Chapter 2
The Growth Deficiency Of A Xanthomonas oryzae pv. oryzae fur
Mutant In Rice Leaves Is Rescued By Ascorbic Acid
Supplementation

2.1 Abstract

Xanthomonas oryzae pv. oryzae (Xoo) causes Bacterial Leaf Blight, a
serious disease of rice. A mutation was isolated in the fur (ferric uptake
regulator) gene of Xoo and shown that the mutation results in the production
of siderophores in a constitutive manner. The fur mutant is hypersensitive to
the metallo-antibiotic streptonigrin, a phenotype that is indicative of
intracellular free-iron overload, and also exhibits a slow growth phenotype on
rich medium. The fur mutant is virulence deficient, hypersensitive to
hydrogen peroxide and exhibits reduced catalase activity. Exogenous
supplementation with ascorbic acid (an anti-oxidant) rescues the growth
deficiency of the fur mutant in rice leaves. The virulence deficiency of the Xoo
fur mutant is proposed to be, at least in part, due to an impaired ability to cope
with the oxidative stress conditions that are encountered during infection.

2.2 Introduction

Xoo causes bacterial leaf blight, a serious disease of rice. Some of the
genes required for Xoo virulence that have been previously described include
gumG and gumM genes involved in extracellular polysaccharide (EPS)
biosynthesis (Dharmapuri & Sonti 1999; Rajeshwari & Sonti 2000); rpfC involved
in regulation of EPS biosynthesis (Tang et al. 1996); hrp and avr genes encoding
components of a type III protein secretion system, it’s effectors and regulators
(Kamdar et al. 1993; Bai et al. 2000; Yang et al. 2000; Zhu et al. 2000); xadA
encoding an adhesin like protein (Ray et al. 2002) and phyA which promotes
utilization of phytic acid as a sole phosphate source (Chatterjee et al. 2003).

One of the critical factors for survival of many pathogens within their
hosts is the availability of iron. In vertebrate hosts, iron sequestration upon
infection is well-known (Weinberg 1984; Payne 1993). In plants, several studies indicate that iron availability is likely to be a limiting factor for bacterial growth within the host (Mila et al. 1996; Neema et al. 1993). Previous work has indicated that Xoo rpfF mutants are deficient for virulence and growth under low iron conditions (Chatterjee & Sonti 2002). Exogenous iron supplementation has been shown to promote in planta growth of the Xoo rpfF mutants. These data suggest that the rpfF gene promotes virulence of Xoo by facilitating iron uptake.

Under aerobic conditions, iron is present in the form of insoluble ferric salts. Many bacteria secrete low molecular weight iron chelators called siderophores, which sequester Fe³⁺ with high affinity (Ratledge & Dover 2000). The siderophore-Fe³⁺ complex is taken up into the bacterial cell by active transport across the outer membrane, and the energy required for this process is transduced by the TonB complex (TonB, ExbB, ExbD) of proteins (Postle & Kadner 2003). In Xcc, a tonB mutant has been reported to be impaired for Fe³⁺ uptake and exhibit increased extracellular siderophore, indicating that TonB plays an important role in Fe³⁺ uptake by this bacterium (Wiggerich et al. 1997). Other methods of iron uptake from heme or host iron binding proteins and direct Fe²⁺ transport may also be employed by pathogenic bacteria (Ratledge & Dover 2000; Velayudhan et al. 2000). However, excess iron in the cell can be toxic due to the ability of Fe²⁺ to catalyze the Fenton reaction, which generates cell damaging hydroxyl radicals (Touati 2000). Hence, the expression of iron uptake functions must be tightly controlled, and this function is performed in many bacteria by the Ferric Uptake Regulator (Fur) protein.

Fur functions as a transcriptional repressor of iron uptake functions and iron regulated genes in bacteria. The repressor binds as a dimer, with Fe²⁺ as corepressor, to specific sequences called Fur boxes that are located in the promoter regions of such genes (Ratledge & Dover 2000). Under conditions of iron starvation, the Fur protein is inactive and the Fur-regulated genes are transcribed. Besides controlling genes involved in iron metabolism, Fur has been shown to regulate functions involved in varied cellular processes such as defense against acid shock & oxidative stress (Bijlsma et al. 2002; Thompson et
al. 2002; Vasil & Ochsner 1999), enzymes involved in energy metabolism (Thompson et al. 2002; Vasil & Ochsner 1999), and production of virulence-associated functions in animal pathogens (Barton et al. 1996; Grifantini et al. 2003). Fur also appears to positively affect expression of certain genes for bacterioferritin, superoxide dismutase and catalase. (Vasil & Ochsner 1999; Hassett et al. 1996). The effect might be through an indirect repressive mechanism because Fur represses the expression of small regulatory RNA molecules, which, in turn, inhibit the expression of target genes (Masse & Gottesman 2002; Wilderman et al. 2004).

