Mutants of *Xanthomonas oryzae* pv. *oryzae* Deficient in General Secretory Pathway Are Virulence Deficient and Unable to Secrete Xylanase

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Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial leaf blight, a serious disease of rice. A virulence- and xylanase-deficient mutant of *Xoo* was isolated following ethyl methane sulfonate (EMS) mutagenesis. A cosmid clone that restored virulence and xylanase secretion was obtained from a genomic library by functional complementation. Transposon mutagenesis and marker exchange studies revealed genes on the cloned DNA that were required for xylanase production and virulence. Sequence analysis with transposon-specific primers revealed that these genes were homologues of *xps F* and *xps D*, which encode components of a protein secretion system in *Xanthomonas campestris* pv. *campestris*. Enzyme assays showed xylanase accumulation in the periplasmic space and cytoplasm of the *xps F* mutant and the complementing clone restored transport to the extracellular space.

Additional keywords: type II protein secretion.

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight, a serious disease of rice. It is a vascular pathogen that grows in the xylem vessels and causes drying of infected leaves. Previously reported virulence-deficient mutants of *Xoo* include spontaneous mutants that lack extracellular polysaccharide (Eps) (Rajeshwari et al. 1997), mutations in *rpfC*, which encodes a regulator of pathogenicity factor production and causes Eps deficiency (Tang et al. 1996), *Hrp*[−] (hypersensitive response and pathogenicity) mutants, which are unable to elicit a hypersensitive response (HR) on nonhost plants (Kamdar et al. 1993; White et al. 1995), mutations in certain avirulence (*avr*) genes (Leach et al. 1996), and particular *Xoo* auxotrophs (Yamasaki et al. 1964). We are using a genetic approach to identify other virulence functions of *Xoo*.

This paper discusses the isolation of a virulence- and xylanase-deficient mutant (referred to as BXO801) of *Xoo* and a genomic clone that restored both xylanase and virulence proficiency to this mutant. Transposon mutagenesis of the cloned DNA with either Tn5 or Tn10 and marker exchange techniques were used to define regions in the cloned *Xoo* DNA that are required for virulence and xylanase production. Se-

quencing of these regions with outwardly directed primer sequences specific to the transposon revealed that the genes involved were homologues of *xpsF* and *xpsD*, which encode components of a protein secretion system in *Xanthomonas campestris* pv. *campestris*. (*Xcc*). We report here the requirement for a general secretory pathway (GSP; also known as type II protein secretion system) in *Xoo* virulence and the secretion of xylanase through this system to the extracellular environment.

RESULTS

Isolation of virulence deficient *Xoo* mutants.

An ethyl methane sulfonate (EMS) mutagenesis protocol that resulted in 99.9% lethality was used to generate a collection of mutants of BXO43 strain (Table 1) of *Xoo*. The frequency of auxotrophs in the collection was 7%, frequency of pigment deficient mutants was 0.1%, and frequency of Eps-deficient mutants was approximately 10%. Ten out of 200 Eps⁺, Pig⁺, colonies were virulence deficient (Vir[−]) following inoculation onto rice leaves (see Materials and Methods). Lesion lengths caused by these mutants (e.g., BXO801; Fig. 1) varied between 2 and 7 cm, 17 days after inoculation (DAI), whereas the lesion caused by the wild-type strain (BXO43) was 20 cm. Three out of the 10 Vir[−] mutants were auxotrophs; reversion analysis proved their virulence was due to auxotrophy (data not shown). The HR phenotype of the seven prototrophs was assessed by infiltrating *Xoo* strains in tomato leaves as described in Materials and Methods. One strain out of the seven (BXO802) did not elicit HR whereas the other six mutants were HR⁺. They also grew as well as the wild type when plated on peptone sucrose agar (PSA) or minimal media, suggesting that the virulence deficiency was not due to any general growth deficiency. Exponentially growing cultures of these six mutants were as motile as wild type when observed under the light microscope. One of these mutants, called BXO801, was taken up for this study.

The BXO801 mutant is xylanase deficient.

The production of extracellular enzymes like cellulases, pectinases, and proteases has been correlated with virulence in several Gram-negative, plant-pathogenic bacteria (Andro et al. 1984; Roberts et al. 1988; Schell et al. 1988; Tang et al. 1987). Our laboratory wild-type strain, BXO1, did not produce detectable levels of extracellular proteases, pectinases, or cellu-

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lases when assayed as described in Materials and Methods. The BXO1 strain, however, produced a secreted xylanase and the BXO801 mutant did not show xylanase activity (Fig. 2). The other EMS-induced, virulence-deficient mutants were all xylanase proficient (data not shown).

Isolation of a clone that restores both xylanase and virulence proficiency to BXO801.

