Role of an In Planta–Expressed Xylanase of *Xanthomonas oryzae* pv. *oryzae* in Promoting Virulence on Rice

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Xanthomonas oryzae pv. *oryzae* is the causal agent of bacterial leaf blight, a serious disease of rice. We demonstrated earlier that the type II secretion system (T2S) is important for virulence of *X. oryzae* pv. *oryzae* and that several proteins, including a xylanase, are secreted through this system. In this study, the *xynB* gene encoding for the secreted xylanase was cloned as a 6.9-kb *EcoRI* fragment (pRR7) that also included a paralog called *xynA*. As in *X. oryzae* pv. *oryzae*, *xynA* and *xynB* are adjacent to each other in *X. axonopodis* pv. *citri*, whereas only the *xynA* homolog is present in *X. campestris* pv. *campestris*. Mutations in *xynB* but not *xynA* affect secreted xylanase activity. Western blot analysis using anti-XynB antibodies on exudates from infected rice leaves indicated that this xylanase is expressed during in planta growth. Another T2S-secreted protein was identified to be a lipase/esterase (LipA) based on the sequence tags obtained by tandem mass spectrometry analysis and biochemical assays. Mutations in either *xynB* or *lipA* partially affected virulence. However, a *lipA-xynB* double mutant was significantly reduced for virulence, and the pRR7 clone containing an intact *xynB* gene could complement the virulence-deficient phenotype of the *lipA-xynB* mutant. Our results suggest that there is functional redundancy among the T2S secreted proteins of *X. oryzae* pv. *oryzae* in promoting virulence on rice.

Additional keywords: cell-wall-degrading enzymes, rice pathogen.

The cellulose and hemicellulose components of plant cell walls, which provide strength and rigidity, also act as a barrier for pathogens. Cell-wall-degrading enzymes, including cellulases, pectinases, xylanases, and proteases, are secreted by phytopathogenic bacteria for breaking down the components of host cell walls. The presence of 30 to 40 genes potentially encoding for cell-wall-degrading enzymes in the genome sequences of *Xanthomonas campestris* pv. *campestris* and *X. axonopodis* pv. *citri* is an indicator of the likely importance of these genes in the plant pathogenesis of xanthomonads (da Silva et al. 2002).

Xylan is an important component of the hemicellulose complex in plant cell walls. The cell walls of rice and other grasses have an unusually high composition of xylan, accounting for

up to 60% of the total cell wall carbohydrate (Caprita and Gibeau 1993; Takeuchi et al. 1994). Xylan is a polymer consisting of a linear backbone of 1,4-linked D-xylanopyranosyl residues and side chains of arabinose, glucuronic acid, or methylglucuronic acid. Additional modifications in the form of acetyl and phenolic esters contribute to the complexity of xylan. Several sets of enzymes are required to degrade xylan to xylose monomers. The endo- or exo- β -xylanases hydrolyze the backbone to yield xylo-oligosaccharides. Another enzyme, β -xylosidase, cleaves the 1-4- β linkages to yield xylose monomers. Additional sets of enzymes are required for cleaving the side-chain residues, which vary depending on the nature of the side chains among different plant types. Of particular relevance to this study are the esterases that are involved in hydrolyzing acetyl ester and phenolic ester bonds in xylan. Xylanases and esterases as well as the other enzymes that cleave the side chains have been shown to act cooperatively in degrading xylan (Bachmann and McCarthy 1991; Biely et al. 1986; Saha and Bothast 1999).

Xylanases have been isolated from a wide range of microorganisms (Kulkarni et al. 1999) and can be assigned to two major families (10 or F and 11 or G) of glycosyl hydrolases, based on the primary sequence classification scheme of Henrissat and Bairoch (1993). The two families differ significantly in molecular mass, isoelectric points, substrate preferences, and the nature of oligoxylosides generated as products (Biely et al. 1997). Family 10 xylanases are larger (30 kDa and above), more complex, produce smaller oligosaccharides, and fold into α/β barrel, with approximately 40% of the secondary structure of the enzyme being α -helix. Family 11 members are much smaller (<30 kDa), fold into structures composed primarily of β -strands, and are more specific for xylan. The differences in their structural domains may have evolved as a result of the abundance and heterogeneity of xylan in plant cell walls.

We had earlier demonstrated that a type II protein secretion system (T2S) is required for virulence of *X. oryzae* pv. *oryzae* and several proteins, including a xylanase, are secreted through this system (Ray et al. 2000). In this article, we describe the characterization of *xynB*, the gene for secreted xylanase, and assess its role in virulence of *X. oryzae* pv. *oryzae*.

RESULTS

Cloning and sequence of *xynB* gene encoding a secreted xylanase.

Two degenerate oligonucleotide primers were used to amplify an 800-bp xylanase gene fragment by polymerase chain reaction (PCR) from genomic DNA of *X. oryzae* pv. *oryzae*. The cloned product (pXyn8) exhibited approximately 60% iden-

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tity at the amino acid level to family 10 xylanases from a wide range of microorganisms, including *Rhodothermus marinus*, *Aspergillus niger*, and *Magnaporthe grisea*. The 800-bp fragment was used to clone a 6.9-kb genomic *Eco*RI fragment (pRR7) which was further sequenced.