Interestingly, a mutation in the fur gene of Rhizobium leguminosarum has been shown to have no effect on the expression of several operons that are involved in iron acquisition (Wexler et al. 2003). Instead, another protein called RirA has been found to regulate genes involved in iron uptake (Todd et al. 2002). Among plant pathogens, reduced virulence has been associated with mutations in the fur gene of Erwinia chrysanthemi, which causes soft rot of African Violets (Franza et al. 1999). In Xanthomonas campestris pv. phaseoli (Xcp), the Fur protein has been characterized with respect to primary structure and iron-dependent expression pattern (Loprasert et al. 1999) and shown to be capable of repressing a Fur-regulated promoter in E. coli. The nucleotide sequences of fur genes from four xanthomonads have been determined (accession numbers AF146020, AF146021, AF146022 & AF146830). However, the role that the Fur protein plays in virulence of any member of the xanthomonad group of plant pathogens is unknown. In this paper, the characteristics of a fur mutant of Xoo are reported.

2.3 Materials And Methods

2.3.1 Bacterial strains, plasmids and culture media

The Xoo strains used were BXO43 (rif-2, rifampicin resistant; laboratory wild-type strain); BXO1801 (fur1::bla rif-2, Ap+ and BXO1802 (fur+ derivative of BXO1801, Ap+). These strains were grown at 28°C in peptone sucrose (PS) medium (Tsuchiya et al. 1982). pSS1 (pMOS Blue + 268-bp PCR product amplified from fur), was generated and maintained in Escherichia coli strain
DH10B (Invitrogen Life Technologies, Carlsbad, CA). *E. coli* cells were cultured at 37°C in LB medium (Miller 1992). The concentrations of antibiotics used were rifampicin (Rf), 50 μg ml⁻¹; ampicillin (Ap), 100 μg ml⁻¹; cycloheximide (Cy), 80 μg ml⁻¹ and cephalexin (Cp), 20 μg ml⁻¹. The iron chelator, 2,2'-dipyridyl, was used at a concentration of 100 μM from a 10 mM stock solution made in sterile water. Streptonigrin was made in sterile water as a 1 mg ml⁻¹ stock solution and the concentration used is 15 μg ml⁻¹.

2.3.2 Generation of a *fur* mutant of Xoo

An internal fragment of the Xoo *fur* gene was amplified using primers FurF1 (5'-GCAGAAGACATCTATCGCCAG-3') and FurR (5'-GCTCTTCGAGTTCATAGCCG-3'). The fragment was cloned into the PCR cloning vector pMOS (Amersham Pharmacia Biotech, Uppsala, Sweden) to obtain the pSS1 plasmid, which was sequenced to verify the identity of the cloned product. Around 500 ng of the plasmid was introduced by electroporation into electro-competent cells of BXO43 (wild-type strain). The transformants were grown in PS for 3 h and then plated on peptone sucrose agar (PSA) containing ampicillin (Ap). Integration of the plasmid into the genome of Ap⁻ colonies was confirmed by PCR using flanking primers FurF (5'-ATGGAAACCCACGACCTGC-3') and FurR1 (5'-GCGCGGACGCTTCTTGC-3') in combination with vector specific primers M13F and T7. Sequencing of the PCR products was done to confirm integration into the *fur* gene.

2.3.3 Plasmid isolation and other molecular techniques

Plasmid isolation was by the alkaline lysis method as described (Sambrook et al. 1989). Restriction digestions were done, as required, with enzymes obtained from NEB (New England Biolabs, Beverly, MA) as per the supplier's instructions. Inverse PCR to obtain the DNA sequences flanking the Xoo *fur* gene was done by digesting 2 μg of wild-type genomic DNA with enzymes *Sau*96I and *Bfa*I. After cleaning of the digested DNA, 50 ng was taken
for overnight ligation in 50 μl volume to circularize the DNA fragments. Different aliquots of the ligated product were taken for inverse PCR using the FR1 primer (5'-CGCTGCAGCGCCAGATTGCC-3') to obtain sequence downstream of fur and the FR2 primer (5'-CTCTTTTGCTCCAGCAATTCC-3') to obtain upstream sequence. Sequencing of the PCR products was performed with an ABI Prism 3700 automated DNA sequencer (Perkin-Elmer, Foster City, CA). Similarity searches were performed in the NCBI database, using the BLAST algorithm (Altschul et al. 1990). ORF searches were done using ORF Finder of the National Center for Biotechnology Information and FramePot 2.3.2 (Ishikawa & Hotta 1999).

