Identification of differences in genome content between *Xanthomonas oryzae* pathovar *oryzae* and *Xanthomoas oryzae* pathovar *oryzicola* by suppressive subtractive hybridization and further analysis

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Identification of differences in genome content between *Xanthomonas oryzae* pathovar oryzae and *Xanthomoas oryzae* pathovar oryzicola by suppressive subtractive hybridization and further analysis

by

Ling Chen

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This is to certify that the master's thesis of

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
# TABLE OF CONTENTS

| LIST OF FIGURES | iv |
| LIST OF TABLES | v |
| ABSTRACT | vi |

## CHAPTER 1. MOLECULAR INTERACTIONS OF PLANTS AND PLANT PATHOGENS - A REVIEW OF LITERATURE RELEVANT TO THE RESEARCH PRESENTED IN THIS THESIS  
1

## CHAPTER 2. GENOMIC SUPPRESSIVE SUBTRACTION HYBRIDIZATION REVEALS CANDIDATE DETERMINANTS OF THE TISSUE SPECIFICITY OF PATHOVARs OF THE RICE PATHOGENIC SPECIES XANTHOMONAS ORYZAE  
23

- Summary
- Introduction
- Results
- Discussion
- Experimental procedures
- References

## CHAPTER 3. SEQUENCE ANALYSIS OF A LIPOPOLYSACCHARIDE SYNTHESIS LOCUS IN XANTHOMONAS ORYZAE PATHOVARs  
54

- Summary
- Introduction
- Results
- Discussion
- Experimental procedures
- References

## CHAPTER 4. TOOLS TO IDENTIFY TYPE III EFFECTORS OF XANTHOMONAS ORYZAE PV. ORYZAE AND X. ORYZAE PV. ORYZICOLA  
72

- Summary
- Introduction
- Results and Discussion
- Experimental procedures
- References

## CHAPTER 5. FUTURE PERSPECTIVE  
84

## ACKNOWLEDGMENTS  
86
LIST OF FIGURES

Figure 2.1: Reverse Southern blot analysis of subtraction libraries. 48
Figure 2.2: Southern blot analysis of select subtraction clones. 49
Figure 3.1: Southern blot analysis of long-range PCR products of LPS locus. 68
Figure 3.2: Genetic organization of the \textit{metB-ettA} locus in Xac strain 306 and part of the locus in Xoc BLS256. 69
Figure 3.3: PCR amplification of an internal fragment of \textit{wxoD}. 70
Figure 4.1: Response of leaves of pepper cvs. ECW-20R and ECW to Xcv strains and Psg. 81
Figure 4.2: Plasmid map of pLC8. 82
**LIST OF TABLES**

Table 2.1: Summary of reverse Southern blot results. .................................................. 50
Table 2.2: Summary of Southern blot analyses. ................................................................. 51
Table 2.3: BLAST results of sequences unique to Xoo. ....................................................... 52
Table 2.4: Bacterial strains and plasmids used in this study. ........................................... 53
Table 3.1: Bacterial strains and plasmids used in this study. ........................................... 71
Table 4.1: Summary of estimates for genomic libraries of Xoo PXO86RA and
Xoc BLS256.  .................................................................................. 83
**ABSTRACT**

*Xanthomonas oryzae* pathovar (pv.) oryzae (Xoo) causes bacterial leaf blight of rice. *X. o. pv. oryzicola* (Xoc) causes bacterial leaf streak of rice. Xoo invades the vascular system, while Xoc colonizes the mesophyll parenchyma. To identify key factors that distinguish vascular from non-vascular pathogens, Xoo and Xoc offer a good model. The work presented here provides specific candidate genes and tools for further study. Suppressive subtractive hybridization (SSH) was used to identify sequences unique to one pathovar or the other. Eight clones specific to the Xoo strain used were detected. Four of these were putative insertion sequences (IS). Two encoded a methyltransferase and a putative O-antigen acetylase, respectively. One matched the upstream sequences of some genes in the *avrBs3* family. No clones specific to the Xoc strain used were found. The putative O-antigen acetylase gene, *wxoD*, is localized in a lipopolysaccharide biosynthesis locus in Xoo. A partial clone of the corresponding locus in Xoc BLS256 was found to be highly conserved with the corresponding region in *X. axonopodis* pv. citri, a non-vascular pathogen, but different from the loci in Xoo and *X. campestris* pv. campestris, a vascular pathogen, consistent with a role in tissue specificity determination. Preliminary work to mutagenize this gene for functional characterization is presented. In a related study, tools to identify and compare type III effectors in Xoo and Xoc were assessed. Type III effectors are proteins involved in interactions of pathogenic bacteria and their hosts that may play a role in host and tissue specificity. The feasibility of screening Xoo and Xoc genomic libraries with a reporter of type III secretion based
on the \textit{avrBs2} gene from \textit{X. c. pv. vesicatoria} was examined. A strain carrying the \textit{avrBs2} gene was demonstrated to elicit a visible hypersensitive response in resistant plants in the presence of four times more cells of a strain not carrying the gene, indicating that a pooling approach would be effective to screen a large number of clones. \textit{Xoo} and \textit{Xoc} genomic libraries were constructed for screening.
CHAPTER 1. MOLECULAR INTERACTIONS OF PLANTS AND PLANT PATHOGENS - A REVIEW OF LITERATURE RELEVANT TO THE RESEARCH PRESENTED IN THIS THESIS

*Xanthomonas oryzae* pathovar (pv.) oryzae (Xoo) and *X. oryzae* pv. oryzicola (Xoc) are bacterial rice pathogens. Xoo attacks the vascular system of the leaves and Xoc multiplies in the non-vascular tissue. The two pathovars offer a good model to identify key factors that distinguish vascular from non-vascular pathogens. Based on the hypothesis that Xoo and Xoc contain specific genomic sequences that might account for tissue specificity in the interactions of the pathogens with rice plants, three approaches were used to identify and characterize candidate determinants (Chapters 2 to 4). In this chapter, I review literature concerning molecular interactions of plants and plant pathogens relevant to the research presented in the thesis.

**Bacterial plant diseases**

Bacteria cause 5-10% of known plant diseases (Sigee, 1993). There are four major types of bacterial plant diseases: 1) non-vascular diseases, such as spots and streaks caused by *Pseudomonas* and *Xanthomonas* spp, which multiply typically in the mesophyll parenchyma; 2) vascular diseases including vascular wilts and blights, in which bacteria invade xylem vessels of plants and move systemically through the hosts; 3) soft rots, in which bacteria produce cell wall degrading enzymes and
macerate plant tissues; and 4) tumor diseases, in which bacteria affect plant growth and development.

Though there are economically significant diseases in each of the four classes, the majority of diseases are caused by either necrogenic vascular or necrogenic non-vascular pathogens. Bacteria colonizing vessels belong to many genera of plant pathogens. In some cases, they are vectored by insects. Vascular pathogens may enter wounds and infect the damaged xylem vessels (Mount and Lacy, 1982). Vascular bacterial pathogens can invade hydathodes as well. At dusk, guttation fluid exudes on the tips of leaves from the hydathodes. Transport via this fluid as it is reabsorbed during the day is one means of entry (Xie et al., 1991). Spread from vessel to vessel may depend on ability of the pathogen to degrade the pit walls of vessels (Mount and Lacy, 1982). However, moving through the vascular system is much more rapid than vertical movement in the parenchyma. Bacteria can be passively transferred by nutrient fluid transportation in vessels. Hence, pathogens can infect systemically (Mount and Lacy, 1982; Sigee, 1993). Vascular diseases cause significant losses of yield in severe cases.

Non-vascular bacterial pathogens mostly invade stomata of leaves. *Xanthomonas campestris* pv. vesicatoria is a typical non-vascular pathogen that causes tomato leaf spots. Non-vascular pathogens move in plant intercellular spaces by swimming and bulk flow of bacterial mass (Sigee, 1993).

Many bacterial plant pathogens are given designations of pathovar (pv.). A pathovar designation refers to a set of strains with similar characteristics on the basis of distinctive pathogenicity or host specificity at the infrasubspecific level. For
example, *Pseudomonas syringae* pv. tomato indicates tomato is the host of the pathogen. The meaning of pathovar in *Xanthomonas oryzae* pv. oryzae (Xoo) and *Xanthomonas oryzae* pv. oryzicola (Xoc) does not refer to host range, but distinctive pathogenicity. Rice is the host of these two pathogens but pv. oryzae is a vascular pathogen and pv. oryzicola is non-vascular. These pathogens together constitute a good comparative model to understand the determinants of tissue specificity in pathogen and plant interactions.

**Xanthomonas oryzae** **pv.** **oryzae** and **X. oryzae** **pv.** **oryzicola**

Xanthomonads are generally host-specific, plant-associated bacteria that collectively infect at least 392 plant species, causing necrosis and vascular or parenchymal diseases (Mew, 1993). *Xanthomonas oryzae* pv. oryzae (Xoo), which was first reported in 1922 (Swings et al., 1990), causes bacterial leaf blight and is a typical vascular pathogen. *Xanthomonas oryzae* pv. oryzicola (Xoc) reported first in 1957 (Swings et al., 1990), causes bacterial leaf streak and is a typical non-vascular pathogen. Blight disease is the most important bacterial disease in rice and causes severe losses, up to 50% in some cases. It is distributed in many places where rice is planted. Streak disease is prevalent in humid tropical regions such as south Asia, but also occurs in Africa. It is particularly aggressive on some hybrid varieties and can cause as much as 15-20% losses (Mew, 1993).

Xoo ooze from infected leaves and can spread into neighboring leaves through hydathodes. Xoo can be disseminated by wind as well. Following entry into hydathodes, Xoo multiplies in the epitheme (the tissue connecting the hydathodes
with the xylem) and progresses towards the xylem vessels. Finally, bacterial cells are transported to the entire plant through the vascular system. Blight disease often causes opaque strips along the vein extending to the edge of leaves.

In contrast, Xoc invades the stomata and vertically moves in the intercellular spaces of the mesophyll parenchyma. Tiny spots can be observed after Xoc infection. These expand into streaks. When the transparent yellowish streaks merge, they can mimic blight (Mew, 1993).

Different inoculation methods have been used to compare the modes of invasion of two pathogens. Smearing a bacterial suspension with a brush on the back of leaves resulted in blight disease only 10% of the time but streak 90% of the time. On the other hand, blight disease had a high incidence of up to 90.5% by inoculation of bacteria to guttation fluid, while streak disease only produced disease via this method 2% of the time (Xie et al., 1991). Niño-Liu, Darnielle and Bogdanove (unpublished) have observed that following clipping the leaf tips with a pair of scissors dipped with bacterial suspensions, Xoo spread rapidly down the leaf, but Xoc was restricted to the edges of the cut tip. Following infiltration of a bacterial suspension into the leaf by a syringe without a needle, however, Xoc could spread more widely than Xoo. Thus, methods are available to compare and differentiate infection modes of Xoo and Xoc.

**Bacterial molecules for virulence and pathogenicity**

The majority of known bacterial plant pathogens are extracellular. They use a variety of molecular tools to attack plant cells.
Extracellular polysaccharide (EPS)

Extracellular polysaccharide is thought to protect bacteria from antimicrobial compounds and water stress, and possibly from plant perception (Alfano and Collmer, 1996). It may also play a role in wilts by physically blocking xylem vessels. EPS mutants of *Pseudomonas solanacearum* (Kao et al., 1992), *Erwinia amylovora* (Steinberger and Beer, 1988), and *X. oryzae* (Dharmapuri and Sonti, 1999) created by transposon mutagenesis were reduced in virulence. Xanthan gum produced by xanthomonads is an EPS that has many industrial applications (Daniels *et al.*, 1993).

