Future plans

The results presented in Chapter two demonstrate that the Xoo fur mutant overproduces siderophores and that it exhibits hypersensitivity to hydrogen peroxide and a growth deficiency under laboratory as well as in planta conditions. The results also show that the in planta growth deficiency of the fur mutant can be rescued by supplementation with ascorbic acid, an antioxidant. This suggests that the in planta growth deficiency of the fur mutant is due to an inability to deal with reactive oxygen species that are encountered during infection. If this hypothesis is correct, one would expect that overexpression of proteins like catalase or superoxide dismutase, that are involved in detoxification of reactive oxygen species, might also promote in planta growth of the Xoo fur mutant. This can be accomplished by cloning the genes for these proteins, derived from either Xoo or heterologous sources, in front of promoters, which are expressed constitutively and at high levels in xanthomonads.

We have obtained evidence (S. Sujatha and R. V. Sonti, unpublished data) which indicates that superoxide dismutase activity is also reduced in a Xoo fur mutant. The mechanistic reason for the reduced level of catalase and superoxide dismutase activity in the Xoo fur mutant needs to be elucidated. In Pseudomonas aeruginosa, the Fur protein has been shown to regulate sodB (encodes superoxide dismutase) expression in an indirect manner by repressing expression of a small regulatory RNA molecule that inhibits sodB gene expression. It remains to be determined whether the Xoo Fur protein also acts in a similar manner to regulate superoxide dismutase and catalase gene expression.

The fur mutant also exhibits a deficiency for growth on peptone sucrose medium. However, ascorbic acid supplementation does not fully suppress this growth defect. One possible explanation for this observation is that ascorbic acid is not taken up very well by Xoo cells. The complete suppression of the in planta growth defect of the fur mutant by ascorbic acid might be because the oxidative stress may be coming from an external source;
i. e. the host plant. It would be interesting to compare ascorbic acid uptake rates by Xoo and *E. coli* and to determine whether there is any difference in the efficiency with which this molecule is taken up by the latter. It is interesting in this regard to note that a specific ascorbic acid uptake system has been identified in *E. coli*. By doing a BLAST search, I have identified homologs of this uptake system that are encoded in the Xoo genome. It remains to be determined whether these Xoo functions are involved in ascorbic acid uptake.

It is also possible that the slow growth phenotype of the Xoo *fur* mutant on laboratory medium is only partly due to oxidative stress. It would be interesting to identify and characterize transposon induced second site suppressors of the slow growth phenotype of the Xoo *fur* mutant. Such a study should provide insights into the reason for the slow growth phenotype of the Xoo *fur* mutant on peptone sucrose medium.

The constitutive production of siderophores by the Xoo *fur* mutant indicates that siderophore production is under the control of the Fur repressor in this bacterium. What are the Xoo genes that participate in siderophore production and are under the control of Fur? It would be interesting to identify and characterize transposon-induced mutants that reduce the amount of siderophores that are produced by the *fur* mutant. Some of these insertions might be in genes that are involved in siderophore biosynthesis.

Using a bioinformatics approach, we have identified a cluster of Xoo genes that are homologous to *Vibrio parahaemolyticus* (a fish pathogen) genes that are involved in siderophore biosynthesis (S. Sujatha, A. Pandey and R. V. Sonti; unpublished results). The expression of these genes is induced upon iron starvation (A. Pandey and R. V. Sonti; unpublished results). It would be interesting to determine if these genes are regulated by Fur and whether they are involved in siderophore biosynthesis.

Finally, it would be interesting to determine the complete set of genes that are under the control of the Fur regulon in Xoo. The availability of the Xoo genome sequence makes it possible to construct microarrays/gene chips
for whole genome expression analysis of Xoo wild type and fur mutant. This would allow the identification of all the genes that are either upregulated or downregulated in a fur mutant background and pave the way for a more complete understanding of the role of the Xoo Fur protein in controlling iron uptake, oxidative stress response as well as growth on laboratory medium and within the rice plant.

