oryzae* pv. *oryzae* strains was done as described previously (Das et al. 2009). In this method, *X. oryzae* pv. *oryzae* cells harboring pMP2464 (expressing EGFP) (Stuurman et al. 2000) were inoculated in PS medium (4 ml) supplemented with appropriate antibiotics and grown to early stationary phase, pelleted down, washed, and resuspended in sterile Milli-Q water at a concentration of 1×10^9 cells/ml. Fresh leaves (with intact and uninjured tips) from 30- to 40-day-old rice plants (*X. oryzae* pv. *oryzae*-susceptible TN-1) were detached and dipped for an hour at 28°C in bacterial suspension up to a distance of approximately 3 cm from the tip. Leaf pieces extending up to a distance of 2 to 3 cm from the leaf tip were cut with scissors, vortexed in water for 10 to 15 s, and observed in a confocal microscope under $\times 63$ water objective (LSM 510, meta; Carl Zeiss). The excitation maximum was at 488 nm (argon laser) and the emission maxima were observed in band pass (BP) 510 to 530 nm (for EGFP fluorescence) and BP 650 to 710 nm (for red autofluorescence of leaf).

Optical sections of the *X. oryzae* pv. *oryzae*-inoculated leaves were taken in a plane parallel to the dorsal leaf surface. Scanning of the leaves was done in three successive regions, each with a field area of approximately 200 by 200 μm (along the x and y axes) up to the first 600 μm from the very tip of the leaf and for a depth of up to a maximum of 90 μm from the dorsal surface of the leaf, with 1- to 3- μm thickness of each optical section. The total number of EGFP spots in this region of the leaf was counted and considered to be representative of the total number of bacterial cells associated with the leaf (Das et al. 2009). For each strain, at least three leaves were observed and the mean number of cells per leaf was calculated. Each set of experiments was carried out at least three times. The images were analyzed using the software Zeiss LSM image examiner (Carl Zeiss).

Expression analysis by semiquantitative and real-time RT-PCR.

For RNA isolation, *X. oryzae* pv. *oryzae* cells were grown to an OD_{600} of 1.3 in PS (rich media), MM9 (minimal medium), and XOM2 medium (plant-growth-mimicking media). Bacteria from infected rice leaves were isolated as described previously (Rajeshwari et al. 2005). Briefly, leaves from rice plants infected with BXO43 wild-type strains of *X. oryzae* pv. *oryzae* (10 leaves) were cut 10 days after inoculation at the region where the lesion ended, and the cut surface was placed in 1 ml of water in Eppendorf tubes for 1 h to allow the xylem sap and bacteria to flow into the water. The bacterial cells were pelleted in a microfuge and processed for RNA isolation. The RNA was isolated by the Trizol (Invitrogen) method as per the manufacturer's instructions. Real-time RT-PCR was done as described previously (Chatterjee et al. 2008).

Raising of polyclonal antibodies to XadM peptide, immunofluorescence microscopy, and FACS analysis.

To predict potential antigenic sites, the XadM protein sequence was analyzed by the Hopp-Woods scale. Out of several predicted regions, we selected peptide N-RPGQNST DSRWR VR-C (peptide-1), because it is located at the unique N-terminal region of the XadM protein (corresponding to amino acids 386 to 399 of the XadM). Peptide-1 conjugated with BSA was obtained from SCL Biosciences (SCL, Bangalore, India) and used for raising polyclonal antibodies in rabbit by standard protocols (Sambrook et al. 1989). The antiserum could detect 1 μg of peptide-1 at a dilution of 1:12000.

Immunofluorescence localization was done as described previously (Buddelmeijer et al. 1998; McNulty et al. 2006). *X. oryzae* pv. *oryzae* strains were grown in XOM2 medium to early stationary phase. Whole-cell trypsin treatment was carried out as described by Lattemann and associates (2000). Cells were resuspended in PBS to a concentration of 1×10^8 CFU/ml. For trypsin treatment, cells were incubated at 37°C for 10 min with trypsin (50 $\mu\text{g}/\text{ml}$) before washing twice in PBS to remove trypsin. Cells were fixed in 2.8% formaldehyde and 0.04% glutaraldehyde in growth medium at room temperature for 15 min. The cells were centrifuged at $8,000 \times g$ for 5 min, washed three times with PBS, and incubated in 0.1% Triton X-100 in PBS for 15 min at room temperature. Nonspecific binding sites were blocked by incubating the cells in 0.5% (wt/vol) blocking reagent (Boehringer Mannheim, Mannheim, Germany) in PBS for 30 min at 37°C. Primary antibody was diluted in blocking buffer (1:1000) and incubation was carried out for 2 h at 37°C. The cells were washed three times with PBS containing 0.05% (vol/vol) Tween-20 (PBST). Incubation with secondary antibody (goat anti-rabbit immunoglobulin G-FITC; Invitrogen) diluted in blocking buffer (1:500) was carried out for 30 min at 37°C. The cells were washed again three times in PBST. The cells were fixed on the glass slides by air drying and observed in a confocal microscope under a $\times 100/1.4$ oil DIC objective (LSM 510, META; Carl Zeiss). Argon and infrared lasers were used for detecting FITC (excitation = 488 nm and emission = BP 500 to 550 nm) fluorescence. Images were analyzed using the software Zeiss LSM image examiner (Carl Zeiss).

For FACS analysis, cells were grown to midlogarithmic phase and harvested, and the pellets were resuspended in 1 ml of PBS as described previously (McNulty et al. 2006). The cells were then diluted 1:2 in PBS and were harvested by centrifugation ($11,600 \times g$, 20 min, room temperature) in a desktop centrifuge (Sigma Mini-Spin). The cells were then resuspended in a final volume of 50 μl in dilution of 1:100 of primary antibody (α -XadM) and incubated overnight at 4°C. Following incubation, cells were centrifuged as before at $11,600 \times g$ and washed three times in 500 μl to PBS to remove any unbound antibody. The cell pellets were resuspended in 50 μl in 1:200 dilution of the FITC-conjugated secondary antibody (Invitrogen) and incubated at room temperature for 1 h. Cells were then washed as before, resuspended in 300 μl of PBS, and sorted on the basis of fluorescence intensity using a FACS Sort fluorescence-activated cell sorter (BD FACS Aria III; BD Biosciences, San Jose, CA, U.S.A.). Blue laser was used for FITC (excitation = 488 nm and emission = 530/30nm). Raw data were analyzed by using software Flow Jo 7.6.4 (Tree Star, Inc.).

Statistical analysis.

P values were determined by an unpaired Student's t test, two-sample, assuming unequal variances, using the Microsoft Office Excel data analysis tool. $P < 0.05$ was considered statistically significant.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ExPASy Bioinformatics PROSITE database: www.expasy.ch/prosite
 Colorado State University Molecular Toolkit Protein Hydrophobicity Plot page: www.vivo.colostate.edu/molkit/hydropathy/index.html