Extracellular enzymes

Bacteria degrade barriers of plants with extracellular enzymes: endoglucanase, pectinase and protease. Cellulose is a significant component of plant cell walls, and pectin polymers are a basic structural unit in the primary cell walls and middle lamellae of plants. Endoglucanase belongs to a common class of cellulase that degrades cellulose and hemicellulose (Gough *et al.*, 1990; Sigee, 1993). Pectinase secreted by pathogens macerates cell walls and causes cell death and tissue collapse (Collmer and Keen, 1986). Plant bacterial pathogens can produce extracellular proteases that play a role in the early stages of infection of the plant, though their specific targets are not known (Dow *et al.*, 1990; Liu *et al.*, 1990). Cellulase and pectinase are different from protease in some characteristics including phase of secretion, specific transport mechanism and internal accumulation. For example, protease of *Erwinia chrysanthemi* was detected at logarithmic growth
phase, but cellulase and pectinase were released at stationary phase in vitro, which suggests that they might function at different times in planta (Wandersman et al., 1987; Sigee, 1993). Xylanase is another extracellular enzyme and can degrade xylan, a component of xylem vessels. Xylanase genes exist in Xanthomonas oryzae pv. oryzae, but have not been found in X. campestris pv. campestris and X. axonopodis pv. citri. Xylanase production may be a unique feature of Xoo (Lee et al., 2005).

**Toxins**

Bacterial toxins are typically secondary metabolites, and are often important virulence factors. Most of them are small peptides: syringomycin from Pseudomonas syringae pv. syringae and 3(methylthio)-proprionic acid from Xanthomonas campestris pv. manihotis are a few examples (Xu and Gross, 1988; Sigee, 1993). Toxins can spread ahead of bacteria by diffusion. They affect plant metabolism in different ways but generally promote successful infection (Sigee, 1993; Alfano and Collmer, 1996).

**Type III secretion proteins**

Type III secretion proteins are essential for pathogenesis both in animal and plant pathogens. At least four kinds of protein secretion systems are known in Gram-negative bacteria. Type I secretion systems secrete some toxins, proteases and lipases. The type I secretion signal resides in the C-terminal 60 amino acids. The type II secretion system is a two-step secretion pathway that relies on the Sec
pathway, a system used to target proteins to the membrane via a N-terminal signal peptide that is usually cleaved upon secretion. Cellulases, proteases, and pectate lyases discussed above are secreted via the type II pathway. The type IV secretion system is homologous to the bacterial conjugation system and T-DNA transfer system of *Agrobacterium*. The type III secretion system (TTSS) requires a large number of proteins. Type III secretion is independent of the Sec system, and takes place in one step with no periplasmic intermediate (Snyder and Champness, 2003).

The TTSS structure is similar to the flagellar biosynthetic complex (Galan and Collmer, 1999). A supramolecular structure was detected related to the type III apparatus in *Salmonella typhimurium* by electron microscopy (Kubori et al., 1998). A needle-like domain protrudes from the cell envelope and a cylindrical base. The base is composed of a member of the secretin family, InvG (anchors outer membrane), and two lipoproteins, PrgH (anchors inner membrane) and PrgK (holds up InvG and PrgH). Other proteins associated with inner membrane components are InvA, InvC, SpaP, SpaQ, SpaR, and SpaS (Kubori et al., 1998). InvA, InvC, SpaP, SpaQ, SpaR, SpaS, and PrgK share sequence similarities with flagellar proteins FlhA, FlhB, FliC, FliI, FliQ, FliR, FliH, and FliF, respectively (He, 1998). In phytopathogens, genes encoding the TTSS are *hrp* (hypersensitive response and pathogenicity) genes clustered in “pathogenicity islands”. At least eight of them are called *hrc* (for “*hrp*, conserved”) because of their similarity with genes of FlhA to FliF mentioned above and their strict conservation in TTSSs of plant and animal pathogens (Bogdanove et al., 1996; He, 1998). The needlelike supramolecular structure
associated with the TTSS of phytopathogens is referred to as the hrp pilus (He, 1998).

hrp genes control pathogenicity in susceptible plants and elicitation of HR (hypersensitive response) in resistant plants. HR is a rapid cell death associated with plant defense that limits pathogen growth (Wright and Beattie, 2004). Some hrp genes control the expression of other genes in the cluster and others perform the actual functions needed to move proteins out of bacteria and into the plant (Lindgren et al., 1986). Phytopathogenic bacteria in which hrp genes have been well characterized can be grouped according to sequence similarity of regulatory genes and gene organization. Pseudomonas syringae and Erwinia amylovora group together. hrp genes in this group are regulated under HrpL, a member of the ECF (extra cytoplasmic function) family of sigma factors (Xiao and Hutcheson, 1994; Wei and Beer, 1995). Xanthomonas campestris and Ralstonia solanacearum form group II, in which hrp genes are controlled by HrpX (HrpB in R. solanacearum), a member of the AraC family of positive activators (Genin et al., 1992; Wengelnik and Bonas, 1996; Alfano and Collmer, 1997).

Chaperones are required for secretion or stabilization of some TTSS secreted proteins (Page and Parsot, 2002). Chaperone genes are usually present near the locus encoding the corresponding secreted proteins.

TTSS proteins of plant pathogenic bacteria can be divided into two classes: avirulence proteins and harpins (Galan and Collmer, 1999). Avirulence genes are defined by their ability to elicit a defense response in a host possessing a corresponding resistance gene (gene-for-gene hypothesis; Flor, 1956). For example,
The product of *avrPto* in *P. syringae* pv. tomato can elicit a hypersensitive response (HR) in tomato containing the *Pto* gene (Tang *et al.*, 1996). Many avirulence genes have demonstrable roles in parasitism (Alfano and Collmer, 1996). Although most avirulence proteins do not share similar physical characteristics, evidence shows they are Hrp dependent and targeted to plant cells. The cloned *hrp* cluster of *Pseudomonas syringae* pv. syringae strain 61 enables saprophytic *E. coli* to secrete avirulence proteins into plant cells (Pirhonen *et al.*, 1996). AvrB of *P. syringae* functions inside cells of *Arabidopsis thaliana* as revealed by a transient expression experiment, which suggested that the Hrp secretion system could deliver AvrB to plant cells (Gopalan *et al.*, 1996). The AvrBs3 family contains nuclear localization signals (NLSs) that direct translocation of these proteins to the plant nucleus (Yang and Gabriel, 1995; Van den Ackerveken *et al.*, 1996). The resistance protein Pto is predicted to be cytoplasmic and interacts with *P. syringae* pv. tomato AvrPto, indicating that AvrPto must enter plant cells (Tang *et al.*, 1996).

In addition to the local HR, several plant responses are observed during plant defense: (1) active oxygen is generated 1.5 to 3 hours post inoculation; (2) rapid K⁺ efflux/H⁺ influx exchange occurs concurrent with active oxygen generation; and (3) defense-related proteins are strongly expressed (Atkinson, 1993; Levine *et al.*, 1994; Ryals *et al.*, 1996). However, the details of the mechanism of Avr and R protein interaction to trigger defense responses are not clear.

Avirulence genes cloned from *Xanthomonas oryzae* pv. oryzae include *avrXa7* and *avrXa10*, members of the *avrBs3* family that correspond to rice R genes *Xa7* and *Xa10* (Hopkins *et al.*, 1992). An avirulence gene *avrRxo1* was
characterized in *X. o. pv. oryzicola*, which has a corresponding *R* gene in maize (Zhao *et al.*, 2004).

Harpins are glycine-rich, cysteine-lacking, hydrophilic, heat stable proteins that can trigger HR when applied exogenously. However, this function does not manifest during plant bacterial interaction (Galan and Collmer, 1999). Harpins lack detectable enzymatic activity. Though they are thought to facilitate delivery of other bacterial proteins into plant cells, the functions of harpins remain a puzzle (Alfano and Collmer, 1996).

**Lipopolysaccharide (LPS)**

Lipopolysaccharide (LPS) is another pathogenicity-related factor, though it has not been demonstrated to be as important as EPS, extracellular enzymes or TTSS proteins. LPS is a ubiquitous component of the outer-membrane of Gram-negative bacteria and is not present on the cell surface of Gram-positive bacteria. LPS is made up of three parts: 1) a glucosamine with fatty acids called lipid A anchors to the outer-membrane; 2) a core oligosaccharide links to lipid A with ketodeoxyoctonate; and 3) polysaccharide chains of monosaccharides termed the O-antigen are surface oriented and species-specific repeating units (Dow *et al.*, 2000). LPS is important in the survival of *Pseudomonas aeruginosa* (an animal pathogen that can cause severe diseases in immunocompromised patients) in different environments. Two forms of LPS O-antigen can alter physico-chemical properties of the bacterial cell to protect the cell from serum killing (Newton, 2001). In plant pathogens, an LPS biosynthesis locus in *Xanthomonas campestris* pv.
campestris contributes to pathogenicity (Dow et al., 1995). Curiously, exogenous LPS from X. c. pv. vesicatoria can induce defense responses in plants, including callose and cellulose deposition and alteration of plant cell walls (Keshavarzi et al., 2004). LPS pretreatment can alter the expression of defense-related genes in plants (Dow et al., 2000). The LPS O-antigen epitope of the nitrogen-fixing symbiont Rhizobium is expressed differentially in different sections of pea root nodules (Kannenberg et al., 1994). But how LPS contributes to the pathogenicity of bacterial phytopathogens is still largely unclear.

Genomic comparisons for understanding plant pathogenesis

Understanding molecular mechanisms of bacterial plant pathogenicity is important because it would provide a chance to develop new, effective controls. A major question that remains to be answered is what are the determinants of vascular and non-vascular pathogenicity. X. oryzae pathovars provide a uniquely valuable comparative model to explore this question. My interest and the topic of this thesis is a genomic comparison between these two pathovars to identify differences that define their different pathogenic characteristics.

DNA similarity between Xoo and Xoc is about 90% as measured by DNA-DNA hybridization (Vauterin et al., 1995). Each has a similar percentage of guanine plus cytosine (%G+C) in the genome: 64.5-65.0% (Swings and Civerolo, 1993). The genomes of Xoo and Xoc carry different copies and patterns of repetitive DNA sequences (Swings et al., 1993).
The genomic sequence of a Xoo strain has recently been published (Lee et al., 2005). A complete Xoc sequence is not yet available however. Nonetheless, there are several approaches possible for a comparative genomics study. Genetic mapping is useful to localize DNA markers on the chromosome and identify major differences in gene content and organization. Restriction Fragment Length Polymorphisms (RFLPs) can be used in genetic mapping. Electrophoretically separated DNA, fragmented by treatment with a restriction endonuclease, is hybridized with a labeled piece of homologous DNA. Single-base-pair differences throughout the genomes cause differences in numbers and locations of restriction sites; another possibility is variation in the form of rearrangements, insertions, and deletions. Marker technologies analogous to RFLP are Amplified Fragment Length Polymorphism (AFLP) and Randomly Amplified Polymorphic DNA (RAPD).

Subtractive hybridization (SH) is a useful method of genomic comparison based on selecting strain-specific DNAs. It was first reported in 1990 (Straus and Ausubel, 1990). After SH was reported, several modifications were applied. Representational difference analysis (RDA) is one of the variations. It employs three rounds of hybridization and PCR to isolate and amplify subtraction products (Becker et al., 2001). Another variation of SH is suppressive subtractive hybridization (SSH), which was first reported in cDNA hybridization in eukaryotes (Diatchenko et al., 1996). The technique was used with genomic DNA in a study of Helicobacter pylori in 1998 (Akopyants et al., 1998). In contrast to SH, which hybridizes tester DNA (from the strain containing specific regions of interest) to excess driver DNA (from the reference strain to be subtracted) directly using only one adaptor ligated to tester
DNA, SSH divides the tester strain into two aliquots each ligated to a different adaptor, and hybridizes tester to tester to excess driver. The adaptors have long terminal repeats that suppress amplification by PCR if matching adaptors exist on both ends of a fragment following hybridization (and fill-in). Thus tester fragments from one aliquot that have not hybridized to a tester fragment in the other aliquot will not be amplified, reducing the recovery of false positives. SSH is not highly efficient, but it can be fairly comprehensive for differences of 100 bp or more. Generally, a subtraction library is constructed after SSH. Southern blot analysis is used to identify and confirm strain specific clones.