In the third chapter of this thesis I have discussed a genome-wide genetic screen that was carried out to identify novel functions that are involved in iron metabolism and to determine their role in Xoo virulence. We describe the colR/colS genes that code for a putative two component regulatory system of Xoo, which appears to be involved in promoting growth on low iron medium and virulence. Through sequence analysis, we have identified the conserved histidine and aspartate residues in the sensor and regulator proteins coded by these genes. Generating site directed mutants of colS and colR at these residues would reveal their function in signal transduction. Purified wild type ColR and ColS proteins and their mutant counterparts may be used in in vitro phosphorylation assays to demonstrate the phosphorelay. Analysis of expression of colR and colS genes can be studied using Gus reporter fusions in the genomic contexts. The conditions that induce the expression of colR and colS genes might provide clues about the external signals that are detected by this two component regulatory system. Identification of genes under the control of ColR/ColS would give new information about the role of this two-component regulatory system in promoting virulence and growth on low iron medium. This can be done by performing microarray experiments using wild type and colR/colS mutants that are defective for signal transduction.

Two of the sop mutants have insertions in the acnB gene, which encodes an aconitase. As discussed in Chaper three, the aconitase may have a regulatory as well as enzymatic role. It would be interesting to identify site directed mutations in the acnB gene, which might affect enzymatic activity of the aconitase without affecting the siderophore overproduction phenotype.
and vice versa. One of the sop mutants has an insertion in the XOO3893 gene, which is a homolog of the metC gene of Ralstonia solanacearum, a bacterial pathogen of plants like tomato and tobacco. The E. coli homolog of this gene encodes a cystathionine gamma-synthase protein, an enzyme that functions in methionine biosynthesis. Only a single insertion was identified in the XOO3893 gene but this insertion mutant exhibits a severe virulence phenotype. There is no ortholog of XOO3893 in either Xcc or Xac. Therefore, this is an interesting gene for further study and we are planning to begin these studies by making additional mutations in the XOO3893 gene in order to ensure that they display the same phenotypes as the mutation that has been identified in this study.

As discussed in chapter three, only two sup mutants with severe phenotypes were identified in this study. Both mutants carry an insertion in the XOO0589 (wzyC) gene, which is homologous to bacterial genes that function in lipopolysaccharide (LPS) biosynthesis. A mutation in the Xcc ortholog of this gene results in a defect in LPS biosynthesis. We need to determine whether the two sup mutants that have been isolated in this study do indeed have defects in LPS biosynthesis. In a previous study, we have identified another Xoo locus that also functions in LPS biosynthesis. Would mutations in the genes at this locus also lead to a sup phenotype? It is also interesting to note that the two wzyC Xoo mutants are able to cause near wild type levels of virulence on rice although they apparently produce no siderophores. How do the wzyC mutants source iron during in planta growth? Does Xoo use siderophore independent methods to source iron during in planta growth? The FeoB protein is part of an important bacterial ferrous uptake system. We are constructing mutations in the Xoo homolog of the feoB gene in order to determine if this leads to a loss in virulence.

A number of the sop mutants that we have identified in this study are mutated in genes that encode conserved hypothetical proteins. How do these proteins promote virulence and/or iron uptake? A detailed bioinformatics analysis of the predicted products of these genes is needed as the starting
point for understanding the biochemical functions of these proteins.

Some clues are already available from the studies described in Chapter three. However, the identification of additional sequence motifs might provide the clues that are needed for designing specific biochemical/physiological tests for assessing function. For several sop mutants, for eg; XOO0007, there is a possibility that the phenotype associated with the mutation is actually due to a polar effect on downstream genes that might be co-transcribed with the gene that has been mutated. Specific complementation tests and isolation of non-polar mutations would be needed to address the issue of polarity. However, it is important to note that genes that control a particular trait are clustered together in bacteria and are often organized as operons. Therefore it is possible that the genes that have been mutated in Chapter three, and the downstream genes with which they may be co-transcribed, are involved in controlling the same traits.

Finally, from the literature review covered in chapter one and the results discussed in chapters two & three, it is clear that at least three regulons have been identified in Xoo that affect siderophore production and virulence. Mutations in the respective regulators of each of the three regulons namely, rpfF, fur and colR/colS exhibit siderophore overproduction phenotype and virulence deficiency. It would be very interesting to investigate whether there is a cross talk between these regulons that facilitates bacterial infection.