2.3.4 Siderophore production assay

Chrome Azurol Sulphonate (CAS) containing siderophore indicator plates were prepared as described by Schwyn & Neilands (1987). Since Xoo grows very poorly on minimal medium, the siderophore indicator plates were made in PS instead of MM9 medium. Xoo strains were patched on PSA-CAS plates and incubated at 28°C. In order to create iron depleted conditions, 2,2'-Dipyridyl (100 μM) was added to PSA-CAS plates. Similarly, PSA-CAS plates were supplemented with ferrous sulphate (100 μM) when required. The colonies were scored for the orange halo phenotype after 36 h.

2.3.5 Sensitivity to hydrogen peroxide and streptonigrin

Xoo strains were grown in 3 ml of PS to a cell density of 10⁸ cfu ml⁻¹. Bacterial cultures were dilution plated on PSA to obtain cell numbers before treatment with either H₂O₂ (Loba Chemie, Mumbai, India) or streptonigrin. H₂O₂ was added to the culture at a concentration of 10 mM and incubated at 28°C and 150 rpm for 1 h. Streptonigrin was added to the culture at a concentration of 15 μg ml⁻¹ and incubated at 28°C and 150 rpm for 2 h. The cells were then pelleted, washed once with sterile distilled water, before plating to count cell number. The logarithmic value of viable cells per ml culture is indicated as mean +/− S.D.
2.3.6 Preparation of cell extracts and catalase assay

Bacterial cultures were grown initially for 30 h in PS (for the fur mutant, ampicillin was added to prevent revertant accumulation) to obtain pre-inoculum. The fur mutant had to be grown for a longer period (48 h) to obtain pre-inoculum with the same cell number as wild-type. Into 30 ml PS in a 100 ml flask, 5% pre-inoculum was added and grown at 28°C, 150 rpm (Chamnongpol et al. 1995). Cells were pelleted at early log phase (after 2 h of growth) by centrifugation at 7000 rpm for 10 min. Cell pellets were suspended in 50 mM potassium phosphate buffer containing 0.1 mM EDTA. The cell suspension was disrupted by sonication with 0.5-s pulses for 2 min with 1 min interval during which the probe is removed from the sample. Tubes containing the samples were always kept on ice. Debris was pelleted by centrifugation at 13000 rpm for 10 min. Total protein concentration was measured by the method of Bradford (1976), with bovine serum albumin as the standard. Catalase was assayed by the method of Beers & Sizer (1951). Briefly, 5 to 25 µl of total protein extract was diluted to 1 ml with double distilled water (DDW). A 59 mM H₂O₂ solution was prepared from a 10 M stock solution by freshly diluting in 50 mM potassium phosphate buffer pH 7.0. To 1 ml of the sample in a cuvette, 0.5 ml of 59 mM H₂O₂ is added and the absorbance at 240 nm was measured every 10-s for 1 min, to calculate the decrease in absorbance in that time period (expressed as ΔA₂₄₀ min⁻¹). The specific activity of catalase was calculated as follows: 1000 × ΔA₂₄₀ min⁻¹/ 43.6 × mg of protein ml⁻¹ of reaction mixture (Visick & Clarke 1997).

2.3.7 Virulence assays on rice plants

Virulence assays were performed on ~40-day-old rice plants of the highly susceptible rice cultivar Taichung Native (TN-1) in the greenhouse. Inoculation was done by clipping leaf tips with scissors dipped in bacterial culture (10⁸ cfu/ml resuspended in sterile double distilled water) (Kauffman et al. 1973). BXO1801 was grown in PS + Ap + 2,2’-dipyridyl to minimize the number of revertants in the culture at the time of inoculation. To maintain similar growth conditions, BXO43 and BXO1802 were also grown in PS + 2,2’-dipyridyl.
Inoculated plants were incubated in the green house and lesion lengths were taken 7 and 14 days after inoculation. Control inoculations were performed with scissors dipped in sterile water and no lesions were observed. In each experiment, 15 leaves were inoculated and the lesion length was calculated as the mean of all individual lesion lengths +/- standard deviation. Similar results were obtained in independent experiments.