SSH will miss single nucleotide polymorphisms or small insertions/deletions. Nevertheless, many differences among closely related species that differ in their pathogenicity are encoded in large pathogenicity islands (Pais) (Hacker et al., 1997). hrp, and many pth and avr genes exist on such islands, which may move from strain to strain by horizontal gene transfer (Gabriel, 1999). In addition to containing genes associated with pathogenicity, Pais are typically characterized by atypical %G+C content and flanking repeats or insertion sequences (IS) (Winstanley, 2002). Pais are an example of differences that might be revealed efficiently by SSH.

**Content of this thesis**

SSH is a cost-efficient and convenient method to compare two genomic DNAs. In chapter 2 of this thesis, I describe the successful execution of SSH between an Xoo strain and an Xoc strain, which revealed candidate genes for determinants of tissue specificity. In chapter 3, I present further characterization of
an LPS biosynthesis locus that contains one of the genes identified by SSH. In this chapter I also describe preliminary work toward mutagenesis of the gene for functional characterization. Finally, in Chapter 4 I describe preliminary work toward conducting a screen for and comparison of type III secreted proteins in Xoo and Xoc.
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CHAPTER 2. GENOMIC SUPPRESSIVE SUBTRACTIVE HYBRIDIZATION
REVEALS CANDIDATE DETERMINANTS OF THE TISSUE SPECIFICITY OF
PATHOVAR S OF THE RICE PATHOGENIC SPECIES XANTHOMONAS ORYZAE

A paper to be submitted to Molecular Plant Pathology

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Summary

*Xanthomonas oryzae* pathovar (pv.) oryzae (Xoo) is a rice vascular pathogen that multiplies in the xylem vessels and causes bacterial leaf blight disease. *X. o. pv. oryzicola* (Xoc) is a rice non-vascular pathogen that multiplies in the mesophyll parenchyma and causes bacterial leaf streak disease. To identify key factors that distinguish vascular from non-vascular pathogens, Xoo and Xoc offer a good model. To identify genome sequences specific to Xoo or Xoc that might account for tissue specificity in the interactions of the pathogens and rice plants, the suppressive subtractive hybridization (SSH) technique was employed. Eight clones specific to the Xoo strain (Xoo PXO86RA) were detected. Four of the eight clones were found to encode putative insertion sequences (IS). Two clones were predicted as a methyltransferase and a putative O-antigen acetylase, respectively. One of the

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remaining clones was found to match the upstream sequences of many avirulence genes in the \textit{avrBs3} family. The last clone did not show significant similarity to any known protein. Clones specific to the Xoc strain (Xoc BLS256) were not found. The Xoo PXO86RA-specific clones include sequences implicated in pathogenesis and represent good targets for future study.

\textbf{Introduction}

Bacteria cause severe losses in many crops. Bacterial pathogens typically invade plants either through the vascular system or by colonizing the mesophyll parenchyma. Vascular diseases can be particularly damaging because they can often lead to systemic infection. Examples of vascular diseases include black rot or black vein of crucifers, and bacterial wilt of bean. Examples of non-vascular diseases include bacterial spot of tomato and pepper, and bacterial leaf streak of rice. The mechanisms that pathogens use to infect plants are relatively little known; a better understanding of those mechanisms would provide opportunities to develop novel, effective control and prevention methods. A good starting point is to identify key factors that distinguish vascular from non-vascular pathogens. \textit{Xanthomonas oryzae} \textit{pathovar (pv.) oryzae} (Xoo) and \textit{X. o. pv. oryzicola} (Xoc) offer a powerful model system to accomplish this goal.

Both Xoo and Xoc are rice pathogens. Xoo causes bacterial blight. It invades leaves through hydathodes or wounds and spreads along the xylem vessels. Xoc causes bacterial leaf streak. It enters through stomata and colonizes the mesophyll parenchyma (Mew, 1993). Xie \textit{et al.} (1991) demonstrated that this specificity is
maintained in artificial inoculation. Following inoculation to guttation fluid, and subsequent entry through hydathodes, Xoo incited disease 90% of the time and Xoc only 2%. Conversely, “painting” bacterial suspension onto the abaxial leaf surface, permitting entry through stomata, resulted in infection by Xoc 90% of the time, but by Xoo only 10% of the time. Xoo and Xoc share some genetic features: moderately high G+C content (63-71%), repetitive elements, and no indigenous plasmid (Swings et al., 1993). At the whole genome level they are greater than 90% identical as measured by DNA-DNA hybridization (Swings et al., 1990).

Several molecular tools that help xanthomonads and other bacteria multiply in plants have been identified, such as extracellular polysaccharide (EPS), proteases, and type III effector proteins (Daniels et al., 1993; Alfano and Collmer, 1996; Dharmapuri and Sonti, 1999). Xoo and Xoc have similar type III secretion system genes (Raymundo and Leach, 1994). Two avirulence genes in Xoo have been isolated (Hopkins et al., 1992) and a gene in Xoc that triggers resistance responses in maize has been characterized (Zhao et al., 2004). One of the _avr_ genes in Xoo, _avrXa7_, has been shown to play a major role in virulence (Yang and White, 2004). However, differences at the genome level that contribute to defining tissue specificity or distinctive pathogenicity remain unknown. Identifying these factors and their functions will enable us to better understand the mechanism of interactions between plant pathogens and their hosts and suggest ways to better control them.

The genome sequence of Xoo strain KACC10331 was recently published (Lee et al., 2005). However, a genome sequence of Xoc is not yet available. To identify unique sequences between Xoo and Xoc, there are several possible
approaches. These include genetic mapping using marker technologies, for example, restriction fragment length polymorphism (RFLP) or amplified fragment length polymorphism (AFLP), and subtractive hybridization (SH) (Krawiec and Riley, 1990; Straus and Ausubel, 1990; Araujo et al., 2004). SH is the more comprehensive approach. It can enrich specific regions in one strain (tester) by subtracting with a reference strain (driver). There are two variations of the SH technique. One is representational difference analysis (RDA), which is usually used to screen cDNA libraries for unique clones (Becker et al., 2001). The other method is suppressive subtractive hybridization (SSH), which was developed to compare eukaryotic transcripts (Diatchenko et al., 1996) and later used in comparing microbial genome differences (Akopyants et al., 1998). In SSH, the tester DNA is divided into two aliquots and each is ligated with a different adaptor prior to hybridization with driver DNA and with each other. The adaptors contain long terminal repeats. In contrast to SH, in which the tester DNA is ligated with only one adaptor without long repeats, this approach “suppresses” amplification of tester that has not undergone hybridization: the long terminal repeats on both ends (following fill-in) favor the formation of hairpin structures that hinder amplification.

An abundance of repetitive sequences, and moderately high G+C content typical of Xanthomonas spp. could be expected to have a negative effect on the efficiency of SSH, and in fact I found that following a standard protocol in a commercial kit was unsuccessful for SSH of Xoo and Xoc genomic DNA. I report here a successful modification of the SSH protocol that was successful for subtraction of genomic DNAs of Xoo and Xoc that may be useful for SSH of other
high-repeat, moderately high G+C content DNAs. Using this protocol, I identified several sequences unique to Xoo, also described here. Some of these are sequences implicated in interactions with the host and may represent determinants of host tissue specificity.

Results

Modification of hybridization parameters enables subtraction of genomic DNAs of *X. oryzae* pathovars

Both Xoo and Xoc have abundant repetitive sequences throughout their genomes and a moderately high G+C content (63-67%, Swings et al., 1993). Use of a standard protocol (PCR-select bacterial genome subtraction kit user manual, Clontech) failed to yield amplifiable products. In order to avoid non-specific subtraction of DNA fragments of the tester strain by the driver, several modifications were made. First, five hybridization temperatures (63°, 65°, 68°, 70°, and 73°C) were tested. The hybridization temperature in the standard protocol is 63°C. Hybridization at 73°C yielded strong bands after PCR amplification (data not shown). Second, use of only one hybridization step instead of two was assayed, and found to yield more distinct bands of PCR product. Finally, the amount of tester DNA aliquots relative to driver was increased from 1:1:50 to 1:1:12.5. This further improved amplification results.

After hybridization and PCR amplification, PCR products were cloned and two subtraction libraries (Xoo as tester and Xoc as tester, respectively) with 480 clones each were constructed. Primers corresponding to the vector were used to amplify
the inserts. 467 clones in the Xoo library and 426 clones in the Xoc library had inserts. The inserts were separated in agarose gels, transferred to membrane by Southern blotting, and hybridized separately with labeled total genomic DNA of Xoo and Xoc ("reverse Southern hybridization"). Four kinds of clones were observed: highly conserved between Xoo and Xoc, weakly conserved, absent from both, and unique to tester (Figure 2.1). The apparently unique clones were tested again by using the clones individually to probe Southern blots of digested Xoo and Xoc genomic DNAs. There were still the same four kinds of results, indicating that the reverse Southern hybridization was prone to yielding artifacts (Figure 2.2).

After reverse Southern blot, the percentage of clones apparently unique to tester strain was 14.6% for Xoo (68 clones) and 8.7% for Xoc (37 clones). The percentage of clones to which both genomic DNA probes hybridized but with different apparent strength was high, 46.25% for Xoo and 64.32% for Xoc. And 10.71% in the Xoo library and 17.37% in the Xoc library showed similar levels of hybridization to both probes (Table 2.1). The 68 clones from the Xoo tester library and 37 clones from the Xoc library that were apparently unique were selected as candidates to sequence. Sequence was obtained from 56 and 32 of these, respectively. The clones were grouped according to sequence similarity into 16 clusters for Xoo and 11 clusters for Xoc. Representative clones for each of these clusters were chosen and hybridized individually to Southern blots of Xoo and Xoc genomic DNA, respectively, cut with EcoRI, SalI or Rsal. Clones representing eight clusters were confirmed to be unique to Xoo (Table 2.2). These eight clusters
contain a total of 27 clones. Sequences specific to Xoc were not identified, but two clones hybridizing strongly to Xoc showed only weak hybridization to Xoo (Table 2.2).

**DNA fragments unique to Xoo include sequences implicated in pathogenesis**

The eight Xoo-specific representative clones were subjected to BLASTN and BLASTX (available through the National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) analysis to identify similar sequences and predicted functions. Half of the clones, Xoo2H12, Xoo4A6, Xoo4C1, and Xoo5D6 were homologous to transposase genes (Table 2.3). They also hybridized to multiple bands in Southern blot analysis (data not shown). This finding is consistent with the large number of IS element families in the published Xoo genome (Lee et al., 2005). The putative IS elements identified here are absent from the other published *Xanthomonas* genomes, *X. campestris* pv. *campestris* (Xcc) and *X. axonopodis* pv. *citri* (Xac) (Da Silva et al., 2002) (Table 2.3).

Using BLASTX, Xoo4H1 was found to be similar to the first 100 bp of the open reading frame of some avirulence genes in Xoo KACC10331 belonging to the *avrBs3/pthA* family (Table 2.3), as annotated in the accession. This sequence was found 77-247 base pairs upstream of the open reading frames of *avrXa10* (Xoo), *avrBs3* (*Xanthomonas campestris* pv. *vesicatoria*, Xcv), *pthA* (Xac), and *avrBs3-2* (*Xcv*), and 82-252 base pairs upstream of *pthN* and *avrb6* (*Xanthomonas campestris* pv. *malvacearum*, Xcm).
Xoo5E6 matched the O-antigen acetylase encoding gene \textit{wxoD}, which is localized in a lipopolysaccharide (LPS) biosynthesis locus in \textit{Xoo} (Table 2.3). Dharmapuri \textit{et al.} (2001) first identified this locus in \textit{Xoo} and later noted that it was absent from a \textit{Xoc} strain examined in their study (Patil and Santi, 2004). The \textit{wxoD} gene plays a role in decoration of the O-antigen of LPS. No homologies of the gene were found in \textit{Xcc} or \textit{Xac} (Lee \textit{et al.}, 2005).