Clones from a cosmid genomic library of BXO1 were mobilized to the BXO801 strain in 26 pools of 12 clones each (as described in Materials and Methods). The transconjugants obtained on selection plates were inoculated on rice plants, and one donor pool that restored virulence to BXO801 was identified. Testing of the individual clones in that pool resulted in isolation of clone pSR1, which restored virulence to BXO801 (Fig. 1). Complete restoration was not observed, probably in part due to instability of the complementing clone in the absence of antibiotic, since only 50% of the bacteria recovered from the leaf 17 DAI retained the clone. The pSR1 clone also restored xylanase production to BXO801 (Fig. 2). Restriction map of the cloned DNA was generated with *EcoRI*, *HindIII*, and *BamHI* (Fig. 3). Four *EcoRI* fragments of 11.0, 9.0, 6.2, and 3.5 kb were present on the insert. Digestion of *Xoo* genomic DNA and pSR1 DNA with *EcoRI* and hybridization with pSR1 revealed identical insert bands, indicating that the cloned DNA was from the *Xoo* genome. The organization of the insert DNA in pSR1 was shown to be same

as in the genomic DNA with other restriction enzymes, such as *BamHI* and *HindIII* (data not shown).

Functional mapping of pSR1 by transposon mutagenesis and marker exchange.

The pSR1 clone was subjected to mutagenesis with mini Tn5 and mini Tn10 elements (see Materials and Methods). The transposon insertions were mapped to the cloned DNA by restriction analysis (Fig. 3). Tn5 mutagenesis of pSR1 resulted in two insertions in the 11-kb (*zxx-102* and *zxx-103*) and two in the 9-kb *EcoRI* fragments (*zxx-101* and *vir-2*). None of these four insertions affected complementation of BXO801 for either xylanase production or virulence. Also, marker exchange of three of these insertions in the wild type background did not affect either xylanase production or virulence. However, one marker exchange mutant (BXO808) with transposon insertion (*vir-2::Tn5*) in the 9-kb fragment was not affected for xylanase production but was reduced for virulence. The mutant showed lesion lengths of approximately 2 and 8 cm 10 and 17 DAI, respectively, compared with 15 and 24 cm caused by the wild-type strain. The mutant strain also exhibited an altered lesion phenotype (data not shown).

Eleven Tn10 insertions were also isolated on cloned DNA in pSR1 and insertions were mobilized into the BXO801 background to study their effect on complementation for virulence as well as xylanase proficiency. One insertion (*xpsF1::Tn 10*) in the 3.5-kb fragment (Fig. 3) affected com-

Table 1. Bacterial strains and plasmids^a

Strain	Relevant characteristics	Reference
<i>Escherichia coli</i> strains		
DH5 α	F', end A1 hsdR17 (rk ⁻ mk ⁺) supE44 thi-1 recA1 gyrA relA1 ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) U169	Lab collection
S17-1	RP4-2-Tc::Mu-Km::Tn7 pro hsdR recA	Simon et al. 1983
LE392	supF supE hsdR galK trpR metB lacY tonA	Lab collection
MC4100	F' Δ (argF-lac) U169 rpsL 150 relA1 araD139 flbB5301 deoC1 ptsF25	Lab collection
MG1655	Wild type	Singer et al. 1995
CAG18431	MG1655 ilv-500::Tn10	Singer et al. 1995
Plasmids		
pUFR034	IncW Nm ^r Mob ⁺ mob (P) lacZa ⁺ Par ⁺ cos (8.7 kb)	DeFeyter et al. 1990
pRK600	pRK2013 npt::Tn9; Cm ^r	Lab collection
PBluescript (KS)	Ap ^r	Stratagene, La Jolla, CA
pSR1	pUFR034 + 30-kb insert from BXO1 genome (complements BXO801 for virulence and xylanase production)	This study
pSR2	pUFR034 + 3.5-kb <i>EcoRI</i> fragment of the insert from pSR1	This study
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> strains		
BXO1	Laboratory wild type	Lab collection (an Indian isolate)
BXO43	<i>rif-2</i> , Rif ^r derivative of BXO1	Lab collection
BXO160	<i>zxx-1::Tn5</i> gusA11 <i>rif-11</i> ; Sp ^r , Gus ⁺ derivative of BXO43	Lab collection
BXO1050	BXO43/pUFR034	Lab collection
BXO801	<i>vir-1 rif-2</i> ; Eps ⁺ , Hrp ⁺ , Vir ⁻ , Xyn ⁻ , derivative of BXO43	This study
BXO802	Eps ⁺ , Hrp ⁺ , Vir ⁻ , Xyn ⁺ derivative of BXO43	This study
BXO803	<i>vir-1 rif-2</i> /pUFR034; derivative of BXO801	This study
BXO804	<i>vir-1 rif-2</i> /pSR1; Vir ⁺ , Xyn ⁺ derivative of BXO801	This study
BXO805	<i>xpsF1::Tn10 rif-2</i> ; Vir ⁻ , Xyn ⁻ derivative of BXO43	This study
BXO807	<i>xpsD1::Tn10 rif-2</i> ; Vir ⁻ , Xyn ⁻ derivative of BXO43	This study
BXO808	<i>vir-2::Tn5</i> gusA40 <i>rif-2</i> ; Vir ⁻ , Xyn ⁺ derivative of BXO43	This study
BXO809	<i>vir-3::Tn10 rif-2</i> ; Vir ⁻ , Xyn ⁺ derivative of BXO43	This study
BXO810	<i>xpsF1::Tn10</i> /pSR1; Vir ⁺ , Xyn ⁺ derivative of BXO805	This study
BXO811	<i>xpsF1::Tn10</i> /pSR2; Vir ⁺ , Xyn ⁺ derivative of BXO805	This study
BXO814	<i>xpsD1::Tn10</i> /pSR1; Vir ⁺ , Xyn ⁺ derivative of BXO807	This study