The 6,904-bp sequence revealed the presence of one incomplete and four complete open reading frames (ORFs) (Fig. 1). Sequence comparisons with the genome sequences of *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* showed high homology to genes in these organisms. The incomplete ORF1 (*sidA*) of 378 bp exhibited 97% nucleotide sequence identity to putative TonB-dependent receptor genes from *X. axonopodis* pv. *citri* (AAM39091) and *X. campestris* pv. *campestris* (AAM43341) as well as 57% identity and 71% similarity at the amino acid level to a TonB-dependent receptor from *Caulobacter crescentus* (accession number AAK24796). ORF2 encoded a 493-amino-acid (aa) protein, which showed substantial similarity to putative ExuT protein from *X. axonopodis* pv. *citri* (accession number AAM39090; identity 84%, similarity 89%, in a stretch of 493 aa) and *X. campestris* pv. *campestris* (accession number AAM43340; identity 79%, similarity 83%). ORF2 also showed 33% identity and 48% similarity in a stretch of 352 aa to ExuT (galacturonate transporter) from *Erwinia chrysanthemi* (accession number U35884) and 26% identity and 45% similarity to ExuT (glucuronate transporter) from *Bacillus subtilis* (accession number NP388130).

ORFs 3 and 4, encoding for 329- and 325-aa proteins, were designated as *xynA* and *xynB*, respectively. The predicted amino acid sequences showed 55% identity and 67% similarity to each other and approximately 50% identity and 60% similarity to the catalytic domain (592 to 889 aa) of a 993-aa xylanase from *R. marinus* (accession number Y11564). A predicted signal peptide of 22 aa, with the most likely cleavage site

Fig. 1. Schematic of the arrangement of open reading frames (ORFs) in a 6,904-bp genomic region of *Xanthomonas oryzae* pv. *oryzae*. ORF1: putative Ton B dependent receptor; ORF2: putative hexuronate transporter; ORF3: putative xylanase A; ORF4: secreted xylanase B; ORF5: putative uronate isomerase. Arrows indicate the direction of transcription. Filled triangles indicate transposon insertions affecting xylanase secretion, whereas open triangles indicate insertions that do not affect xylanase secretion. E and B represent sites for restriction by *Eco*RI and *Bam*HI, respectively.

between A22 and G23, was detected only in XynB. A National Center for Biotechnology Information (NCBI) conserved domain database search showed the presence of the catalytic domain of xylanase in both XynA and XynB but the absence of a cellulose-binding domain present in several other xylanases. The predicted *xynA* product indicated 71% identity and 75% similarity to putative xylanase from *X. axonopodis* pv. *citri* (accession number AAM39089) and 61% identity and 73% similarity to putative xylanase from *X. campestris* pv. *campestris* (accession number AAM43339). The predicted *xynB* product showed substantial similarity to a putative xylanase of *X. axonopodis* pv. *citri* (accession number AAM39087; identity 95%, similarity 98% in a stretch of 292 aa). The six conserved regions found in the predicted sequences of the genes for family 10 xylanases from other bacteria (Gilkes et al. 1991) also were present in the XynB sequence of *X. oryzae* pv. *oryzae*, indicating that XynB belongs to family 10 of xylanases (Fig. 2). The two glutamic acid residues reported as essential for the activity of xylanaseA in *Streptomyces lividans* (Moreau et al. 1994) were found in the conserved region (Fig. 2, marked with an asterisk).

ORF 5 encoded for a 472-aa protein, which is homologous (92% identity, 95% similarity across 100% coverage of the protein) to putative uronate isomerase of *X. axonopodis* pv. *citri* (accession number AAM39086) and *X. campestris* pv. *campestris* (accession number AAM43338; 85% identity, 89% similarity in a stretch of 467 aa). The 286 nucleotides at the 3' end of pRR7 exhibited 90% identity at the nucleotide level to the noncoding 3' region of *pthB* pathogenicity gene from the cassava pathogen, *X. campestris* pv. *manihotis* (AFO12325).

The gene organization in the *xynA-xynB* region of *X. oryzae* pv. *oryzae* was compared with that of the corresponding region in the genomes of *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* (da Silva et al. 2002). The number, type, and orientation of the ORFs was similar in both *X. axonopodis* pv. *citri* and *X. oryzae* pv. *oryzae*. However, the intergenic region between *xynA* and *xynB* in *X. oryzae* pv. *oryzae* was noncoding; whereas, in *X. axonopodis* pv. *citri*, an incomplete 426-bp ORF showing 66% identity to the ORF2 from *X. oryzae* pv. *oryzae* was present between the two xylanase ORFs. The genome organization in *X. campestris* pv. *campestris* differed from *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *citri* by the absence of the *xynB* ORF in that region.

Functional mapping of pRR7 by transposon mutagenesis and marker exchange.