\textit{Xoo3H1} was highly similar to a predicted methyltransferase gene of \textit{Xoo KACC10331}. This gene is homologous to adenosine-specific DNA methylases of the \textit{mod} family of type III restriction modification systems (Bickle and Kruger, 1993) (Table 2.3).

The last clone, \textit{Xoo5B4}, corresponded to a roughly 800 bp intergenic region between a copy of the \textit{ISXo8} transposase and a predicted homolog of the \textit{acrD} transporter gene in \textit{Xoo KACC10331}. It did not show significant similarity to any other known sequences in the nucleic acid or protein databases (Table 2.3).

\textbf{Subtraction clones have relatively low \%G+C content}

Among the eight \textit{Xoo} clones, the highest G+C content is 59%; the lowest is 47% (Table 2.3). The average \%G+C content is only 55%. The average for the \textit{Xoo} genome is 63.7\% (Lee \textit{et al.}, 2005).

\textbf{Subtraction from \textit{Xoc} reveals divergent but not unique sequences}

The two \textit{Xoc} clones that hybridized weakly to \textit{Xoo} were \textit{Xoc2C2} and \textit{Xoc2E11}. \textit{Xoc2C2} is 248 base pairs in length and has 56\% G+C content. The
closest BLASTX match was a hypothetical protein in *Xylella fastidiosa* (Genbank accession: ZP_00341653.1) with an e-value of $2 \times 10^{-11}$, conserved in several other bacterial species. Xoc2E11 is 297 base pairs and has a G+C content of 60%. Its closest match using BLASTX was a hypothetical protein in *Bradyrhizobium japonicum* (Accession: NP_774956.1) with an e-value of $2 \times 10^{-12}$, but it also aligned with IS element related ORFs in numerous bacterial species.

**Discussion**

As an initial step toward identifying genome differences that account for the characteristic tissue-specificity of *Xanthomonas oryzae* pathovars Xoo and Xoc, I carried out reciprocal suppressive subtractive hybridizations (SSH) of genomic DNAs to identify sequences unique to one pathovar or the other. A standard protocol successful with genomic DNA of *Helicobacter pylori*, *E. coli*, and *Pseudomonas fluorescens* strains (PCR-select bacterial genome subtraction kit user manual, Clontech; Akopyants *et al.*, 1998; Mavrodi *et al.*, 2002) failed to yield subtraction products with the high repeat, and relatively high G+C content DNAs of *Xanthomonas oryzae* pathovars. I modified the protocol by raising the hybridization temperature, increasing the tester to driver ratios and by carrying out the hybridization in only one step. With these modifications, PCR products from both subtractions were successfully amplified. Among these, not only multiple copy sequences, but also single copy sequences unique to tester were isolated, indicating that these modifications were effective. Successful SSH of high repeat, moderately high G+C content DNAs has not been reported previously. I anticipate that the
protocol presented here will be useful for SSH of DNA from other organisms with similar characteristics. My results suggest also that for other DNAs for which the standard protocol is sub-optimal, the parameters I modified would be appropriate targets for optimization.

Clones from both subtractions were assayed by reverse Southern blot analysis to determine whether they were in fact unique to the pathovar used as tester: Xoo and Xoc genomic DNAs were labeled and hybridized with clone inserts that had been PCR amplified, separated by agarose gel electrophoresis, and Southern blotted to membrane. This approach was undertaken in lieu of the commonly used colony hybridization in order to avoid potential false positives due to hybridization of Xanthomonas genomic DNA to residual E. coli genomic DNA. Nevertheless, the reverse Southern blot in my hands yielded both negative and positive artifacts, as determined by subsequent probing of genomic DNAs with individual, apparently unique clones in standard Southern blots. I surmise that negative artifact arose due to uneven labeling of genomic DNA used as probe, resulting in weak or undetectable hybridization signal. Positive artifact might be explained by weak, non-specific hybridization of a clone to total labeled genomic DNA. In no case were there clones apparently unique to tester in the reverse Southern analysis that proved subsequently to be unique to driver in the standard Southern blot analysis. It is probably safe to assume that most of the actual unique clones reside among those apparently unique in the reverse Southern blot analysis, and that some may reside among those that failed to show any hybridization at all by reverse Southern and those that hybridized to both genomic DNA probes. In
summary, the reverse Southern blot is a useful but likely not comprehensive method to detect candidate unique clones, and confirmation of clones by standard Southern blot analysis or other means is essential.

Eight clusters of sequences were identified as unique to Xoo, among these, four were related to IS elements and present in multiple copies in the Xoo genome. These sequences are not present in the published Xcc and Xac genomes. The Xoo genome has more than twice the number of mobile elements as the Xcc or Xac genomes. Many of these flank strain-specific genes (Lee et al., 2005), especially some virulence and avirulence genes. IS elements may contribute indirectly to host and tissue specificity by vectoring new genes that serve as specificity determinants. Additionally, IS elements may affect host and tissue specificity by disrupting or altering expression of genes upon insertion into the genome. Mapping the distribution of the elements identified in this study and characterization of the genes near them is an important goal.

Two of the four remaining clusters of clone sequences from the subtraction from Xoo represented single copy genes. One of these, represented by clone Xoo3H1, is a putative DNA methylase. It is absent from Xac, but present in the Xcc genome (Table 2.3). It is also present in Xylella fastidiosa, a fastidious plant pathogenic species closely related to the xanthomonads. Therefore, its absence from Xoc and Xac is somewhat puzzling. Distinct from the previously characterized XorI1 locus shown to be unique to Xoo (Choi and Leach, 1994), it is most similar to adenosine-specific DNA methylases of the mod family of type III restriction modification (RM) systems, which are relatively rare (Bickle and Kruger, 1993). RM
systems of bacteria are one factor affecting sensitivity to phage attack. Methylases in RM systems modify the bacterial DNA and protect it from cleavage by corresponding bacterial endonucleases. Foreign (e.g., phage), unmethylated DNA, if it contains cleavage sites corresponding to the endonucleases, is destroyed upon entry into the cell. If Xoo3H1 represents part of a fully functional restriction-modification system, it may contribute to differential phage sensitivity between Xoo and Xoc. Phage sensitivity has not been well characterized in Xoc however.

The second clone of a single copy gene unique to Xoo represents a putative O-antigen acetylase gene, wxoD, previously characterized by Dharmapuri et al. (2001). The gene is in a large locus that is involved in lipopolysaccharide (LPS) and extracellular polysaccharide (EPS) biosynthesis and is required for full virulence. The locus is between metB, a putative cystathionine gamma-lyase gene and etfA, a putative electron transferring flavoprotein alpha subunit gene. These flanking genes are conserved in many xanthomonads. Patil et al. (2004) reported six genes including wxoD in the locus. These are wxoA, a putative sugar nucleotide epimerase/dehydrase gene, wxB, a putative glycosyl transferase gene, wxC, a putative glycosyl transferase gene, wxoD, wzt, a putative ATP binding protein gene, and wzm, a putative ABC transporter system integral membrane protein gene (Dharmapuri et al., 2001; Patil and Sonti, 2004). Patil et al. (2004) determined that the contents of the locus, including wxoD, are not conserved in Xoc, Xcc, or Xac. The locus between metB and etfA in Xac contains 14 genes from wzm (XAC3601), an ABC transporter permease gene, to XAC3588, an integral membrane protein gene (GenBank accession: NC_003919). The locus between metB and etfA in Xcc
contains 15 genes from \textit{wxA} (XCC0599), glycosyltransferase gene, to \textit{wxcH} (XCC0618), WxcH protein gene (GenBank accession: NC_003902).

\textit{wxoO} is predicted to function in modification of the O-antigen of lipopolysaccharide (LPS) based on homology (42\% sequence identity of the predicted proteins) with the well-characterized \textit{wbiA} gene in the human pathogenic species \textit{Burkholderia thailandensis}. (DeShazer \textit{et al.}, 1998). The O-antigen of bacterial LPS plays a role in triggering animal immune response. The roles of O-antigen in plant bacterial interactions have been studied in symbiotic relationships between bacteria and plants (Kannenberg \textit{et al.}, 1994), but the picture is not yet entirely clear. An LPS biosynthetic locus in \textit{Xanthomonas campestris} was shown to be required for full virulence (Dow \textit{et al.}, 1995), yet, purified LPS can induce the expression of defense-related genes in plants, or in some cases prevent the defense-associated hypersensitive response (Newman \textit{et al.}, 1997; Dow \textit{et al.}, 2000). Different pathovars within \textit{X. campestris} have antigenic variation (Swings \textit{et al.}, 1993). In sum, the evidence points to a potential role for O-antigens in contributing to host range determination or pathogenicity. It is intriguing to speculate that \textit{wxoD} may modify O-antigen in a way that contributes to the tissue specificity of \textit{Xoo} relative to \textit{Xoc}.

\textit{Xoo4H1} corresponds to a region present upstream of some but not all of the many members of the \textit{avrBs3} family in \textit{Xoo} (Leach \textit{et al.}, 1993), including \textit{avrXa10}. The sequence is conserved in \textit{Xac}, \textit{Xcv}, and \textit{Xcm}, and therefore, does not correlate with tissue specificity alone. In the \textit{Xoo} KACC10331 annotation, the sequence corresponds to the first 100 bases of five predicted, \textit{avrBs3/pthA} homologous ORFs
that are distributed throughout the genome. Each of these ORFs starts with the alternative codons TTG or GTG and lacks a Shine-Dalgarno consensus ribosome binding site sequence (RBS) upstream. Furthermore, each contains an internal ORF starting with ATG, preceded by an RBS, and aligning exactly with the start of characterized avrBs3/pthA family members. These observations suggest that the sequence of Xoo4H1 in fact may not be part of the structural portion of the genes, but rather part of the promoter region. There are an estimated eighteen or more avrBs3/pthA family members in Xoc (Yang and White, 2004). The fact that none of these contains this sequence suggests the possibility that avrBs3/pthA-like genes are differently regulated in Xoo and Xoc. Whether this is indeed the case and has an impact on tissue specific pathogenicity in rice remains to be determined.

The eight sequences unique to Xoo were generally lower in G+C content than the average for the genome, 63.7%. The average G+C content was 55%, with no sequence showing greater than 59% G+C content. This observation could reflect a bias of the subtraction for sequences relatively less G+C rich than the bulk of the genome, or it could indicate that sequences unique to Xoo relative to Xoc are in fact lower in G+C content than the average for the genome. The average G+C content for the eleven representative sequences for Xoc and eight sequences for Xoo that were demonstrated to be the false positives by standard Southern blot were 60.64% and 60.25%. The highest %G+C content of the false positives was 64%. The fact that these percentages are much closer to the average for the genome than those for the unique clones argues against the possibility of technical bias, and in turn
suggests that sequences unique to Xoo may have been acquired relatively recently through horizontal transfer.

The estimated efficiency of the subtraction with Xoo as tester was about 6% (27 unique clones out of a total of 467 clones tested). In the subtraction with Xoc as tester, no sequences unique to Xoc were isolated. This result could be indicative of a true paucity of sequences unique to Xoc. The absence from the subtraction library of clones representing the **avrRxo1** gene previously shown to be specific to Xoc (Zhao *et al.*, 2004), however, indicates that the screen was not saturating. Two clones from the Xoc subtraction hybridized only weakly to Xoo genomic DNA, suggesting that the subtraction may have been relatively sensitive with the hybridization parameters used. The sequences of these Xoc clones, however, do not immediately suggest potential roles in tissue specific pathogenesis.