^a *rif* indicates a mutation that confers rifampicin resistance. *vir* indicates a mutation that causes virulence deficiency. Vir⁺ indicates proficiency for virulence. Eps⁺ indicates proficiency for extracellular polysaccharide production. Xyn⁺ indicates proficiency for xylanase production. The chromosomal location of the *zxx-1* insertion has not been determined.

plementation for virulence as well as xylanase production. This insertion also caused xylanase and virulence deficiency when marker exchanged in the BXO43 background (see Figure 1 for data obtained for virulence with BXO805, the *xpsF1::Tn10* mutant). The full clone pSR1 and the subclone pSR2 containing the 3.5-kb *EcoRI* fragment restored virulence (Fig. 1) as well as xylanase proficiency when introduced into BXO805. Two other *Tn10* insertions were found by sequence analysis to be at the same site as the *xpsF1::Tn10* and behaved identically to this insertion in complementation and marker exchange studies. The other eight *Tn10* insertions did not affect complementation in BXO801 for either xylanase production or virulence. The marker exchange mutant with the insertion in the 6.2-kb *EcoRI* fragment (*xpsD1*; Fig. 3) exhibited virulence as well as xylanase deficiency (BXO807), similar to what was observed in BXO805 (data not shown). The full clone pSR1 restored virulence as well as xylanase secretion to BXO807. However, this insertion had no effect on the complementation ability of pSR1 to BXO801, suggesting that the mutation in the 6.2-kb region was in a different complementation group than the insertions in the 3.5 kb region. Only five of the six *Tn10* insertions (*zxx-105* to *108* and *vir-3*; Fig. 3) in the 11-kb region could be marker exchanged and four of the marker exchange mutants remained proficient for virulence and xylanase secretion. However, one mutant (BXO809) was

reduced for virulence, causing lesion lengths of 3 and 7 cm, 10 and 17 DAI, respectively, whereas the wild-type strain caused 15 and 24 cm lesions during the same time intervals. The xylanase secretion in the mutant was not affected. One insertion (*isr-1*) could not be marker exchanged (Fig. 3).

Fig. 1. Lesion lengths caused by strains of *Xanthomonas oryzae* pv. *oryzae* on susceptible rice cultivar TN-1, 10 and 17 days after inoculation. BXO43: the wild-type strain; BXO801: ethyl methane sulfonate (EMS)-induced *Vir⁻*, *Xyn⁻* mutant; BXO804: BXO801 strain containing pSR1; BXO805: *xpsF-1::Tn10* marker exchange mutant; BXO810: *xpsF1::Tn10/pSR1*; BXO811: *xpsF1::Tn10/pSR2*. Mean and standard deviation of 10 replicate measurements of lesion lengths are given. Similar results were obtained in independent experiments.

Fig. 2. Xylanase activity in strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Peptone sucrose agar (PSA) + Km plates containing RBB-xylan with colonies of *Xoo* strains: 1, BXO1050 (wild-type strain carrying pUFR034); 2, BXO803 (*vir-1/pUFR034*); 3, BXO804 (*vir-1/pSR1*). Presence of halo around colony in BXO1050 and BXO804 indicates xylanase proficiency. This halo is very much reduced in BXO803.

Fig. 3. Restriction map of pSR1 showing restriction sites for *EcoRI* (E), *BamHI* (B), and *HindIII* (H) and sites of *Tn5* and *Tn10* insertions. Filled squares, insertions that affect xylanase production and virulence; open circles, insertions that had no effect on either virulence or xylanase production; filled circles, insertions that affect only virulence; open square, an insertion that could not be marker exchanged. (3) indicates that three independent insertions of *Tn10* were obtained at the same site in the *xpsF* gene.

Sequence analysis.