The 18 transposon insertions obtained in the pRR7 insert were marker exchanged in the wild-type background, and the marker-exchange mutants were assessed for xylanase secretion. Two transposon insertions in the *xynB* gene affected xylanase secretion and all other insertions were xylanase proficient, in-

XynB	53	V	P	E	N	A	G	K	59	91	Q	F	H	V	G	L	W	G	S	Q	Q	P	T	W	103	133	M	Q	V	A	N	E	138	★
RM	30	T	P	E	N	A	G	K	36	67	N	F	H	V	L	L	W	G	A	Q	Q	P	A	W	80	114	V	Q	V	V	N	E	119	
TA	40	T	P	E	N	R	M	K	46	77	Y	G	H	T	L	V	W	S	Q	L	P	P	W	90	108	W	D	V	V	N	E	113		
FS	56	T	A	E	N	G	C	K	62	93	K	F	H	A	L	V	W	G	S	Q	Y	P	N	W	106	136	I	D	V	V	N	E	141	
SL	83	T	A	E	N	E	M	K	89	120	R	G	H	T	L	A	W	H	S	Q	Q	P	G	W	133	164	W	D	V	V	N	E	169	
ST	82	T	A	E	N	E	M	K	88	119	R	G	H	T	L	A	W	H	S	Q	Q	P	Y	W	132	163	W	D	V	V	N	E	168	

★

XynB	185	T	K	L	M	I	N	D	Y	N	193	218	I	D	A	I	G	I	Q	G	H	226	252	I	T	E	F	D	-	I	257
RM	164	T	R	L	M	I	N	D	Y	G	172	196	I	D	V	I	G	V	Q	G	H	204	229	V	T	E	M	D	-	I	234
TA	133	A	K	L	Y	I	N	D	Y	N	141	167	I	I	G	I	G	N	Q	T	A	175	221	I	G	E	-	D	Y	I	226
FS	189	A	I	L	I	Y	N	D	Y	N	197	220	V	D	A	Y	G	L	Q	A	H	228	269	I	S	E	Y	D	-	I	274
SL	206	A	K	L	C	Y	N	D	Y	N	214	239	I	D	C	V	G	P	Q	S	H	247	275	I	T	E	L	D	-	I	280
ST	205	A	K	L	C	Y	N	D	Y	N	213	239	I	D	C	V	G	F	Q	S	H	247	274	I	T	E	L	D	-	I	279

Fig. 2. Comparison and alignment of the conserved regions of XynB with those of family 10 xylanases. The amino acid residues that are identical in all the sequences are boxed. Active site residues of family 10 xylanases are marked by asterisks. XynB and RM-Xyn1 = xylanase B from *Xanthomonas oryzae* pv. *oryzae* and *Rhodothermus marinus*, respectively; TA = *Thermomonospora aurantiacus*; FS = XynB from *Fibrobacter succinogenes*; SL = XynA from *Streptomyces lividans*; ST-Xyn1 = xylanase B from *Streptomyces thermoviolaceus*.

cluding the eight insertions in *xynA*. The extracellular protein profiles of *xynB* strains were marked by the absence of a 32-kDa band, whereas the *xynA* mutant strains were similar to the wild-type strain when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3A). The complementing pRR7 clone restored the 32-kDa product (data not shown) and xylanase activity (Fig. 3B) to the *xynB* mutants, indicating that the 32-kDa protein is the product of the *xynB* gene. Neither secreted xylanase activity nor a protein band could be associated with the *xynA* gene.

XynB protein is expressed

during in planta growth of *X. oryzae* pv. *oryzae*.

Xylanase was purified from the culture supernatants of peptone sucrose (PS)-grown *X. oryzae* pv. *oryzae* strains. Passage through MonoS columns resulted in separation of at least four protein fractions upon elution with 0 to 0.2 M NaCl gradient. Xylanase activity was associated with fraction number 1, which eluted with approximately 0.12 M NaCl (Fig. 4). The purified xylanase appeared as a single band of 32 kDa on SDS-PAGE gels upon staining with Coomassie Blue R250 (Fig. 4) or with silver nitrate (data not shown). Polyclonal anti-

bodies were raised against the purified protein. Western blot analysis of the xylem sap collected from infected leaves was performed to examine whether xylanase is secreted within the plant. A band corresponding to 32 kDa, which is the expected size of the processed XynB protein, was detected only in xylem sap of leaves infected with the wild-type and *xynA* mutant strains but not in the leaves infected with the *xynB* mutant (Fig. 5).

Fraction number 3 resolved as a single band of 39-kDa protein when analyzed in SDS-PAGE. Two sequence tags, GYVVVSTDYLG^L and TSNFLT^SYT, generated by tandem mass spectrometry (MS-MS) analysis of this band, matched conserved hypothetical proteins from *X. axonopodis* pv. *citri* (accession number AAM35390) and *X. campestris* pv. *campestris* (accession number AAM42229). The amino acids in the sequence tags (except for those underlined) were identical to the corresponding sequence of the *X. axonopodis* pv. *citri* homologue. An NCBI conserved domain database search (Marchler Bauer et al. 2003) with the predicted protein sequences of homologues from *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* revealed the presence of an LIP domain found in secreted lipases. The purified 39-kDa protein tested positive for lipase/esterase when spotted on peptone sucrose agar (PSA) containing either CaCl₂ and Tween 80 or Tributyrin as a substrate. The purified protein exhibited lipase/esterase activity (6.65 nmoles p-nitrophenol released/min/μg)

Fig. 3. A, Analysis of extracellular protein fractions from *Xanthomonas oryzae* pv. *oryzae* strains by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1: BXO43 (wild-type); lane 2: BXO201 (*xynB1::Ty2*); lane 3: BXO203 (*xynA1::Ty2*); lane 4: low molecular mass markers (Pharmacia). Note the 32-kDa band is absent in lane 2. Proteins were stained with Coomassie Blue R250. Arrow indicates the 32-kDa product of the *xynB* gene. **B,** Xylanase activity in *X. oryzae* pv. *oryzae* strains on peptone sucrose agar plates containing Remazol Brilliant Blue-xylan. 1, BXO43 (wild-type strain); 2, BXO201 (*xynB1::Ty2*); 3, BXO204 (BXO201/pRR7). Presence of halo around the colony indicates xylanase proficiency.