2.3.8 Re-isolation of bacteria from rice leaves and fur\(^{+}\) revertant identification

Infected leaves were surface sterilized by dipping in 1% (vol/vol) sodium hypochlorite (Loba Chemie, Mumbai, India) for 1 min and washing thrice in distilled water. The leaves were cut at the leading edge of the lesion and dipped in 1 ml of sterile water for 5 min. Bacteria that exuded from the cut edge of the leaf were isolated by plating for individual colonies on PSA. fur\(^{+}\) revertants were identified by their larger colony size and these colonies were then patched on PSA-CAS plates for halo phenotype (no halo was detected as these colonies would behave like wild-type) and on PSA + Ap plates to detect loss of the previously integrated construct (such colonies were sensitive to ampicillin). Colonies of smaller size were also examined, and, as expected were found to have characteristics of the fur mutant strain.

2.3.9 Detached leaf inoculation experiment with exogenous ascorbic acid supplementation

Leaves of 40-day-old greenhouse-grown rice plants of the susceptible rice cultivar TN-1 were cut with scissors 2 cm above the junction of the leaf blade and sheath, and were dipped in 250-ml conical flasks (20 leaves per flask) containing 100 ml of 0.1% glucose in DDW, as described previously (Chatterjee & Sonti 2002), with or without 500 \(\mu\)M ascorbic acid (Sigma-Aldrich). The leaves were maintained for 24 h on a laboratory bench top prior to inoculation with different strains of Xoo by leaf-clip method (Kauffman et al. 1973). At desired time intervals, the infected leaves were surface sterilized as mentioned above. 1 sq. cm of leaf material from the point of inoculation was taken, crushed using a
mortar and pestle in 1 ml sterile DDW and dilution plated on PSA media containing Rf + Cp + Cy. After 3-4 days, colonies were counted and mean +/- S.D. of the logarithmic value of cell number obtained from 3 different leaves was calculated. Similar results were obtained in independent experiments.

2.3.10 Growth experiments

Bacterial cultures were grown initially in PS to obtain pre-inoculum and 0.1% pre-inoculum of wild-type and mutant cells was added in PS, with or without 500μM ascorbic acid, and grown at 28°C, 200 rpm. To determine the cell numbers per ml of culture, appropriate dilutions were plated on PSA plates at regular intervals. For the BXO1801 strain, in addition to PSA medium (to determine the total cell number in the culture), plating was also done on ampicillin containing medium (to determine the total number of fur-1::bla mutant cells in the culture). After 4 days colonies were counted and expressed as mean +/- S.D. of logarithmic value of cell number per ml at different intervals. An estimation of number of fur+ revertants in the BXO1801 culture was obtained by subtracting the cell numbers obtained on ampicillin containing medium from that on PSA medium. A representative number of the Ap^r and Ap^s cells were checked for the siderophore over-production phenotype. As expected, the Ap^r colonies exhibited excess siderophore secretion while the Ap^s colonies were similar to the wild-type strain.

2.3.11 Nucleotide sequence accession no.

Nucleotide and aminoacid sequence data are available in the GenBank database under accession number AY688951.

2.4 Results

2.4.1 Gene organization in the fur region of Xoo

A partial sequence (408-bp) of the Xoo fur gene (available as accession No. AF146830) was used to obtain, through inverse PCR, the sequence
Figure 2.1 Schematic of the arrangement of open reading frames (ORFs) in a 1.248-kb genomic region that encodes the Xoo fur gene. 'M' and '.' represent the start and stop codons of ORFs. Large arrows represent the direction of transcription. Only partial sequences of ORFs 2 and 3 are available. The striped region indicates the internal fragment of ORF1 (fur) which was cloned in pMOS vector. Small arrows indicate the positions of different primers: Primers 1 and 2 were used to amplify the internal fragment of ORF1, primers 3 and 4 flank this region and were used to confirm mutation in ORF1. Arrow-head indicates the orientation of the lacZ promoter of the vector.
of a 1.25-kb genomic region containing the entire Xoo fur gene (411-bp) and flanking sequences. The sequenced region contained one complete open reading frame (ORF1) and two partial ORFs (ORF2 and ORF3) (Figure 2.1). ORF1 (fur) is 411-bp long and potentially encodes a protein of 136 amino acids. A BLAST search (Altschul et al. 1990) with the deduced amino acid sequence of ORF1 (fur) revealed that it exhibits high similarity to the Fur proteins of Xcp (accession number AAD44804, 98% similarity, 97% identity); Xcc (accession number NP636842, 99% similarity, 98% identity) and Xac (acces sion number NP641850, 98% similarity, 97% identity).