To improve recovery of sequences unique to Xoc, additional modification of the protocol might be necessary. This modification could include the use of multiple restriction enzymes to fragment the DNAs before hybridization (Agron *et al.*, 2002), and increasing further the ratio of tester to driver. SSH is ultimately limited, however, to detection of relatively large insertion-deletions (indels). Small indels and single nucleotide polymorphisms may play critical roles in defining tissue specific interactions of *X. oryzae* pathovars with rice plants. In order to uncover these and other differences comprehensively, whole genome sequence comparisons are required. Such analysis awaits the determination of a Xoc genome sequence.

One might predict that differences in tissue specificity could involve differences in type III effector content or structure, in components of the outer
membrane, or in metabolic or environmental adaptation. In fact, SSH between Xoo and Xoc revealed 1) a difference in sequences upstream of members of a large family of type III effectors, 2) a putative O-antigen acetylase, and 3) numerous putative IS elements that via gene-disruption or horizontal transfer could affect various functions. Thus, the present work provides important targets for further study. It should be noted though, that so far, the differences identified can only be regarded as strain-specific. Determination of the extent of the conservation of these differences among diverse isolates of Xoo and Xoc is the next important step.

Experimental procedures

**Bacterial strains and plasmids used**

The bacterial strains and plasmids used are listed in Table 2.4. *Xanthomonas* strains were cultured in Glucose Yeast Extract (GYE) medium at 28°C. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. For selection of the plasmid vector, kanamycin (25 µg/ml) was added to the LB medium.

**DNA isolation**

Genomic DNA was isolated from 24 hr liquid cultures of *Xanthomonas* strains by using the DNeasy Tissue Kit (QIAGEN, Valencia, CA). Plasmid DNA was extracted from *E. coli* by using the FastPlasmid Mini Kit (Eppendorf, Westbury, NY).
Suppressive Subtractive Hybridization (SSH)

SSH was carried out first with Xoo genomic DNA as tester and Xoc as driver and subsequently with Xoc genomic DNA as tester and Xoo genomic DNA as driver, resulting in two subtraction libraries. The procedure was carried out using the PCR-Select Bacterial Genome Subtraction Kit (Clontech, Palo Alto, CA), essentially according to the protocol supplied by the manufacturer, with three important exceptions: only one hybridization was carried out; the ratio of tester-1:tester-2:driver was 1:1:12.5; and tester and driver DNAs were hybridized overnight at 73°C. A detailed description follows.

15 µg genomic DNA of tester and driver strains were digested with Rsal (Fisher, Fair Lawn, NJ), which produced a smear of fragments <3 kb in size. Two different adaptors (adaptor1, CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT, ACC TGC CCG G and adaptor2R, CTA ATA CGA CTC ACT ATA GGG CAG CGT GGT CGC GGC CGA GGT, ACC TCG GCC G) were ligated to digested tester DNA in two separate pools. Adaptor ligation efficiency was assessed by comparing PCR amplification from the pools and unmodified tester DNA using a primer specific to the adaptors (Primer1, CTA ATA CGA CTC ACT ATA GGG C).

The two adaptor-modified tester pools (12 ng each) were then denatured and hybridized with an excess of denatured, unmodified driver (150 ng) as described above. Following hybridization, PCR amplification was performed on the subtracted hybridization mix using Primer 1 in two rounds of PCR. Manual hot-start PCRs were carried out in a standard PCR buffer (Invitrogen, Carlsbad, CA) with an Mg$^{2+}$
concentration of 1.5 mM, deoxynucleoside triphosphates at 0.2 mmol, primers at 100 nM each, and 0.2 U of Taq polymerase (Invitrogen, Carlsbad, CA) per reaction. The design of the adaptors permits efficient amplification of only those double-stranded tester sequences that have a different adaptor at either end (those with the same adaptor at either end are likely to form hairpin structures). Such sequences are likely to form only when hybridization occurs between tester ssDNA present in both adaptor pools but absent from the driver pool.

PCR products were cloned into the pCR2.1-TOPO vector and transferred to E. coli TOP10, according to the protocol supplied with pCR2.1-TOPO (Invitrogen, Carlsbad, CA). For each library, 480 clones were arrayed into 96-well plates and cultured overnight at 37°C in LB medium supplemented with kanamycin (25µg/ml) and 15% glycerol for storage at -80°C.

**Reverse Southern blot analysis**

Clone inserts were amplified from 2 µl of cultured library clones by PCR using M13 forward (GTA AAA CGA CGG CCA G) and reverse (CAG GAA ACA GCT ATG ACC) primers. PCR products were subjected to electrophoresis in 1.0% agarose gels. Following electrophoresis, agarose gels were soaked in 0.25M HCl for 10 min, and then DNA was transferred to Nytran Supercharge nylon membranes (Schleicher & Schuell Inc., Keene, N.H.) overnight by Southern blotting using 0.4 M NaOH. Membranes were then washed in 2X standard saline citrate (0.3 M sodium citrate, 3 M NaCl [pH 7.0]) for 5 min. The blots were then crosslinked with a UV Stratalinker1800 (Stratagene, Cedar Creek, TX). Two replicate blots were prepared
for all samples. The membranes were prehybridized for 2-3 hr in Rapid-hyb buffer (Amersham, UK) at 65°C. Replicate blots were probed separately with 25 ng each of radiolabeled Xoo and Xoc genomic DNA by hybridization at 65°C in Rapid-Hyb buffer overnight. Xoo and Xoc genomic DNAs were radiolabeled with 5'-α-32P dCTP (Amersham, UK) by using the Prime-a-Gene Labeling System (Promaga, Madison, WI) following digestion with Rsal (Fisher, Fair Lawn, NJ) and purification by using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Labeled DNAs were hybridized with the membranes at 65°C in Rapid-Hyb buffer overnight. Membranes were washed at 65°C with 5X, 2X, then 0.2X SSC plus 0.1% SDS for 10 minutes each. Label was visualized by exposing blots to a Storage Phosphor Screen (Amersham, UK) for 5 hr and scanning the screens with a STORM Phosphor Imager (GE Healthcare, Piscataway, NJ).

**DNA sequencing and analysis**

Plasmids were isolated using the FastPlasmid Mini Kit (Eppendorf, Westbury, NY), and inserts were sequenced using M13 forward and reverse primers by the Iowa State University DNA Sequencing and Synthesis Facility. Sequences were analyzed by searching GENBANK for similar sequences or predicted motifs using the BLASTN and BLASTX algorithms available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).
Confirmation of subtraction clones by Southern blot analysis

Xoo and Xoc genomic DNAs were individually digested at 37°C for 6 hr with EcoRI, Sall (Invitrogen, Carlsbad, CA) and Rsal (Fisher, Fair Lawn, NJ). 5 µg of DNA and 2.5 units of enzyme were used for each digest. Digested DNA was separated by 1.0% agarose gel electrophoresis and transferred to membranes as described for the reverse Southern blot above. Blots were probed with clone inserts cut from pCR2.1-TOPO with EcoRI and labeled with 5'-α32P dCTP (Amersham, UK) by using the Prime-a-Gene Labeling System (Promaga, Madison, WI). Washes were as for the reverse Southern blot, except an additional wash with 0.1XSSC plus 0.1% SDS for 10 min was included.
References


Figure 2.1: Reverse Southern blot analysis of subtraction libraries.

Library inserts were amplified by PCR, separated on agarose gels, transferred to nylon membranes, and probed with fragmented and labeled total genomic DNA of Xoc BLS256 (1 and 3) or Xoo PXO86RA (2 and 4). Shown are results of hybridization to 48 inserts from the subtraction library made using Xoo PXO86RA as tester (a) and 48 inserts from the library made using Xoc BLS256 as tester (b). Results suggest four classes of inserts: those representing sequences highly conserved between the two pathovars (A), sequences not present in either pathovar (B), sequences unique to one pathovar (C), or shared but weakly conserved sequences (D).
Figure 2.2: Southern blot analysis of selected subtraction clones.

EcoRI digests of genomic DNA of Xoo PXO86RA (lanes marked 1 in each panel) and Xoc BLS256 (lanes marked 2 in each panel) were probed with labeled inserts of library clones that appeared to be unique to one pathovar based on reverse Southern blot analysis. In each panel, ethidium bromide (EtBr) stained gels prior to Southern blotting are shown at left; autoradiograms (A) are shown at right. Results representative of four observed classes are shown: (a) clone Xoo5E6, unique to tester; (b) clone Xoo3B11, shared but weakly conserved; (c) clone Xoc1F9, shared, highly conserved; and (d) clone Xoc2F9, absent from both pathovars. Lane 3 in each panel contains purified plasmid clone (a) for Xoo5E6, or insert (b, c, d) for Xoo3B11, Xoc1F9, and Xoc2F9, respectively, as positive controls. Lane M in each panel contains 1kb ladder (Gibco BRL).
Table 2.1: Summary of reverse Southern blot results.

<table>
<thead>
<tr>
<th>Tester</th>
<th>Total clones</th>
<th>Highly conserved $^a$</th>
<th>Weakly conserved</th>
<th>No signal</th>
<th>Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xoo</td>
<td>467</td>
<td>50 (10.71%)</td>
<td>216 (46.25%)</td>
<td>133 (28.48%)</td>
<td>68 (14.56%)</td>
</tr>
<tr>
<td>Xoc</td>
<td>426</td>
<td>74 (17.37%)</td>
<td>274 (64.32%)</td>
<td>41 (9.62%)</td>
<td>37 (8.69%)</td>
</tr>
</tbody>
</table>

$^a$ Numbers in parentheses are the percent of total clones represented.
Table 2.2: Summary of Southern blot analyses.

<table>
<thead>
<tr>
<th>Tester</th>
<th>Total clones tested&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conserved strong</th>
<th>Conserved weak</th>
<th>No signal</th>
<th>Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xoo</td>
<td>16</td>
<td>2 (12.5%)</td>
<td>3 (18.75%)</td>
<td>3 (18.75%)</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>Xoc</td>
<td>11</td>
<td>5 (45.45%)</td>
<td>2 (18.18%)</td>
<td>4 (36.36%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Single clones were chosen from clusters of clones grouped based on sequence similarity.
Table 2.3: BLAST results of sequences unique to Xoo.

<table>
<thead>
<tr>
<th>Representative clone</th>
<th>Insert size (bp)</th>
<th>Number of similar clones</th>
<th>Closest informative match/ GenBank ID</th>
<th>BLAST E-value</th>
<th>G+C content</th>
<th>Similar sequence in Xcc</th>
<th>Similar sequence in Xac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xoo2H12</td>
<td>302</td>
<td>7</td>
<td>Possible transposase [Xanthomonas oryzae pv. oryzae KACC10331]/YP_203218.1</td>
<td>5e-33</td>
<td>56%</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Xoo3H1</td>
<td>491</td>
<td>1</td>
<td>Methyltransferase [Xanthomonas oryzae pv. oryzae KACC10331]/YP_202850.1</td>
<td>1e-73</td>
<td>54%</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Xoo4A6</td>
<td>523</td>
<td>13</td>
<td>P38K [Xanthomonas oryzae pv. oryzae KACC10331]/YP_201530.1</td>
<td>3e-83</td>
<td>59%</td>
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<tr>
<td>Xoo4C1</td>
<td>263</td>
<td>1</td>
<td>Transposase and inactivated derivatives [Xanthomonas oryzae pv. oryzae KACC10331]/YP_201714.1</td>
<td>4e-20</td>
<td>57%</td>
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<tr>
<td>Xoo4H1</td>
<td>259</td>
<td>2</td>
<td>Avirulence gene [Xanthomonas oryzae pv. oryzae KACC10331]/YP_200918.1</td>
<td>3e-21</td>
<td>58%</td>
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<tr>
<td>Xoo5B4</td>
<td>213</td>
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<td>6e-15</td>
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<tr>
<td>Xoo5D6</td>
<td>312</td>
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<td>Putative ISXo8 transposase [Xanthomonas oryzae pv. oryzae KACC10331]/YP_203230.1</td>
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<tr>
<td>Xoo5E6</td>
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<td>Putative o-antigen acetylase [Xanthomonas oryzae pv. oryzae]/AAO20851.2</td>
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\(^a\) Xcc = Xanthomonas campestris pv. campestris; Xac = Xanthomonas axonopodis pv. citri
Table 2.4: Bacterial strains and plasmids used in this study.