Fig. 4. Elution profile of *Xanthomonas oryzae* pv. *oryzae* secreted proteins from a MonoS column. The proteins were precipitated from culture filtrates with 55% (wt/vol) ammonium sulfate, desalted by dialyzing against 5 mM Tris-Cl buffer, pH 6.5, and loaded onto the column. After a wash in buffer, the proteins were eluted with a 0 to 0.2 M NaCl gradient in the same buffer (solid line). Fractions showing absorbance at 280 nm (A₂₈₀) were collected and assayed for xylanase (dashed line). Inset shows sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified xylanase appearing as a single band of approximately 32 kDa (lane 2), along with molecular mass standard (lane 1). The gel was stained with Coomassie Blue R250.

Fig. 5. Western analysis for in planta expression of XynB protein in infected rice leaf exudates using anti-XynB antibodies. Xylem sap from lane 1: uninoculated leaves or leaves inoculated with lane 2: wild-type strain; lane 3: BXO201 (*xynB1::Ty2*); and lane 4: BXO203 (*xynA1::Ty2*).

when assayed using p-nitrophenyl oleate as a substrate, thereby confirming it to be a lipase/esterase (LipA).

xynB and *lipA* together affect virulence of *X. oryzae* pv. *oryzae*.

Degenerate primers (5'-CGCGACGCCAAGggaaygaycc-3' forward and 5'-GCGGAGGCCACCAGCtkraaytcytt-3' reverse) designed using the CODEHOP program (Rose et al. 1998), based on the *lipA* homologues of *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris*, amplified a 308-bp fragment from the genomic DNA of BXO43. The sequence of this fragment (accession number AY627913) exhibited 91 and 80% sequence identity to the homologous genes of *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris*, respectively. The fragment was cloned into the pMOS-Blue vector to obtain pGJ1. A *lipA* mutant (*lipA1::bla*; BXO2001) was generated by electroporating pGJ1 in the wild-type background. The *lipA* insertion site was confirmed by PCR analysis of this region using a vector-specific primer and another primer flanking the inserted region. The resulting mutant strain did not show lipase activity in enzyme assays, and the analysis of its secreted proteins revealed the absence of the peak associated with LipA in the fast protein liquid chromatography (FPLC) elution profiles and the 39-kDa band in SDS-PAGE gels (data not shown).

The purified plasmid pGJ1 was introduced into the *xynB* mutant strain BXO201 to generate *lipA-xynB* (BXO2004) double mutant, which was deficient for secreted xylanase and lipase activities (data not shown). The plasmids pRR7, which contains the *xynB* gene, and pRR9, which has a transposon insertion in *xynB*, were introduced into BXO2004 to obtain BXO2005 and BXO2006, respectively. The BXO2001 (*lipA* mutant), BXO201 (*xynB* mutant), BXO2004 (*lipA xynB* double mutant), BXO2005, and BXO2006 strains were assayed along with BXO43 for virulence on rice. BXO2004 was severely affected for virulence, causing lesions that were only approximately 15% of those of BXO43. The BXO2001 and 201 strains were only partially affected for virulence, causing lesions that were 65 and 70% in length, respectively, compared with BXO43 (Fig. 6). Complementation with the pRR7 clone containing *xynB* could restore the lesion lengths to the wild-type levels in BXO2005. The virulence was severely affected in BXO2006 (approximately 14% lesion length), which contains a clone with a transposon insertion in the *xynB* gene (Fig. 6). All values were statistically significant at $P < 0.05$, except for the pairwise combinations of BXO201 versus BXO2001 and that of BXO43 versus BXO2005.

DISCUSSION

Here, we report the cloning, sequencing, and mutational analysis of the gene for XynB, the secreted xylanase of *X. oryzae* pv. *oryzae*. The predicted 325-aa sequence of XynB is homologous to several members of family 10 xylanases, showing the presence of six conserved regions that are characteristic of this family (Gilkes et al. 1991; Tsujibo et al. 1997), including the two glutamic acid residues reported as essential for the activity of xylanase A in *Streptomyces lividans* (Moreau et al. 1994). XynB has a xylanase catalytic domain; however, unlike several other family 10 xylanases, it is smaller in size and lacks a cellulose-binding domain. The xylanase from *R. marinus*, to which XynB shows approximately 60% homology to the catalytic domain at the amino acid level, consists of 993 aa with a molecular mass of approximately 110-kDa and five different domains and belongs to family 10 of xylanases (Karlsson et al. 1997). The substrate specificity of XynB toward xylan from various sources and the nature of the xylan breakdown products are yet to be determined.