The flanking regions of the transposon insertions that caused virulence and xylanase deficiency when marker exchanged were sequenced with primers specific to the ends of the transposon (Fig. 4). Computer-based homology search was performed with the BLAST program (Altschul et al. 1990). The homology search showed that one insertion in the 3.5 kb *EcoRI* fragment and another insertion on the 6.2-kb *EcoRI* fragment were in the *Xoo* homologs of the *xpsF* and *xpsD* genes of *Xcc*, respectively. The nucleotide sequence and the derived amino acid sequence of the *xpsFXo* are shown in Figure 4A, with region of transposon insertion. For *xpsFXo*, the homology was about 85% at the nucleotide as well as at the protein level with *xpsFXc*. The partial nucleotide and derived amino acid sequence of *xpsDXo* is shown in Figure 4B. The homology at the nucleotide level for *xpsDXo* was not significant within the sequenced region except for a short stretch of 50 nucleotides, whereas there was 79% homology at the protein level within the stretch of 124 amino acids compared. The *xpsF* and *xpsD* genes from *Xcc* encode components of a type II protein secretion system that is required for the secretion of pectinases, proteases,

and cellulases (Dums et al. 1991; Hu et al. 1992). Ongoing sequence analysis indicates that, as in *Xcc*, the *xpsE* homologue of *Xoo* is located adjacent to the *xpsF* gene (data not shown).

Assay for xylanase activity and analysis of protein profiles in various cell fractions of wild-type and mutant strains of *Xoo*.

To show that the *xpsF* gene from *Xoo* was involved in secretion of xylanase, the activity of xylanase was assayed in extracellular, periplasmic, and cytoplasmic fractions of the following strains: BXO803 (BXO801/pUFR034), BXO1050 (BXO43/pUFR034), BXO804 (BXO801/pSR1), BXO805 (*xpsF*-1::Tn10), BXO810 (*xpsF*-1::Tn10/pSR1), and BXO811 (*xpsF*-1::Tn10/pSR2). The results are shown in Figure 5. Most of the xylanase activity was detected in the extracellular fraction of the wild-type strain (BXO1050), whereas periplasmic and cytoplasmic fractions showed very little activity. On the other hand, the mutant strain (BXO803) showed high activity in the periplasmic and cytoplasmic fractions and very little in the extracellular fraction. The BXO805 strain also exhibited a deficiency in xylanase transport. Xylanase activity was restored in the extracellular medium of BXO804, 810, and 811 strains (which contain the complementing clones). We introduced pUFR034 into the wild-type and mutant strains to show that the vector DNA does not affect xylanase secretion. We have performed similar assays on the *xpsD* mutant and find that it exhibited similar deficiency for xylanase export (data not shown). These data suggest that xylanase is secreted by the type II protein secretion system in *Xoo* and that mutations in the *xps* genes affect its secretion, resulting in its accumulation in the periplasm and cytoplasm.

The proteins isolated from the extracellular and periplasmic fractions of BXO1050, BXO803, and BXO804 were analyzed

A

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atgctcgacggccagatggaagcggccagcgcacccgaggtggcg
M L D G Q M E A A S D T E V A
ttcgctctcaggaagccacggcgaaacgattcaccatcgctg
L R L Q E A T G E N D S P S L
cgcatgttcttgcgaagaagccgttcgataacgcggcactggtg
R M L L R K K P F D N A A L V
caatttaccagcaactggcgacgttgatcgg▼ggcggcgacggcg
Q F T Q Q L A T L I G A G Q P
ctggatcgcgcgctgtcgattctgatggatctgcccgaagacgaa
L D R A L S I L M D L P E D E
aaaagcggcggtgatcgcgatgtgcgcgataccgtgcgcggcg
K S R R V I G D V R D T V R G
ggtgcgccattgtcgtccgcactgcagcgcagcggcggtgtt
G A P L S S A L E R Q H G L F
tccaagctgtacatcaacatggtgcgcgcgggcgaagcggcgcg
S K L Y I Q R L A N M V R A G
agcatgcaggacacgctgcaacggctggcggattatctggagcgc
E A G G S M Q D T L D Y L E R
agcgtgctcggcgggcggtgatcaacgcg
S R A L R G K V I N A

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B

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accttgctggtgcgtccacgcgcgagcctggagctcgatccgc
T L L V R S T P Q A W S S I R
gatgtcatcgaaaagctcgacgtgatgcgatgcagggtgcatac
D V I E K L D V M P M Q V H I
gaagcgcaggtggcggaggtgaatttgactggcaagctgcagtat
E A Q V A E V N L T G K L Q Y
ggtgtgaattggtacttcgag▼aactcgggtgaatgctgcagcggat
G V N W Y F E N S V N A A A D
tcggcgccaatagcaccgcttgctggtgcccgttggtgca
S A A N S T G I G A G A G L A
agcgcagcagggagaaacatttggggagatatcgtgggaaatc
S A A G R N I W G D I A G K I
accggtgaaaaagcgctcagtggtgacgttcttgggcaagaatgcg
T G E K G A Q W T F L G K N A
gcctcgatcatccatgcacttgatgaggtgactaatgtgcgtctt
A S I I H A L D E V T N V R L
ctgcaaacgcct
L Q T P

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Fig. 4. A, Partial nucleotide sequence of the *xpsF* gene of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Deduced amino acid sequence is shown by single letter codes below. Triangle indicates site of the *xpsF*::Tn10 insertion. Partial nucleotide sequence is available at the GenBank database under accession number AF190908. **B**, Partial nucleotide and derived amino acid sequence of the *xpsD* gene of *Xoo*. Triangle indicates site of the *xpsD*::Tn10 insertion. Partial nucleotide sequence is available at the GenBank data base under accession number AF190907.