ORF2 (a partial sequence of 102-bp) is immediately upstream of the fur gene and exhibited 97, 96 and 94% nucleotide sequence identity, respectively, to smpA (small protein A) genes of Xac (accession number AE011783); Xcp (accession number AF146829) and Xcc (accession number AE012247). ORF3 (a partial sequence of 192-bp) is immediately downstream to the fur gene, and has a transcriptional orientation in the opposite direction. ORF3 exhibited 94 and 92% nucleotide sequence identity, respectively, to recN (putative DNA repair protein) genes of Xac (accession number AE011783) and Xcc (accession number AE012247). The gene organization of the fur region of Xoo (Figure 2.1) is conserved in Xcc and Xac, except for the presence of an ORF encoding a conserved hypothetical protein of 104 amino acids in between fur and recN genes in Xac (da Silva et al 2002). A 440-bp intergenic region lies between the fur and recN genes in Xoo. The 440-bp region was analyzed using the ORF Finder program as well as FramePlot (Ishikawa & Hotta 1999), and no ORF with significant homology or length was detected. The intergenic region also exhibited no significant similarity against the corresponding regions of Xcc and Xac when analyzed by Blast2 (Tatusova & Madden 1999).

2.4.2 Generation of a Xoo fur mutant

An internal 268-bp fragment of the fur gene was cloned into pMOS and used to generate a fur mutant (strain BXO1801) of Xoo (strain BXO43) by homologous recombination (Figure 2.1). A fur+ revertant (designated as
BXO1802) was obtained by growing the *fur* mutant in peptone-sucrose (PS) medium without ampicillin and screening for Ap<sup>+</sup> colonies that are expected to have arisen by excision of the integrated construct. Revertants were distinguished by sensitivity to ampicillin, and a bigger colony size. PCR was performed with the flanking primers (FurF & FurR1; primers 3 and 4 in Figure 2.1) to obtain a product of about 408-bp in the revertant as in the wild-type strain.

**2.4.3 Xoo fur mutant produces siderophores in an unregulated manner and is hypersensitive to streptonigrin**

The chemical assay for siderophore production using Chrome Azurol Sulphonate (CAS) was employed (Schwyn & Neilands 1987). The BXO43 strain does not produce siderophores on PSA-CAS (peptone sucrose agar + CAS) plates. However, under conditions of iron depletion caused by addition of 100 μM dipyridyl (a ferrous chelator), BXO43 was found to produce siderophores (seen as a yellowish orange halo surrounding the colonies) (Figure 2.2). The *fur* mutant, BXO1801, produced siderophores even on PSA-CAS plates. Addition of increasing concentrations of iron in the form of ferrous sulphate (up to 100 μM) failed to abrogate siderophore production by BXO1801. BXO1802, the *fur*<sup>+</sup> revertant, behaves like the wild-type strain in the above assays.

The overproduction of siderophores by BXO1801 indicated the possibility that excess free iron might be present within these cells. To verify this possibility, we performed the streptonigrin sensitivity assay. Streptonigrin is an antibiotic exhibiting iron dependent catalytic activity resulting in cell death (Ming 2003). PS grown cells of BXO1801, given a 2 h exposure to streptonigrin (15 μg ml<sup>-1</sup>), showed an approximately 10 to 100-fold reduction in viability as compared to BXO43 (Figure 2.3).
Figure 2.2. Xoo fur mutant produces siderophores in an unregulated manner. The Universal CAS (Chrome Azurol-S) assay for detection of secreted siderophores was performed by growing Xoo strains for 36 h on (A) PSA-CAS + 100 μM 2,2'-Dipyridyl and (B) PSA-CAS + 100 μM FeSO₄. The halo is indicative of siderophore production. 1 & 4-BX043 (wild-type), 2-BXO1801 (fur1::bla) and 3- BXO1802 (fur+ derivative of BXO1801). The fur mutant produces siderophores under both growth conditions. The wild-type and fur+ revertant strains produce siderophores only under conditions of iron starvation.
Figure 2.3 The Xoo fur mutant exhibits hypersensitivity to streptonigrin. Xoo strains were treated with 15 μg ml⁻¹ streptonigrin for 2 h and the viable cell number was estimated by plating bacterial culture before and after treatment. BXO43 = wild-type strain; BXO1801 = fur1::bla
2.4.4 BXO1801 is hypersensitive to hydrogen peroxide and exhibits reduced catalase activity