Overall, the organization of the genomic region encoding *xynB* is conserved between *X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri*, and *X. campestris* pv. *campestris* with the only major difference that *xynB* is absent from *X. campestris* pv. *campestris*. The *xynA* as well as genes for uronate isomerase and hexuronate transporter are present at this locus in all three xanthomonads. The absence of a signal peptide suggests that *xynA* may be an intracellular xylanase. A putative intracellular xylanase, which is encoded in a gene cluster along with the gene for a secreted xylanase, has been postulated to be involved in xylan breakdown by *B. stearotheophilus* (Shulami et al. 1999). A gene for uronate isomerase also is located in this gene cluster in *B. stearotheophilus* along with several other genes involved in glucuronic acid utilization. The xylan backbone frequently is substituted with D-glucuronic acid residues, and the uronate isomerase of *B. stearotheophilus* is involved in metabolism of the glucuronic acid that is released from substituted xylooligosaccharides. The hexuronate transporter genes of *E. chrysanthemi* and *B. subtilis* have been shown to be involved in uptake of galacturonates and glucuronates, respectively (Haseloff et al. 1998; Mekjian et al. 1999).

Several xanthomonads were assayed for secreted xylanase activity on Remazol Brilliant Blue (RBB)-xylan plates. *X. campestris* pv. *translucens* and *X. campestris* pv. *mangiferaeindicae* assayed positive, whereas *X. campestris* pv. *vesicatoria*, *X. axonopodis* pv. *citri*, *X. campestris* pv. *holcicola*, *X. campestris* pv. *pelargonii*, *X. campestris* pv. *campestris*, and *X. oryzae* pv. *oryzicola* assayed negative for xylanase activity (data not shown). *X. campestris* pv. *translucens* is a pathogen of grasses like wheat, rye, barley, and similar plants and *X. campestris* pv. *mangiferaeindicae* is a pathogen of mango. Constitutive xylanase production may be an advantage for the former because the cell walls of grasses have a high proportion of xylan. The nature of the advantage that might accrue to *X. campestris* pv. *mangiferaeindicae* due to constitutive production of xylanase is not known. Certain dicots such as muskmelon have been shown to possess considerable amounts of xylose-containing polymers in their cell walls (Doux-Gayat et al. 1978). The genome sequences of *X. axonopodis* pv. *citri* and *X. campestris* pv. *cam-*

Fig. 6. Virulence deficiency of an *xynB-lipA* double mutant of *Xanthomonas oryzae* pv. *oryzae*. BXO43 (wild-type), BXO2001 (*lipA1::bla*), BXO201 (*xynB1::Ty2*), BXO2004 (*xynB1::Ty2 lipA1::bla*), BXO2005 (BXO2004/pRR7-*xynB*⁺), and BXO2006 (BXO2004/pRR9-*xynB1::Ty2*) strains of *X. oryzae* pv. *oryzae* were inoculated on rice leaves at a concentration of 1×10^8 cells/ml and lesion lengths were measured 7 days postinoculation. Error bars indicate the standard deviation of readings from 10 inoculated leaves. Similar results were obtained in three independent experiments. A Student's two-tailed *t* test for independent means was performed in pairwise combinations for all the values. Values with same letter are not significantly different at $P < 0.05$ level.

pestris reveal the presence of four and three xylanases respectively, with *X. axonopodis* pv. *citri* having the same ORF that encodes XynB in *X. oryzae* pv. *oryzae*. However, neither *X. axonopodis* pv. *citri* nor *X. campestris* pv. *campestris* exhibit xylanase activity when tested on RBB-xylan plates. It is possible that the xylanases in these two bacteria are either specific for a particular type of xylan other than RBB-xylan or that they are induced under specific growth conditions.

Mutations that affect the export of extracellular proteins across the outer membrane have been shown to cause a severe virulence deficiency in *X. campestris* pv. *campestris* (Dow et al. 1987; Hu et al. 1992), *E. chrysanthemi* (Andro et al. 1984; Thurn and Chatterjee 1985), *E. carotovora* pv. *carotovora* (Murata et al. 1990), *Ralstonia solanacearum* (Kang et al. 1994), and *X. oryzae* pv. *oryzae* (Ray et al. 2000). In addition to xylanase, at least three other major proteins are secreted through the type II protein secretion system of *X. oryzae* pv. *oryzae*. One of these proteins is the lipase/esterase whereas the other two are cellulase and putative cellobiosidase (G. Jha, R. Rajeshwari, and R. V. Sonti, unpublished data). These enzymes are constitutively secreted into the extracellular medium in vitro, and Western analysis indicates that the XynB protein also is released into the xylem sap during in planta growth of *X. oryzae* pv. *oryzae*.

Mutations that affect either the secreted xylanase or lipase/esterase have slight effects on virulence, which may be due to redundancy caused by the production of multiple cell-wall-degrading enzymes in *X. oryzae* pv. *oryzae*. Therefore, mutations that affect a single gene would not have a significant effect on virulence. The rice pathogen *M. grisea* has been reported to produce at least five different xylanases (Bucheli et al. 1990; Wu et al. 1995), and mutations in genes that encode two of these proteins do not affect virulence (Wu et al. 1997). A xylanase-deficient mutant of *E. chrysanthemi* also retained full virulence on corn (Keen et al. 1996). Most studies with extracellular proteins secreted by *X. campestris* pv. *campestris* (Dow et al. 1989; Tang et al. 1987) and *Erwinia* spp. (Barras et al. 1994; Py et al. 1998) suggest that, individually, the proteins have either partial or no effect on virulence. However, an exopolysaccharide (PehB) and endo-polygalacturonase (PehA) have been shown to contribute substantially to the virulence of *R. solanacearum* because double mutants are significantly reduced for virulence when compared with the individual mutants (Huang and Allen 1997). Purified xylanase from *E. chrysanthemi* acts synergistically with pectate lyase in causing cell killing and tissue maceration in corn and oat tissues (Braun and Rodrigues 1993).