Fig. 5. Distribution of xylanase activity in various cellular fractions of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains. Proteins were precipitated with ammonium sulfate from various cellular fractions of the following *Xoo* strains and assayed for xylanase activity as described in Materials and Methods. BXO1050: wild-type strain carrying pUFR034; BXO803: BXO801/pUFR034; BXO804: BXO801/pSR1; BXO805: *xpsF*1::Tn10; BXO811: *xpsF*1::Tn10/pSR2; BXO810: *xpsF*1::Tn10/pSR1. Similar results were obtained in three independent experiments (data not shown).

by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Several prominent protein bands (approximately 55, 42, 35, 20, and 16 kDa bands) were observed in the extracellular fraction of BXO1050 whereas the BXO803 strain showed very few proteins (Fig. 6). Some of these proteins were restored in the extracellular fraction of BXO804. These proteins were, however, present in the periplasmic fraction of BXO803, the most prominent being the 42-kDa band (lane 6), which was absent in the periplasmic fraction of the wild-type strain (lane 5). The BXO804 strain showed bands similar to those of the mutant in the periplasmic fraction but the intensities of the bands, especially the 42 kDa band, were comparatively low. This confirms the results of xylanase assays that show some xylanase activity in the periplasmic fraction of BXO804, suggesting that pSR1 is not fully restoring transport. The extracellular and periplasmic protein profiles of the *xpsF1::Tn10* and *xpsD1::Tn10* insertion mutants were similar to that of BXO801 (data not shown).

Lack of in planta complementation of *xps* mutants by the wild-type strain of *Xoo*.

Differentially marked wild-type and mutant strains of *Xoo* were mixed in a 1:1 ratio and coinoculated in various combinations onto rice leaves by the leaf clip method described in Materials and Methods. The bacterial colonies were recovered from the lesion ends 15 DAI (see Materials and Methods) and assayed for the respective markers. In one combination, a β -glucuronidase (GUS)-marked derivative of BXO43 (BXO160, *Xps*⁺ and *Vir*⁺) was coinoculated with BXO801 at a ratio of 1:1. The recovered colonies were assayed for *gus* activity as described in Materials and Methods. Of the 150 individual colonies tested, all were found to be *gus* positive, indicating the presence of only the *Xps*⁺ strain. On the other hand, when the *Hrp*[−] mutant, BXO802, was coinoculated with BXO160,

for every 150 colonies assayed for *gus* activity 30 colonies were found to be *gus* negative and therefore of the *Hrp*[−] mutant type. In planta complementation of an *Hrp*[−] mutant of *Xoo* by an *Hrp*⁺ strain has been previously demonstrated by Leach et al. (1996).

In another set of experiments, the BXO1 (*Rf*⁺) strain was coinoculated at a ratio of 1:1 with either BXO801 (*Rf*⁺) or BXO805 (*xpsF1::Tn10*; *Rf*⁺ and *Tc*^r). Fifteen DAI, bacteria were isolated from the leading edge of the lesion (see Materials and Methods) and plated on PSA and PSA plus *Rf* plates. About 10 colonies on PSA + *Rf* plates and approximately 10,000 colonies on PSA plates were obtained from leaves inoculated with the BXO1 + BXO801 mixture. This indicates the presence of one colony of *Xps*[−] mutant type for every 1,000 colonies of the *Xps*⁺ strain. Similar ratios were obtained when BXO1 was coinoculated with the marker-exchange *Xps*[−] mutant, BXO805. A mixture of BXO1 and BXO43 resulted in a 1:1 ratio of *Rf*⁺: *Rf*[−] colonies, indicating that *Rf* marker did not affect growth in planta. However, when BXO1 was coinoculated with the *Hrp*[−] mutant BXO802, 2,000 *Rf*⁺ colonies were observed for every 10,000 colonies, indicating a ratio of 1:4 of the *Hrp*[−] mutant type to the *Hrp*⁺ strain. These data suggest that the virulence function defective in BXO801 and BXO805 could not be efficiently complemented by any factor produced in *trans* by the wild-type cell and is therefore likely to affect individual bacterial cells.