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<thead>
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<th>Bacterial strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td><em>Xanthomonas oryzae pv. oryzae</em> PXO86RA</td>
<td>Azacytidine treated; rifR mutant; race 6</td>
<td>J. E. Leach Colorado State University, derived from Philippines isolate PXO86</td>
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<td><em>Xanthomonas oryzae pv. oryzicola</em> BLS256</td>
<td>Wild type, Philippines isolate</td>
<td>J. E. Leach Colorado State University</td>
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<td><em>Escherichia coli</em> TOP10</td>
<td>Similar to the DH10B™ strain, hsdR for efficient transformation of unmethylated DNA from PCR amplifications</td>
<td>Invitrogen Corporation, Carlsbad, CA</td>
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<td>pCR2.1-TOPO</td>
<td>3.9kb cloning vector Ap⁺ Km⁻</td>
<td>Invitrogen Corporation, Carlsbad, CA</td>
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CHAPTER 3. SEQUENCE ANALYSIS OF A LIPOPOLYSACCHARIDE SYNTHESIS LOCUS IN XANTHOMONAS ORYZAE PATHOVARS

Summary

Lipopolysaccharide (LPS) is an essential component of the outer membranes of Gram-negative bacteria. LPS plays important roles in interactions of animal pathogens with their hosts, and has been implicated in both eliciting and suppressing defense in phytopathogenic bacterial interactions with plants. Suppressive subtractive hybridization between Xanthomonas oryzae pathovar (pv.) oryzae (Xoo) and X. o. pv. oryzicola (Xoc) identified a gene, wxoD, encoding a putative O-antigen acetylase unique to Xoo and residing in an LPS biosynthesis locus. The locus was found to be highly variable among Xoo, X. campestris pv. campestris (Xcc), and X. axonopodis pv. citri (Xac). In order to characterize the sequence of the loci in Xoc and in another Xoo strain, long-range PCR was carried out to amplify the loci in Xoc BLS256 and Xoo PXO86RA. A 12 kb PCR product from Xoo PXO86RA was demonstrated to have the same EcoRI restriction pattern as predicted from published sequences of two other Xoo strains. A 6 kb PCR product from Xoc BLS256 was cloned and sequenced and found to be highly conserved with the locus in Xac and different from the corresponding loci in the two vascular pathogens Xoo and Xcc. Both Xoc and Xac are non-vascular pathogens. This correlation suggests that the LPS locus could play a role in tissue specificity. Toward determining the role of wxoD in pathogenesis, an internal fragment was cloned from
three Xoo strains to make a construct for gene disruption by single homologous recombination. The sequences of the clones from the three strains were identical.

**Introduction**

Lipopolysaccharides (LPSs) are essential components of Gram-negative bacterial cell membranes that contain polysaccharides and phospholipids. The phospholipid chains of LPS insert into the phospholipid-protein bilayer of the bacterial cell membrane. The polysaccharide is composed of an inner core, an outer core and a variable O-specific chain (Rietschel et al., 1994). The O-chains (O-antigens) of animal pathogens induce immunological responses in mammalian hosts.

Though the role of LPS in animal pathogenesis is well characterized, roles for LPS in plant bacterial interactions are less well understood. Mutants of *Xanthomonas oryzae* pv. oryzae (Xoo) deficient in extracellular polysaccharide (EPS) were reduced in virulence to rice and altered in the mobility of LPS in tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Dharmapuri et al., 2001). LPS of *X. campestris* pv. campestris (Xcc) can prevent hypersensitive reaction (HR) triggered by avirulent strains (Newman et al., 1997). LPS pretreatment can alter the expression of defense-related genes induced in plants by pathogens (Dow et al., 2000). Effector proteins secreted by the type III secretion system could suppress basal defenses in pepper induced by LPS in *X. c. pv. vesicatoria* (Keshavarzi et al., 2004). The LPS O-antigen epitopes of symbiotic nitrogen fixing *Rhizobium* bacteroids are expressed differently in different sections of pea root nodules (Kannenberg et al., 1994).
Patil and Sonti (2004) found by Southern blot analysis that a locus involved in LPS biosynthesis of Xoo strain BXO1 is conserved in all but two of several Xoo strains examined, but different in *X. oryzae* pv. oryzicola (Xoc) strain BXOR1. The locus is also different from the loci in the sequenced isolates Xcc and *X. axonopodis* pv. citri (Xac). The locus in Xoo BXO1 is flanked by *metB* (putative cystathionine gamma-lyase gene) and *etfA* (putative electron transferring flavoprotein alpha subunit gene), which are highly conserved among Xoo, Xoc, Xcc and Xac (Dharmapuri et al., 2001; Da Silva et al., 2002; Patil and Sonti, 2004). Patil and Sonti (2004) cloned and sequenced DNA fragments flanking this locus (*metB* to *wzm* and *int* to *etfA*, respectively) from Xoc BXOR1. The sequence between *wzm* (putative ABC-transporter permease) and *int* (putative integral membrane protein) was not characterized. By suppressive subtractive hybridization against Xoc strain BLS256 (Chapter 2 of this thesis), I isolated a DNA fragment unique to Xoo strain PXO86RA that corresponds to the O-antigen acetylase gene-*wxoD* found in the LPS locus identified by Dharmapuri *et al.* In this chapter, I report the amplification of the LPS locus in Xoo PXO86RA, the cloning and sequence analysis of a 6 kb fragment of the corresponding locus from Xoc BLS256, and preliminary work toward mutagenesis of *wxoD* in three Xoo strains.
Results

Amplifying a LPS locus of Xoo PXO86RA and cloning part of the corresponding locus of Xoc BLS256

The sequences of *metB* (putative cystathionine gamma-lyase gene) and *etfA* (putative electron transferring flavoprotein alpha subunit gene) are highly conserved among Xoo, Xoc, Xcc, and Xac, but the region between the two genes is variable. In order to characterize and compare this region between Xoo and Xoc, four pairs of primers were used to carry out long-range PCR on Xoo PXO86RA and Xoc BLS256. Three pairs of them were kindly provided by R. Sonti (Centre for Cellular and Molecular Biology, Hyderabad, India) based on the sequences of *metB* (forward primers) and *etfA* (reverse primers) of Xoo BX01 (Accession: AF337647). The fourth pair of primers was based on the sequences of *metB* (forward primer, Accession: AY319940) and *etfA* (reverse primer, Accession: AY319941) of Xoc BXOR1. In Xoo BXO1, the locus between *metB* and *etfA* is 12 kb in size. A 12 kb PCR product was amplified from Xoo PXO86RA. Digestion with *EcoRI* yielded the exact pattern of fragments predicted from the sequence of this locus in Xoo BXO1 (Patil and Sonti, 2004) and Xoo KX085 (Lee et al., 2005). Ten different combinations of the primers were tested in long-range PCR amplification with Xoc BLS256 and multiple products were amplified in each case. To find the correct fragment, Southern blots of the products were probed with a fragment containing the Xoc *metB* gene and part of the neighboring *wzm* gene cloned from Xoc BLS256 by PCR amplification based on a partial sequence of the region obtained by Patil *et al.* (Genbank accession: AY319940), and with another fragment containing part of the integral membrane
protein gene \( (int) \) and its neighbor \( etfA \), also cloned by PCR amplification based on a partial sequence of the region obtained by Patil et al (Genbank accession: AY319941). Though probe \( metB-wzm \) hybridized to multiple bands, probe \( int-etfA \) gave a relatively clear signal, hybridizing strongly to a product of about 6 kb amplified by two of the ten primer combinations (Figure 3.1). Cloning and sequence analysis of the 6 kb product revealed that it is very similar to part of the LPS locus, close to the \( etfA \) end, of Xac strain 306 (Genbank accession: NC_003919). Roughly the first 1kb of the sequence encodes a predicted amino acid sequence with 59% identity to a hypothetical protein encoded by the gene in the same position in the Xac sequence (XAC3592, Accession: NP_643899.1). The rest of the sequence is nearly identical to that of Xac from the gene encoding the short chain dehydrogenase to \( etfA \). The fragment did not contain the \( metB-wzm \) region. In Xac, there are still eight ORFs between \( wzm \) to the gene encoding the hypothetical protein (Figure 3.2).

**Cloning an internal fragment of \( wxoD \) for mutagenesis in three Xoo strains**

Primers against \( wxoD \) in Xoo KXO85 (Accession: NC_006834) were designed to amplify a 500 bp internal fragment (base pairs 149 to 652 of the open reading frame). Amplification of \( wxoD \) from Xoo PXO86RA, Xoo PXO99A, and Xoo KXO85 yielded fragments of the anticipated size (Figure 3.3). As expected, these primers failed to amplify product from either of two Xoc strains, BLS256 and BLS303 (Figure 3.3). The fragments amplified from three Xoo strains were cloned into
pCR2.1-TOPO. Sequence analysis showed that the sequences of the three clones were identical. Since the pCR2.1-TOPO vector does not replicate in Xoo, selection with kanamycin following introduction of the plasmid into Xoo strains can be used to disrupt the gene by single homologous recombination.

Discussion

I carried out a long range PCR to amplify a 12 kb fragment corresponding to the *metB-etfA* locus from Xoo PXO86RA and demonstrated that the locus in Xoo PXO86RA had the same restriction pattern with the locus in Xoo BXO1 and Xoo KXO85. By using the same method, I cloned an approximately 6 kb fragment of the locus from Xoc BLS256, which was highly similar to the corresponding region in Xac strain 306. I also cloned an internal fragment amplified from *wxoD* in three Xoo strains that can be used to disrupt *wxoD* by single homologous recombination.

The locus between *metB* and *etfA* is highly variable among Xoo, Xac, and Xcc (Da Silva *et al.*, 2002; Patil and Sonti, 2004; Lee *et al.*, 2005), however, the sequence of the fragment of the locus cloned from Xoc BLS256 is highly conserved in Xac. Both Xoc and Xac are non-vascular pathogens. It is intriguing to speculate the locus is specific to non-vascular pathogens. The locus is predicted as an LPS biosynthetic gene cluster. As noted in the introduction, LPS is implicated in compatible and incompatible interactions of bacterial pathogens with their plant hosts. The variation in sequence of the LPS loci among xanthomonads could indicate a role in specificity of interaction with different hosts and host tissues.
The region between \textit{metB} and \textit{etfA} in Xac strain 306 is about 20 kb. I was only able to amplify a fragment from Xoc BLS256 of about 6 kb (Figure 3.2). Possible reasons for this result are as follows: failure of the protocol to amplify the entire locus, and/or a rearrangement of \textit{metB-wzm} in Xoc, and/or a duplication of \textit{metB} in the middle of the region in Xoc. Isolation and sequencing of a cosmid clone spanning the entire region (or complete genome sequencing) will be necessary to distinguish among these possibilities and fully characterize the locus.

Both Xoc BLS256 and Xoc BLS303 cosmid libraries are in hand (gifts from Dr. Frank White and Dr. Jan Leach, respectively). The sequences in \textit{metB-wzm} and \textit{int-etfA} in Xoc BLS256 are similar to the corresponding regions in Xoc BLS303, and so were used as probes to screen these libraries. Three cosmid clones from the BLS303 library that hybridize to the \textit{int-etfA} probe were identified, but none hybridized with the \textit{metB-wzm} probe. Further screening should enable isolation of the complete locus for sequencing and functional characterization.