The xylan backbone and side-chain modifications frequently are substituted with acetyl and phenolic esters. An endoxylanase and esterase from *Schizophyllum commune* have been shown to act cooperatively in birchwood xylan degradation (Biely et al. 1986). LipA may hydrolyze ester bonds in rice xylan, and this action may promote xylan degradation by *X. oryzae* pv. *oryzae* xylanases. The fact that a *xynB* clone restores full virulence to a *lipA xynB* double mutant suggests that an additional xylanase other than XynB might be acting cooperatively with LipA to degrade rice xylan. In such a scenario, the action of either XynB alone or LipA and the other xylanase would be required for normal virulence of *X. oryzae* pv. *oryzae*. The severe virulence deficiency observed in the *lipA xynB* double mutant may be due to its inability to degrade xylan because it lacks XynB as well as LipA, which is required by the other xylanase. Alternatively, LipA may act on a lipidic component of rice cell walls, and the loss of either this activity or XynB activity has only a slight effect on virulence, whereas loss of both activities (in the double mutant) must somehow compromise virulence. The restoration of virulence of the *lipA xynB* double mutant to a wild-type level, as opposed to the

level of the *xynB* mutant, by pRR7 plasmid might be due to the extra copies of the *xynB* gene. The slight reduction in virulence of the *lipA xynB* double mutant containing pRR9, in comparison to the *lipA xynB* double mutant, might be due to a detrimental effect associated with the presence of this plasmid, which becomes evident only in the absence of a functional *xynB* gene on the plasmid. Our results, therefore, indicate an important role for xylanase in colonization of rice by *X. oryzae* pv. *oryzae* and point toward a functional redundancy amongst T2S-secreted proteins in promoting *X. oryzae* pv. *oryzae* virulence. We are pursuing these observations by constructing multiple mutations affecting the genes coding for the four T2S-secreted proteins in various combinations and assessing their effects on virulence of *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids used in this study are listed in Table 1. *X. oryzae* pv. *oryzae* strains were grown at 28°C in PS medium (Tsuchiya et al. 1982) and *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (Miller 1992). Antibiotics and their concentrations used in this study are as follows: kanamycin (Km) at 50 mg/liter for *E. coli* and 25 mg/liter for *X. oryzae* pv. *oryzae*, Trimethoprim (Tm) at 60 mg/liter, and rifampicin (Rf) at 50 mg/liter.

PCR amplification.

Genomic DNA (50 ng) of *X. oryzae* pv. *oryzae* was used for PCR as described by Rajeshwari and Sonti (2000) using two degenerate primers (forward primer: 25 mer, CACGGATC CGA(C/T)GCIACICA(A/G/T/C)T(A/G/T/C)TA; and reverse primer: 26 mer, CCAGAATTCTA(C/T) GCIACITGGTGGAT). Our original intent was to amplify the gene for a sigma factor of *X. oryzae* pv. *oryzae*. An error in designing the reverse primer resulted in the fortuitous amplification of the gene for secreted xylanase from the genomic DNA of *X. oryzae* pv. *oryzae*. The primer sequences were present within the ORF encoding XynB. The PCR product of 800 bp was gel purified using a Qiaquick nucleotide purification kit (Qiagen Inc., Chatsworth, CA) and ligated into pMOS T-cloning vector (Amersham-Pharmacia, Uppsala, Sweden) to generate pXyn8.

Molecular biology techniques.

Genomic DNA was isolated as described by Leach and associates (1990) and the plasmid DNA by the alkaline lysis method (Sambrook et al. 1989). Southern hybridizations were performed as described by Rajeshwari and Sonti (2000). Restriction digestions were done with required enzymes from NEB (New England Biolabs, Beverly, MA, U.S.A.) as per the supplier's instructions. Sequencing was performed using transposon-specific primers supplied in the Primer Island Transposon kit (PE Applied Biosystems, Foster City, CA, U.S.A.) and M-13 forward and reverse primers. The sequencing reactions, electrophoresis, and sequence data analyses were performed using an ABI Prism 3700 automated DNA sequencer (Perkin-Elmer, Foster City, CA, U.S.A.). Homology and conserved domain searches were performed in the NCBI database by using the BLAST algorithm (Altschul et al. 1997). The signal peptide prediction program, Signal P server (Nielsen et al. 1997), and an ORF Finder program were used for the prediction of signal peptide and ORFs, respectively.

Bacterial conjugation, transposon mutagenesis, and marker exchange.