DISCUSSION

Phytopathogenic bacteria produce extracellular enzymes like cellulases, pectinases, proteases, and xylanases. These enzymes are secreted to the extracellular environment by the general secretory pathway (GSP) (Dow et al. 1987; Hu et al. 1992; He et al. 1991), which is also called the type II protein secretion system (Salmond et al. 1993; Russel 1998). The GSP is the protein secretion system that secretes proteins from cytosol to extracellular environment in two steps. In step 1, the proteins are secreted from the cytoplasm to the periplasm by *sec* gene products. This is called the general export pathway (GEP). In step 2, the proteins are secreted from the periplasm to the extracellular medium through the outer membrane by a complex assembly of proteins. This is called the main terminal branch (MTB) of the GSP (Pugsley 1993; Pugsley et al. 1997). Mutants of *X. campestris* unable to secrete various enzymes to the extracellular medium due to mutation in the MTB of the GSP are virulence deficient and accumulate enzymes in the periplasm (Dow et al. 1987; Hu et al. 1992), similar to BXO801 and BXO805 (*xpsF1::Tn10*) mutants of *Xoo* reported in this study. It is not known whether the virulence deficiency in *X. campestris* is due to the absence of these enzymes in the extracellular milieu or some other virulence factors secreted by the GSP. The direct involvement of these enzymes in virulence is not defined because several studies (Dow et al. 1989; Tang et al. 1987) have shown that mutants defective in production of particular extracellular enzymes are virulence proficient. However, one possible reason for the virulence proficiency of these mutants may be that other extracellular enzymes performed the necessary functions.

Rice plant cell walls contain 60% xylan (Takeuchi et al. 1994) and the ability to degrade xylan may be an important

Fig. 6. Analysis of extracellular (lanes 2–4) and periplasmic (lanes 5–7) protein fractions from different strains of *Xanthomonas oryzae* pv. *oryzae* by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained by silver staining as described in Materials and Methods. Lane 1, molecular mass markers (Bio-Rad, Hercules, CA). Lanes 2 and 5, BXO1050 (wild-type strain carrying pUFR034); lanes 3 and 6, BXO803 (BXO801/pUFR034); lanes 4 and 7, BXO804 (BXO801/pSR1). Similar results were obtained in three independent experiments and when *xpsF::Tn10* or *xpsD::Tn10* insertion mutants were used instead of BXO801.

attribute of a rice pathogen. However, from the previous reports the role of xylanase as a virulence factor is not very clear. The fungal pathogen of rice, *Magnaporthe grisea*, produces xylanase, and xylanase knockout mutants of this pathogen had no effect on virulence but it was found that other isozymes were also being produced by the pathogen (Wu et al. 1995). Also, xylanase-deficient mutants of *Erwinia chrysanthemi*, which affects corn (Keen et al. 1996), have been shown to be virulence proficient. Interestingly, xylanase from *Trichoderma viridae* and *T. reesei* has been shown to have elicitor functions since it is able to induce hypersensitive cell death in cell cultures of certain tobacco lines (Yano et al. 1998). We have cloned the xylanase structural gene of *Xoo* (data not shown) and are constructing specific marker exchange mutants to determine the role of xylanase in virulence of *Xoo*.

As shown by protein profiles, xylanase is not the only protein (enzyme) whose secretion is affected by a mutation in the MTB of *Xoo*. Some of these other proteins may also be playing an important role in virulence. The observation that the *xpsF* mutants are not complemented for virulence by coinoculation with the wild type suggests that some other virulence functions might be secreted through the MTB that are restricted to cells that possess them. One possibility may be that MTB is required for secretion of a pilus or adhesin that is essential for *Xoo* virulence.

The Tn5-induced MTB mutants of *Xcc* were shown to accumulate polygalacturonate lyase and α -amylase enzymes in their periplasm but no enzyme was detected in the cytoplasm (Hu et al. 1992). In our study, we find that xylanase not only accumulates in the periplasm but also can be detected in the cytoplasmic fraction. This suggests the possibility that mutation in the *xps* genes of *Xoo* may affect, either directly or indirectly, the transport of certain secreted proteins from the cytoplasm to the periplasm. Alternatively, the possibility of contamination between the two fractions during extraction of cellular fractions cannot be ruled out.

Earlier workers had reported a cluster of genes from *Xoo* that were iso-functional with those encoding type II secretion proteins in *Xcc* and could complement for pathogenicity and production of proteases in *Xcc* mutants (Todd et al. 1990). They, however, did not characterize these genes for their role in virulence of *Xoo*. In our study, we conclusively show that mutations in the MTB of the GSP affect virulence as well as secretion of xylanase and several other proteins in *Xoo*. The role of specific secreted proteins in virulence of *Xoo* remains to be determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media.

The source and characteristics of the bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* cells were cultured on LB medium (Miller 1992) at 37°C and *Xoo* cultures were grown on peptone sucrose agar (PSA; Tsuchiya et al. 1982) medium at 28°C. The minimal medium used for growing *Xoo* was modified Miller's minimal medium (M4; Kelemu and Leach 1990). Nutrient agar (NA) medium contained 23 g of nutrient agar (Difco, Detroit, MI) per liter of water.