Variation at this LPS locus suggests the possibility that the locus is involved in interactions with the host. In order to determine whether this is the case and specifically whether the locus contributes to the difference in tissue specificity between Xoo and Xoc, generation of mutants and differential pathogenicity assays should be carried out. Also of interest is whether this locus has an effect on bacterial elicitation of plant defense responses such as the hypersensitive reaction in the incompatible interaction. Dharmapuri et al. (2001) demonstrated that mutagenesis of \textit{wxoA}, \textit{wxoB} and the upstream region of \textit{wxoC} in Xoo BXO1 had an effect on EPS, LPS and reduced virulence. Changes in LPS biosynthesis could affect EPS
production by altering the pool of shared substrates or by affecting transportation or cell-surface properties. \textit{wxoD}, however, is predicted as an O-antigen acetylase. It is 42\% identical at the protein level to the \textit{wbiA} gene in the human pathogenic species \textit{Burkholderia thailandensis} which has been well characterized (DeShazer \textit{et al.}, 1998). Because it is likely involved in O-antigen modification and not LPS synthesis \textit{per se}, mutation of \textit{wxoD} might be expected not to affect EPS.

For characterization of pathogenicity and tissue specificity, two kinds of inoculation methods can be used, clipping and syringe inoculation. These methods are differential for \textit{Xoo} and \textit{Xoc}. \textit{Xoo} can easily infect the whole plant following clip inoculation but spreads only slowly following syringe infiltration. In contrast, \textit{Xoc} spreads rapidly through the mesophyll following syringe infiltration, but does not infect well upon clip inoculation (Niño-Liu and Bogdanove, unpublished). The effect of mutations on HR defense elicitation can be measured by using \textit{wxoD} mutants of strain PXO86RA, which carries several well-characterized avirulence genes, e.g. \textit{avrXa7} and \textit{avrXa10} (Hopkins \textit{et al.}, 1992) for which near isogenic lines of rice carrying corresponding resistance genes are available. The \textit{wxoD} construct described here will be a useful tool to generate mutants and begin to functionally characterize this locus.

**Experimental procedures**

**Bacterial strains and plasmids used**

The bacterial strains and plasmids used are listed in Table 3.1. Growth conditions are as described in Chapter 2.
DNA isolation

Genomic DNA was isolated from 24 hr liquid cultures of *Xanthomonas* strains by using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Plasmid DNA was extracted from *E. coli* by using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI).

Long range PCR and normal PCR

Long range PCR was carried out using the TripleMaster PCR System (Eppendorf, Hamburg, Germany). For each reaction, 175 ng of genomic DNA of Xoo or Xoc strains were mixed with one of the primer pairs CgIL1 and Etfl1, CgIL2 and Etfl2, or CgIL3 and Etfl3 (Patil and Sonti, 2004) or with the primers metBf (CCG TTT TGA CCC ACG CCT CCA T) and etfAr (CCG ATA TTG GGC TGG TGG GT). The PCR mix contained 1X Tuning Buffer with Mg$^{2+}$, deoxynucleoside triphosphates at 2 mM, primers at 100 nM each, and 0.04 U of TripleMaster Polymerase Mix. The PCR program consisted of an initial denaturation for 3 min at 93°C, ten cycles of 15 sec at 93°C, 30 sec at 60°C, 10 min at 68°C, then 16 cycles of 15 sec at 93°C, 30 sec at 60°C, and 10 min plus 20 sec per cycle at 68°C.

Standard PCR was performed as described in Chapter 2 by using primers metBf and wzmr (ATA TCA CCG TCA CCG GCA ACG TCC) or intf (CGC CCA ATG TGG GAA ATA CC) and etfAr.
Southern blot

Southern blotting was performed as described in Chapter 2.

Cloning of long range PCR product

PCR product was separated by agarose gel electrophoresis and purified from the gel by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). PCR product was treated with Taq polymerase (Invitrogen, Carlsbad, CA) to add adenosine to each 3' end. The resulting DNA was then cloned in pCR-XL-TOPO and transferred to E. coli TOP10 competent cells, according to the protocol supplied with TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA).

EZ::TN insertion and sequencing

Long range PCR clones were sequenced using a transposon insertion based strategy. The EZ::TN <TET-1> Insertion Kit (Epicentre, Madison, WI), was used according to the manufacturer protocol to create a library of transposon insertions for each plasmid insert to be sequenced. 48 insertion clones were isolated and insertions mapped by digestion with EcoRI, HindIII (Invitrogen, Carlsbad, CA), and NotI (New England Biolabs, Beverly, MA), respectively. 27 clones with insertions spaced evenly across the insert were sent to Iowa State University DNA Sequencing and Synthesis Facility and sequenced in both directions from the insert using primers corresponding to the transposon sequence (TET-1 FP-1 Forward Primer, GGG TGC GCA TGA TCC TCT AGA GT; RP-1 Reverse Primer, TAA ATT GCA CTG AAA TCT AGA AAT A). Sequences were assembled by using ContigExpress
of Vector NTI (Invitrogen, Carlsbad, CA) and characterized by homology search using the BLAST algorithms available through NCBI (www.ncbi.nlm.nih.gov).

**Construction of plasmid clone for mutagenesis of wxoD**

An 500 bp internal fragment of wxoD was amplified from Xoo PX086RA, Xoo PX099A and Xoo KX085 by using primers wxoD-mf (GGG TCT TTA TTT TCT TTT CG) and wxoD-mr (GAA CAC CAG AGA AGA AGA AA), cloned in pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and transferred to *E. coli* TOP10 cells. Plasmids were isolated as described above, and inserts sequenced using M13f and M13r primers corresponding to the vector.
References


Figure 3.1: Southern blot analysis of long-range PCR products of LPS locus.

Fragments were amplified by long-range PCR, separated on agarose gel (a), transferred to nylon membranes, and probed with purified int-etaA of Xoc BLS256 clone insert (b). Lane 1 contains the purified clone of metB-wzm of Xoc BLS256 as a negative control. Lane 2 contains 1 kb ladder (Gibco BRL). Lane 3 contains amplification product with Xoo PXO86RA as template and primers CgIL1 and EtfL1. Lane 4 to 13 contain amplification products with Xoc BLS256 as template and primer pairs CgIL1 and EtfL1, CgIL1 and EtfL2, CgIL1 and EtfL3, CgIL2 and EtfL2, CgIL2 and EtfL1, CgIL2 and EtfL3, CgIL3 and EtfL3, CgIL3 and EtfL1, CgIL3 and EtfL2, and metBf and etfAr, respectively. Lane 14 contains the purified clone of int-etaA of Xoc BLS256 as a positive control. A product of about 6 kb is hybridized strongly (arrow).
Figure 3.2: Genetic organization of the metB-etfA locus in Xac strain 306 and part of the locus in Xoc BLS256.

Individual ORFs, denoted by bold arrows, are (a) metB (putative cystathionine gamma-lyase gene), (b) wzm (ABC transporter permease gene), (c) wzt (ABC transporter ATP-binding protein gene), (d) hypothetical protein gene, (e) rfbC (truncated O-antigen biosynthesis protein gene), (f) hypothetical protein gene, (g) hypothetical protein gene, (h) hypothetical protein gene, (i) phytoene desaturase gene, (j) NAD dependent epimerase / dehydratase / dehydrogenase gene, (k) hypothetical protein gene, (l) short chain dehydrogenase gene, (m) oxidoreductase gene, (n) integral membrane protein gene, (o) integral membrane protein gene, and (p) etfA (putative electron transferring flavoprotein alpha subunit gene), respectively.
Figure 3.3: PCR amplification of an internal fragment of \textit{wxoD}.

Shown are PCR amplification products, separated by agarose gel electrophoresis, of reactions containing primers \textit{wxoD-mf} and \textit{wxoD-mr} and \textit{Xoo} PXO86RA (\textit{Xoo86}), \textit{Xoo} PXO99A (\textit{Xoo99}), \textit{Xoo} KXO85 (\textit{Xoo85}), \textit{Xoc} BLS256 (\textit{Xoc256}), or \textit{Xoc} BLS303 (\textit{Xoc303}) as template, respectively. 1 kb ladder (Gibco BRL) is shown at right.
Table 3.1: Bacterial strains and plasmids used in this study.

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<td>PXO99A</td>
<td>Azacytidine treated; rifR mutant; race 6</td>
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<td>KXO85</td>
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<td>BLS303</td>
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CHAPTER 4. TOOLS TO IDENTIFY TYPE III EFFECTORS OF XANTHOMONAS ORYZAE PV. ORYZAE AND X. ORYZAE PV. ORYZICOLA

Summary

The observation that the N-terminal signal domain of type III secretion proteins is necessary for secretion and translocation of the proteins into plant cells has led to development of a genetic screen to identify new type III effectors. An avirulence gene lacking its N-terminal secretion signal is fused to target DNA and used as a reporter to determine if the target DNA encodes a type III secretion and translocation signal by expressing the fused construct in a bacterium inoculated to a host with a corresponding resistance gene. Delivery is indicated by the plant resistance-associated hypersensitive response (HR). Based on the hypothesis that type III secretion proteins might be involved in the tissue specificity of Xanthomonas oryzae pathovar (pv.) oryzae (Xoo) and X. oryzae pv. oryzicola (Xoc), the feasibility of this approach to identify and compare Xoo and Xoc genes that encode the type III secretion signal was assessed. The avrBs2 gene from X. campestris pv. vesicatoria (Xcv) was chosen for a translational fusion approach to screen genomic libraries. To determine whether genomic library clones might be pooled, Xcv 85-10 (containing the avrBs2 gene) was mixed with Xcv 85-10ΔavrBs2 (lacking the gene) in different ratios and inoculated into leaves of pepper plants containing the Bs2 resistance gene. Xcv 85-10 elicited a HR in the presence of four times more cells of Xcv 85-10ΔavrBs2, indicating that a pooling approach would be an effective way to screen
large numbers of clones using this reporter. Genomic libraries of Xoo PXO86RA and Xoc BLS256 were constructed toward carrying out a screen.

**Introduction**

The N-terminal signal domain of effector proteins secreted by the type III secretion pathway of bacterial phytopathogens is necessary and sufficient for translocation of these proteins into plant cells (Mudgett *et al*., 2000; Guttman *et al*., 2002). This observation has led to the development of powerful genetic screens to identify new type III effectors (Nomura and He, 2005). In these screens an effector avirulence domain lacking its N-terminal secretion signal is fused to target DNA and used as a reporter to determine if that target DNA encodes a type III secretion and delivery signal by expressing the fused construct in a bacterium inoculated to a host with a corresponding resistance gene. If the target DNA encodes a secretion and delivery signal, the fusion protein is delivered into the plant cell and elicits a resistance gene-mediated hypersensitive reaction (HR) (Mudgett *et al*., 2000). In order to carry out this type of screen for effectors of *Xanthomonas oryzae* pathovar (pv.) oryzae (Xoo) and *X. oryzae* pv. oryzicola (Xoc), I assessed the feasibility of using an approach modified from a recently published secretion-delivery screen that employs a truncated AvrBs2 protein lacking its N-terminus as a secretion-delivery reporter (Roden *et al*., 2004). AvrBs2 is an avirulence protein from *Xanthomonas campestris* pv. vesicatoria (Xcv). Amino acid (AA) sequence 1 to 58 is required for secretion and translocation. AA sequence 62 to 497 is sufficient to trigger resistant responses mediated by the pepper Bs2 resistance gene (Mudgett *et al*., 2000). I
found that a strain carrying the gene could elicit HR in pepper plants containing Bs2 in the presence of four times more bacteria of a strain without the gene, suggesting that a pooling approach to screening large numbers of clones would be feasible. Therefore, I then subcloned an appropriate avrBs2 truncation to be used in constructing the reporter, and I prepared genomic libraries of one strain of each pathovar that would be suitable for screening using this approach.