Matings between *E. coli* strains and *E. coli* (donor) with *X. oryzae* pv. *oryzae* (recipient) strains were carried out as de-

scribed previously (Ray et al. 2000). Transposon mutagenesis of pRR7 plasmid DNA was done using the Primer Island Transposition Kit according to the supplier's instructions. Eighteen different Ty2 transposable element insertions were obtained following in vitro mutagenesis of pRR7 with AT-2 (Ty2), a derivative of Ty1 transposable element from yeast. The distribution of the transposon insertions at different regions of pRR7, as revealed by restriction mapping using single as well as double digestions with *EcoRI* and *BamHI* enzymes, is shown in Figure 1. Using outwardly directed, transposon-specific primers, the sequences flanking each of the 18 insertions were obtained. Vector-specific primers were used to obtain the end sequences of pRR7. The overlapping sequences were identified and the complete sequence of pRR7 clone was obtained (accession number AF331922, submitted in December 2000).

The Ty2 element encodes for Trimethoprim resistance. Those plasmids containing insertions in the 6,904-bp insert were mobilized individually into BXO43 and marker-exchange mutants were obtained by growing the transconjugants in PS-Tm medium for more than 30 generations by serial passage. Colonies that were Tm^r Km^s were analyzed by Southern hybridization to confirm that marker exchange had occurred as expected.

Virulence assays.

Virulence assays were performed on 40- to 60-day-old greenhouse-grown plants of the susceptible rice cv. Taichung Native-1 (TN-1) by the leaf clip method of inoculation

(Kauffmann et al. 1973), and lesion lengths were measured at regular intervals.

Enzyme assays.

Assay for xylanase. Quantitation of xylanase activity was performed according to the procedure described by Biely and associates (1988). Assays for xylanase secretion were performed by spotting colonies of bacteria on PSA plates containing 0.2% RBB-xylan (Sigma-Aldrich, St. Louis) as described by Keen and associates (1996).

Assay for lipase/esterase. Lipase/esterase activity of bacterial colonies or purified enzyme spotted in wells was measured on PSA containing either 0.01% CaCl₂ and 1% Tween 80 or 1% Tributyrin (Holding and Collee 1971). White crystals surrounding colonies on medium containing CaCl₂ and Tween 80 or a zone of clearing on medium containing tributyrin indicated lipase activity. Lipase/esterase activity also was performed using p-nitrophenyl oleate as described (Acharya and Rao 2002).

Xylanase purification.

Bacterial cultures were grown for 48 h at 28°C and the culture supernatant was obtained by centrifugation at 12,000 × g for 20 min at 4°C. All subsequent steps were carried out on ice. Ammonium sulfate fractionation was performed and the 55% fraction was collected by centrifugation at 10,000 × g for 30 min. The precipitate was taken up in 1/20 original volume and salt was removed following extensive dialysis against 5 mM Tris-Cl (pH 6.5). Cation-exchange chromatography was

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics ^a	Reference or source ^b
<i>Escherichia coli</i> strains		
DH5α	F'/endA1 hsdR17 (rk ⁻ mk ⁺) supE44 thi-1 recA1 gyrA relA1 f80dlacZDM15 (lacZYA-argF) U169	Invitrogen
S17-1	RP4-2Tc::Mu-Kn::Tn7 pro hsdR recA	Simon et al. 1983
Plasmids		
pBluescript(KS)	Ap ^r	Stratagene
pMOS	Ap ^r	Amersham
pUFR034	Km ^r IncW Nm ^r Tra ⁻ Mob ⁺ mob(P) LacZα ⁺ Par ⁺ cos	DeFeyer et al. 1990
pXyn8	pMOS + 800-bp polymerase chain reaction (PCR) product	This study
pRR7	pUFR034 + 6.9-kb <i>EcoRI</i> fragment containing <i>xynB</i> gene of <i>X. oryzae</i> pv. <i>oryzae</i>	This study
pRR8	pBluescript + 6.9-kb <i>EcoRI</i> fragment containing <i>xynB</i> gene of <i>X. oryzae</i> pv. <i>oryzae</i>	This study
pRR9	pRR7-xynB1::Ty2; pRR7 containing an insertion in <i>xynB</i> gene of <i>X. oryzae</i> pv. <i>oryzae</i>	This study
pGJ1	pMOS-Blue + 308-bp PCR-amplified fragment of <i>lipA</i> gene of BXO43	This study
<i>Xanthomonas</i> spp.		
<i>X. axonopodis</i> pv. <i>citri</i> # F1	...	J. Leach
<i>X. campestris</i> pv. <i>translucens</i> # 105	...	J. Leach
<i>X. campestris</i> pv. <i>vesicatoria</i> # 65-2	...	J. Leach
<i>X. campestris</i> pv. <i>holcicola</i> # 86	...	J. Leach
<i>X. campestris</i> pv. <i>mangiferaeindicae</i> #2913	...	J. Leach
<i>X. campestris</i> pv. <i>pelargonii</i> # S1	...	N. Sakthivel
<i>X. campestris</i> pv. <i>campestris</i> # 8004	...	M. Dow
<i>X. oryzae</i> pv. <i>oryzicola</i> #BXOR1	...	Lab Collection
<i>X. oryzae</i> pv. <i>oryzae</i> strains		
BXO1	Laboratory wild type; Indian isolate	Lab collection
BXO43	<i>rif</i> ⁻ 2; derivative of BXO1	Lab collection
BXO201	<i>xynB1</i> ::Ty2 <i>rif</i> ⁻ 2; Tm ^r Xyn ⁻ derivative of BXO43	This study
BXO202	<i>xynB2</i> ::Ty2 <i>rif</i> ⁻ 2; Tm ^r Xyn ⁻ derivative of BXO43	This study
BXO203	<i>xynA1</i> ::Ty2 <i>rif</i> ⁻ 2; Tm ^r Xyn ⁺ derivative of BXO43	This study
BXO204	BXO201/pRR7	This study
BXO2001	<i>lipA1</i> :: <i>bla rif</i> ⁻ 2; Lip ⁻ , Ap ^r derivative of BXO43	This study
BXO2004	<i>xynB1</i> ::Ty2 <i>lipA1</i> :: <i>bla rif</i> ⁻ 2; Tm ^r Ap ^r Xyn ⁻ Lip ⁻ Vir ⁻ derivative of BXO 201	This study
BXO2005	BXO2004/pRR7	This study
BXO2006	BXO2004/pRR9	This study