Antibiotics used in this study were as follows: kanamycin (Km) 50 mg/liter for *E. coli* and 25 mg/liter for *Xoo*;

cephalexin (Cp) 20 mg/liter; tetracycline (Tc) 10 mg/liter for *E. coli* and 5 mg/liter for *Xoo*; rifampicin (Rf) 50 mg/liter.

EMS mutagenesis of *Xoo*.

EMS (Sigma Chemical, St. Louis, MO) mutagenesis of *Xoo* was as described for *E. coli* by Miller (1992). Twenty microliters of a cell suspension from mutagenized and washed *Xoo* cells was inoculated in 2 ml of PS and incubated at 28°C for 48 h before the cells were dilution plated for single colonies on PSA plates. Individual Eps-proficient colonies that appeared on the plates were used for virulence assays.

Virulence assays.

Virulence assays were performed on 40- to 60-day-old plants of the highly susceptible rice cultivar Taichung Native -1 (TN-1) by the leaf clipping method of inoculation (Kauffman et al. 1973). Saturated cultures of *Xoo* grown in PS medium were pelleted down, resuspended in water at a density of 10^9 cells per ml. Scissors dipped in this inoculum were used to clip the tips of rice leaves. Symptoms were scored by measuring lesion lengths at regular intervals. Strains that either did not cause lesions or formed smaller lesions on TN-1 leaves after repeated inoculation were considered to be virulence deficient (*Vir*⁻).

Reisolation of bacteria from infected rice leaves.

Infected leaves were surface sterilized by dipping in 2% (vol/vol) sodium hypochlorite (Loba Chemie, Mumbai, India) for 2 min and washing twice in sterile water. The leaves were cut at the leading edge of the lesion and dipped in 1 ml of sterile water for 2 min. Bacteria that exuded from the cut edge of the leaf were isolated by plating for individual colonies on appropriate medium.

HR test on tomato leaves.

Saturated cultures of *Xoo* prepared as for virulence assays were infiltrated into leaves of greenhouse-grown tomato plants of cv. Pusa Ruby (suppliers: Maharashtra Hybrid Seeds, Jalgaon, India) with a needle and hypodermic syringe. HRs elicited by *Xoo* in the form of necrotic regions at the site of inoculation were observed within 24 to 48 h of infiltration.

GUS assays.

GUS activity of bacterial colonies was assayed by staining with 4-methyl umbelliferyl β -glucuronide (MUG, Sigma Chemical) and visualized under UV light as described by Jefferson et al. (1987).

Bacterial conjugation.

Matings between *E. coli* strains were done by growing cultures of each of the donor and recipient strains in LB medium containing the required antibiotics for 24 h at 37°C, washing the cells with sterile water, and resuspending in the original volume of sterile water. The cells were mixed in the ratio of 1:1 (vol/vol) and 0.1 ml of the mixture was spotted on N⁺ membrane (Amersham Life Sciences, Buckinghamshire, UK) placed on LB agar. Following incubation for 24 h at 37°C, the cells were removed from the membrane with sterile toothpicks into 1 ml of sterile, distilled water, plated on selection media, and incubated at 37°C for 24 h to obtain transconjugants.

For mating between *E. coli* as the donor and *Xoo* as the recipient strain, *E. coli* cells were prepared as described above. *Xoo* cultures were grown to saturation in PS medium for 48 h at 28°C, following which the cells were pelleted and resuspended in one-hundredth the original volume of sterile water. Matings were performed by mixing the above cultures of donor and recipient at a ratio of 1:10 (vol/vol) and spotting 0.1 ml on N⁺ membrane overlaid on NA. After 48 h of incubation at 28°C, cells were removed from the membrane and plated on selection media.

Isolation of complementing clone by functional complementation.

A partial *Eco*RI-digested genomic library of our laboratory wild-type *Xoo* strain, having an average insert size of 30 kb, has been made (Rajagopal et al. 1995) in the broad-host-range cosmid cloning vector pUFR034 (Km^r; DeFeyter et al. 1990). Nine hundred sixty clones from this library were transferred from *E. coli* strains DH5 α to S17-1 with pRK600 as a helper (L. Rajagopal, S. Dharmapuri, and R. V. Sonti, *unpublished results*). Genomic clones from the above library in S17-1 were mobilized, in pools of 12 clones each, into the virulence-deficient mutant by biparental matings. Transconjugants that appeared on PSA plus Km and Rf selection plates were pooled together in 1 ml of sterile water and inoculated on TN-1 rice plants to identify clones that restored virulence.

Assay for extracellular enzymes.

Assays for xylanase secretion were performed as described by Keen et al. (1996). *Xoo* colonies were spotted on PSA containing 0.2% RBB-xylan (Sigma Chemical). A white halo surrounding the colonies against the blue background of the plate indicated secretion of xylanase. *Xoo* strains unable to produce xylanase did not show the halo. Quantitation of xylanase in different cellular fractions was done according to the procedure described by Biely et al. (1988).