Results and Discussion

**Feasibility of using truncated ‘avrBs2 as a secretion-delivery reporter to identify effectors of Xoo and Xoc**

I found that both Xoo and Xoc elicit HR on pepper plants carrying Bs2, but not on pepper plants without Bs2, suggesting that they carry functional homologs of the avrBs2 gene. Therefore, the approach used by Roden et al. (2004), creating a truncated ‘avrBs2 genomic fusion library by transposon mutagenesis, using an avrBs2 mutant background, is not possible without first disrupting the avrBs2 genes in Xoo and Xoc. I sought to evaluate whether expression of Xoo and Xoc genomic libraries fused to ‘avrBs2 in a plasmid, expressed in an Xcv strain lacking avrBs2 and assayed on pepper plants carrying Bs2 would be a feasible alternative. Assuming an average insert size of 1.75 kb, a 1X coverage of the ~5 Mb Xoo or Xoc genome would require screening 2,860 clones, but if six possible reading frames for any given fusion were considered, the number of clones would be 17,000. I first tested therefore whether pooling clones would be possible. I tested co-inoculation of different ratios of Xcv 85-10 (which carries the HR-elicitor avrBs2) and virulent Xcv
85-10ΔavrBs2 (lacking avrBs2) to the pepper cv. Early Cal Wonder (ECW, bs2/bs2) and the near isogenic cv. ECW-20R (Bs2/Bs2). Inoculation of Xcv 85-10 and some co-inoculations of Xcv 85-10 and Xcv 85-10ΔavrBs2 to ECW-20R a elicited HR, but the HR appeared only after 48hr and seemed atypical, somewhat black instead of the typical tan, and resulting in less than complete tissue collapse. Inoculation of Xcv 85-10 to ECW, co-inoculation of Xcv 85-10 and Xcv 85-10ΔavrBs2 to ECW, and inoculation of Xcv 85-10ΔavrBs2 to either ECW or ECW-20R elicited no symptoms by 48 hr post-inoculation. Pseudomonas syringae pv. glycinea (Psg) race 4 elicited a typical HR on ECW-20R, but a relatively weaker HR on ECW (Figure 4.1). Xoo PXO99A and Xoc BLS256, elicited HR on pepper Bs2, but failed to elicit HR on ECW (data not shown). Experiments were repeated at least two times with similar results. Xcv 85-10 to Xcv 85-10ΔavrBs2 ratios of 100:1, 50:1, 10:1, 1:1, 1:5, 1:10, 1:15, 1:50 and 1:100 were tested. Inoculation at a 1:5 ratio resulted in the blackish HR (Figure 4.1). At 1:10, a lighter colored but apparent HR resulted (data not shown). At 1:15, 1:50, and 1:100, the O.D. value of the Xcv 85-10 could be too low to elicit HR. Raising the O.D. value of the wild type in these mixtures might elicit HR, but the tests have not been carried out. In sum, though care must be taken in scoring because the phenotype is not as robust as a typical HR, the avrBs2 reporter gene strategy should be effective, and for high-throughput, strains carrying different clones can be pooled in groups of at least five.
**Reporter gene construction**

pTnavrBs2 that contains a portion of the *avrBs2* gene encoding aa 62-574 of the original protein, followed by the HA-tag, was obtained from M. Mudgett (Stanford University). Using custom primers the entire reporter gene with added flanking SalI and Xhol restriction sites was amplified by PCR and subcloned into pCRII-TOPO yielding pLC8 to facilitate cloning into a broad host range vector suitable for fusion with the genomic libraries and expression in Xcv (Figure 4.2). Bohling and Bogdanove (unpublished) have recently done this cloning and shown that fusions with N-terminal portions of the Xoo effector AvrXa10 direct secretion and translocation and result in HR in ECW-20R, confirming the utility of the reporter for Xoo effector screening.

**Xoo and Xoc genome library construction**

Due to the relatively small size of sequences encoding type III secretion signals, I constructed libraries of genome fragments 0.5 kb to 3.0 kb in size. It was necessary to gel purify and clone DNA fragments in three fractions (0.5-1 kb, 1-2 kb, 2-3 kb; see Experimental procedures), because the TOPO vector used preferentially accepts smaller inserts. This vector contains Gateway recombination sites, which allow straightforward transfer of clones into other vectors without loss of orientation or representation. The estimated number of clones and percentage of recombinants in each library are given in Table 4.1. Based on these data, coverage of the Xoo genome is approximately 3.4X and coverage of the Xoc genome 1.6X. Thus, with
these libraries, screens will likely not be saturating, and additional libraries may be required.

If further optimization of the assay is successful, the reporter construct subclone and genomic libraries should facilitate an undertaking of screens to identify type III effectors of Xoo and Xoc.

**Experimental procedures**

**Hypersensitive reaction assays on pepper and pooled inoculations**

Six week-old plants of near isogenic pepper cultivars ECW and ECW-20R were used. *Xanthomonas campestris* pv. vesicatoria (*Xcv*) strains 85-10 and *Xcv* 85-10Δ*avrBs2* (kindly provided by Dr. Ulla Bonas, U. of Halle, Germany; Mudgett *et al.*, 2000), *X. oryzae* pv. *oryzae* PXO99A, *X. o. pv. oryzicola* BLS256 and *Pseudomonas syringe* pv. glycinea (*Psg*) race 4 (Bisgrove *et al.*, 1994) were assayed individually by suspension to an OD520=0.5 in 10 mM MgCl₂ and infiltration into leaf panels using a needleless syringe. *Xcv* 85-10 and *Xcv* 85-10Δ*avrBs2* strains suspended to this O.D. were mixed at 100:1, 50:1, 10:1, 1:1, 1:5, 1:10, 1:15, 1:50, 1:100, respectively. HR was scored at 48 hr post inoculation.

**Reporter gene construction**

pTnavrBs2 (Roden *et al.*, 2004) was a gift from Dr. Mary Beth Mudgett, (Stanford University). Primers 'avrBs2f1 (CTA GGT CGA CAT GAC CGG CAA GCC GGC CCT GG) and 'avrBs2r1 (CTA GCT CGA GTT ACG CAT AGT CAG GAA CAT CGT AT) were used to amplify the ‘*avrBs2*-HA fragment from pTnavrBs2 with *Taq*
polymerase (Invitrogen, Carlsbad, CA). Following PCR, the construct was ligated with pCRII-TOPO (Invitrogen, Carlsbad, CA) and transferred to E. coli TOP10 (Invitrogen, Carlsbad, CA). Sequence was confirmed by using primers corresponding to the vector and custom primers internal to the gene, avrBs2-854r (GCG ACA CCA ACC GGT TCT GCG GGT CGC CT) and avrBs2-767f (GAA GTC AGT TCC GAT GGC GTG CCG GTG).

Construction of Xoo and Xoc genomic libraries

Genomic DNA of Xoo PXO86RA and Xoc BLS256 separately was extracted by using the DNeasy Tissue Kit (QIAGEN, Valencia, CA) and sheared by nebulizing with an Invitrogen nebulizer according to a protocol supplied by TOPO Shotgun Cloning Kit (Invitrogen, Carlsbad, CA). DNA fragments were separated (in triplicate samples each) by gel electrophoresis and extracted from the gel in three size fractions 0.5-1 kb, 1-2 kb, 2-3 kb. For each fraction individually, DNA ends were repaired by using T4 polymerase (Invitrogen, Carlsbad, CA) and dephosphorylated by treatment with CIAP (Fisher, Fair Lawn, NJ), and finally amended with 3' adenosine tail by treatment with Taq polymerase (Invitrogen, Carlsbad, CA). The resulting fractions were ligated into the pCR8/GW/TOPO vector (Invitrogen, Carlsbad, CA) and transferred into E. coli TOP10 competent cells according to the manufacturer protocol to construct a total of six libraries. The number of clones per library was estimated by dilution plating of aliquots. Thirty-two plasmid clones of each library were isolated (except the Xoc 2-3 kb library, for which only 23 clones
were assayed) and cut with EcoRI (Invitrogen, Carlsbad, CA) to check the size of inserts. Libraries were preserved as DNA and glycerol stock.
References


Figure 4.1: Response of leaves of pepper cvs. ECW-20R and ECW to Xcv strains and Psg.

Suspensions of approximately $1.6 \times 10^8$ cfu/ml of Xcv 85-10 (1), Xcv 85-10 and Xcv 85-10ΔavrBs2 at a ratio of 1:5 (2), Xcv 85-10ΔavrBs2 (3), and Psg race 4 (4) were infiltrated into the leaves of pepper cvs. ECW-20R (a) and ECW (b). The areas of infiltration are indicated by the arrows. Pictures were taken 48 hr post inoculation.
Figure 4.2: Plasmid map of pLC8.

pLC8 is based on the plasmid pCRII-TOPO (Invitrogen, Carlsbad, CA). A 1.6 kb subclone of pTnavrBs2 (see text) amplified with primers adding flanking SalI and XhoI sites, was inserted. Bold arrows represent the open reading frames indicated. The origin of replication is denoted by a bold bar.
Table 4.1: Summary of estimates for genomic libraries of *Xoo* PXO86RA and *Xoc* BLS256.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of clones</th>
<th>Percentage of recombinations</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xoo</em> PXO86RA</td>
<td>13806</td>
<td>80.21%</td>
<td>3.42X</td>
</tr>
<tr>
<td><em>Xoc</em> BLS256</td>
<td>11010</td>
<td>80.46%</td>
<td>1.59X</td>
</tr>
</tbody>
</table>
CHAPTER 5. FUTURE PERSPECTIVE

The work presented in this thesis identifies several Xoo PXO88RA-specific clones by SSH as important targets for further study. It provides initial in-depth characterization of a lipopolysaccharide biosynthesis locus that includes one of these, the putative O-antigen acetylase gene wxoD. Finally, it provides an assessment of a secretion-delivery reporter to isolate and compare type III effectors of Xoo and Xoc, and describes the construction of genomic libraries of these two pathogens that can be used for this purpose.

Four of the specific SSH clones are putative IS elements. The Xoo genome has more than twice the number of mobile elements as the Xcc or Xac genomes. Many of these flank strain-specific genes, especially some virulence and avirulence genes. Mapping the distribution of the elements identified in this study and characterization of the genes near them is an important goal for future work.

The clone predicted as an O-antigen acetylase is localized in an LPS biosynthesis locus specific to some Xoo strains. O-antigen acetylase is involved in O-antigen decoration, which is important in animal pathogens. The roles of O-antigen acetylase in plant pathogens are less well understood, but LPS has been implicated in both eliciting and suppressing plant defense. In light of the specificity of the wxoD gene to Xoo and the potential for a role of LPS in pathogenicity, a reasonable hypothesis is that the gene is related to the tissue specificity of Xoo interactions with rice plants. Mutagenesis of the O-antigen acetylase gene and
functional tests using differential inoculation methods in leaves of rice will be important to further explore this possibility.

Sequence analysis showed that part of the corresponding LPS locus in Xoc is highly conserved with the LPS locus in Xac. Both Xoc and Xac are non-vascular pathogens. This correlation bolsters the hypothesis that the LPS locus is involved in tissue specificity. To further test this idea, sequencing the complete LPS locus in Xoc, and characterization of the locus in several strains is necessary.

Type III effectors may be involved in determining the outcome of interactions of X. oryzae pathovars with different rice tissues. The feasibility of screening Xoo and Xoc genomic libraries with a reporter of type III secretion based on the avrBs2 gene from Xcv was examined. The reporter construct subclone and genomic libraries should facilitate an undertaking of screens to identify type III effectors of Xoo and Xoc. Screening Xoo and Xoc genomic libraries is the next essential step.
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