^a Ap^r, Km^r, and Tm^r indicate resistance to ampicillin, kanamycin, and trimethoprim, respectively; *rif*⁻ indicates a mutation that confers rifampicin resistance; Vir⁻ and Xyn⁺ indicate proficiency in virulence and xylanase activity, respectively; and Xyn⁻ and Lip⁻ indicate deficiency in xylanase and lipase/esterase activities, respectively.

^b Invitrogen = Invitrogen Life Technologies, Carlsbad, CA, U.S.A.; Stratagene = Stratagene, La Jolla CA, U.S.A.; and Amersham = Amersham Pharmacia Biotech, Uppsala, Sweden.

performed using the MonoS (HR 5/5) columns in FPLC (Pharmacia Biotechnology, Uppsala, Sweden). The desalted sample was applied to the columns equilibrated with 5 mM Tris-Cl (pH 6.5) and proteins were eluted with a 0 to 0.2 M NaCl gradient in the same buffer. The peaks obtained were assayed for enzyme activity and xylanase active fractions were pooled and concentrated in a Speed-vac concentrator (Savant Instruments, Inc., Farmingdale, NY, U.S.A.) to one-fifth of original volume and dialyzed against 5 mM Tris-Cl (pH 6.5). Trypsin digestion and MS-MS analysis of the purified xylanase yielded sequence tags that matched with the XynB sequence from *X. oryzae* pv. *oryzae*, thus confirming it to be the *xynB* gene product. The enzyme was stored at 4°C until further use.

The prominent xylanase peak was reproducibly obtained upon FPLC separation of *X. oryzae* pv. *oryzae* culture supernatants during a 3-month period in which the enzyme was purified and used to raise anti-XynB antibodies as detailed below. In more recent purifications, the xylanase peak was not prominent even though the *X. oryzae* pv. *oryzae* strain continued to produce the secreted xylanase as observed by halo formation on RBB-xylan plates and SDS-PAGE gels; we are not certain about the reasons for this.

Analysis of protein by SDS-PAGE and MS/MS.

Proteins were analyzed by SDS-PAGE following the procedure of Laemmli (1970) and stained with either Coomassie Brilliant Blue or silver nitrate as described by Sambrook and associates (1989). Low molecular weight markers obtained from Pharmacia Biotechnology were used as standards.

For MS-MS analysis, approximately 500 ng of each purified protein was electrophoresed on 10% SDS-PAGE and stained with Coomassie Brilliant Blue R250 (Sambrook et al. 1989). The gel slice containing each purified protein was cut into approximately 1-mm³ pieces, washed three times (15 min each) with 400 µl of 50% acetonitrile in 25 mM ammonium bicarbonate (NH₄HCO₃), and soaked in 100% acetonitrile for 5 min. The bands were dried to completion in a speed-vac concentrator. Trypsin (20 µl of sequencing grade modified, 20 µg ml⁻¹ in 25 mM ammonium bicarbonate; Promega Corp., Madison, WI, U.S.A.) was used for digestion at 38°C for 16 to 20 h. Extraction of digested fragment was done by soaking the gel pieces in 100 µl of a solution containing 50% acetonitrile and 5% trifluoroacetic acid (TFA) by vigorous vortexing for 1 min and dried in speed-vac to complete dryness. The dried samples were further processed in the CCMB Proteomics facility to obtain sequence tags using electrospray ionization-based MS/MS with a PE-SCIEX-API-QSTAR PULSAR instrument.

Antibody production and Western blots.

Polyclonal antibodies against the purified XynB protein were raised in a rabbit using standard protocols (Sambrook et al. 1989). The antiserum could detect XynB protein in culture supernatants of XynB⁺ *X. oryzae* pv. *oryzae* samples at a dilution of 1:2,000. Leaves from rice plants infected with various strains of *X. oryzae* pv. *oryzae* (10 leaves for each strain) 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. The supernatant was concentrated to 100 µl in a speed-vac and checked for protein concentration by method of Bradford (1976). The xylem exudates (supernatant) were separated by SDS-PAGE and blotted onto a nitrocellulose membrane by the semi-dry method. Western blot analysis was performed using anti-XynB antibodies with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-

chloro-3-indolyl phosphate substrate for detection, as described by Sambrook and associates (1989).

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