Assays for extracellular cellulases, pectinases, and proteases were done by the procedure described by Tsuchiya et al. (1982), with some modifications as per Andro et al. (1984).

Transposon mutagenesis and marker exchange.

Transposon mutagenesis of the cloned DNA was performed with either mini Tn5 (Wilson et al. 1995) or mini Tn10 dTet derivative (Kleckner et al. 1991). Tn5 mutagenesis of the cloned DNA was done by mobilizing a suicide plasmid carrying the transposon Tn5*gus* A40 (Wilson et al. 1995) into the DH5 α strain having the cloned DNA (pSR1), by conjugation. Transconjugants were selected on LB agar plates containing kanamycin and spectinomycin. To identify insertions on plasmid DNA, transconjugants were conjugated with another *E. coli* strain (CAG18431, strain list) and selected on LB agar plates containing spectinomycin, kanamycin, and tetracycline. For Tn10 mutagenesis, lysates of λ NK1323 containing the mini Tn10 transposon (Kleckner et al. 1991) were prepared on the LE392 strain of *E. coli* as described by Miller (1992). The MC4100 strain of *E. coli* carrying the pSR1 plasmid was grown for 24 h at 37°C in 1 ml of LB medium containing 0.4% maltose and kanamycin. The cells were subcultured in 10 ml of the above medium and grown at 37°C till A₆₀₀ was 0.6 to 0.8. To 2 ml of this culture containing approximately 1 $\times$ 10⁹ cells, 10 ml of λ NK1323 lysate (with a titer of 1 $\times$ 10¹⁰

per ml) was added and the culture was incubated at 37°C for 20 min. The culture was centrifuged at 10,000 $\times$ g for 5 min and the pellet was washed with 0.1 M citrate buffer (pH 5.5). The cells were resuspended in 5 ml of LB medium containing 20 mM sodium pyrophosphate, incubated at 37°C for 2 h, diluted in 40 ml of LB medium containing kanamycin and tetracycline, 25 mM sodium pyrophosphate, and incubated overnight at 37°C. Cells were pelleted and then conjugated with DH5 α with a helper strain containing pRK600. Transconjugants were obtained on LB agar plates containing kanamycin, nalidixic acid, and tetracycline. Plasmid was isolated from these transconjugants and digested with *Eco*RI to localize the transposon insertions on either vector or insert DNA. Transposon insertions in the insert DNA were further mapped with *Hind*III and *Bam*HI. These pSR1 derivatives were mobilized into BXO801 for complementation assays and BXO043 for marker exchange. Marker exchange was done by growing the cells in either PS+Tet medium (for Tn10 dTet) or in PS+Sp (for mini Tn5) for more than 30 generations by serial passage. Tet or Sp resistant and Km sensitive (Km is the marker on the vector) colonies were analyzed by Southern hybridization to confirm that marker exchange had occurred as expected.

Plasmid isolation and DNA sequencing.

Plasmid DNA was isolated by the alkaline lysis method as described in Sambrook et al. (1989). Restriction digestions were done, as required, with enzymes obtained from NEB (New England Biolabs, Beverly, MA), as per the supplier's instruction. Sequencing of the Tn10 insertions was performed with primers 5'-TGGTCACCAACGCTTTTCCCGAG-3' and 5'-CTGTTGACAAAGGGAATCATAG-3', directed outwardly from Tn10. The sequencing reactions, electrophoresis, and sequence data analysis were performed with the ABI Prism 377 automated DNA sequencer (Perkin Elmer, Norwalk, CT).

Cellular fractionation.

Different cellular fractions were obtained as described by Hu et al. (1992). Saturated cultures of *Xoo* strains were centrifuged at 17,000 $\times$ g for 10 min. The supernatant was taken as the extracellular fraction. The pellet was washed twice with equal volumes of water and then treated on ice for 2 h with lysozyme (200 mg/ml) in a solution made up of 20% sucrose, 30 mM Tris-HCl (pH8.0), and 1 mM EDTA. The lysozyme-treated cells were pelleted by centrifugation at 25,000 $\times$ g for 10 min. The supernatant was collected as the periplasmic fraction. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8.0), passed through a 24-gauge needle, and centrifuged at 25,000 $\times$ g for 15 min. The supernatant was collected as the cytoplasmic fraction. Each of the above fractions was precipitated by 50% (wt/vol) ammonium sulfate and centrifuged at 12,000 $\times$ g for 10 min. The pellets were dissolved in one-tenth the original volume of acetate buffer and assayed for xylanase activity as described above.

Analysis of proteins from various cellular fractions by SDS-PAGE.

Proteins isolated from various cellular fractions were analyzed by SDS-PAGE by the method of Laemmli (1970). The gels were stained with silver nitrate as described by Sambrook et al. (1989). The molecular mass markers used were obtained from Pharmacia Biotechnology (Uppsala, Sweden).

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