Another feature of defects in iron regulation are concomitant changes in sensitivity to reactive oxygen species. To assess possible changes in the fur mutant, the viability of BXO43, BXO1801 (fur mutant) and BXO1802 (fur+ revertant) were measured after treatment with 10 mM hydrogen peroxide for 1 h (see materials and methods). BXO43 and BXO1802 exhibited a 10-fold reduction in cell number, whereas BXO1801 showed almost a 1000-fold reduction in viability after H₂O₂ treatment (Figure 2.4). Catalase activity was measured in order to determine whether hypersensitivity to H₂O₂ was associated with impaired antioxidant functions. Early log phase cultures were used since Xoo cultures have maximum catalase activity at this phase of growth (Chamnongpol et al. 1995). Cell extracts were prepared from BXO43, BXO1801 and BXO1802 at early log phase and activity was determined by spectrophotometry. Catalase activity of BXO43 and BXO1802 was found to be ~18 units per mg protein, which was ~3 fold more than BXO1801 (5.5 units per mg protein) (Figure 2.5).

2.4.5 BXO1801 is virulence deficient and also exhibits a slow growth phenotype on PS medium

Virulence was assessed by measuring lesion lengths caused on rice leaves. As compared to BXO43 and BXO1802 (fur+ revertant), the lesion lengths on rice leaves caused by the BXO1801 (fur mutant) strain were reduced significantly (~15% of fur+), 7 days after inoculation (DAI) (Figure 2.6). Interestingly, lesions were found to be about 50% of the fur+ strains, at 14 DAI. BXO1801 reverts to fur+ at a frequency of 1 fur+: 10⁴ fur− cells (data not shown).

To determine if the increase in lesion length is caused by a selection for fur+ revertants in planta, bacteria were isolated from leaves inoculated with BXO1801. At 7 DAI, the frequency is 1 fur+: 10²-10³ fur− cells while at 14 DAI it changes to 1 fur+: 1-10 fur− cells. This drastic increase in the number of revertants indicated an in planta growth advantage for the fur+ revertants and illustrated the importance of the fur gene for pathogenicity and survival in rice leaves.
Figure 2.4 The Xoo fur mutant is hypersensitive to hydrogen peroxide. Xoo strains were treated with 10 mM hydrogen peroxide for 1 h and the viable cell number was estimated by plating bacterial cultures before and after treatment. BXO43 = wild-type strain; BXO1801 = fur1::bla; BXO1802 = fur+ derivative of BXO1801.
Figure 2.5 The Xoo fur mutant exhibits reduced catalase activity. The Xoo wild-type (BXO43), fur mutant (BXO1801) and fur+ revertant (BXO1802) were grown in PS at 28°C and cell extracts prepared at early log phase as described in Materials and Methods. Catalase activity was measured by the method of Beers and Sizer (1952). The specific activity of catalase is expressed in terms of units (micromoles of H₂O₂ decomposed per minute at pH 7.0) per milligram of total protein.
Figure 2.6 The Xoo fur mutant is virulence deficient. Virulence assays were done by wound inoculation of leaves of the susceptible rice cultivar Taichung Native-1 (TN-1). Lesion lengths were measured, 7 and 14 days after inoculation. Mean and standard deviation of fifteen replicate measurements of lesion lengths are given BXO43 = wild-type strain; BXO1801 = fur1::bla; BXO1802 = fur\(^+\) derivative of BXO1801. Similar results were obtained in independent experiments.
Growth curves were measured for BX043 and BXO1801 in PS medium. BXO1801 exhibits a severe growth deficiency as compared to BXO43 (Figure 2.7). The relative proportion of fur+ revertants to fur - cells were estimated, and a strong selection for fur+ revertants was observed. The ratio is 1 fur+: 10^1-10^2 fur- cells at the time of inoculation and is approximately 1 fur+: 10^3-10^4 fur - cells after 48 h in PS medium. Between 36-48 h after inoculation, a near 100-fold reduction in the total number of fur cells in the BXO1801 culture was observed. The BXO1802 strain (fur+ revertant) behaves similar to BXO43 in these assays (data not shown).

2.4.6 In planta growth defect of the Xoo fur mutant is rescued by ascorbic acid supplementation

Detached leaf inoculation experiments were carried out to determine if the in planta growth defect of BXO1801 could be alleviated by supplementation with ascorbic acid, an anti-oxidant. Survival of bacteria in rice leaves was monitored by determining the viable cell number of the re-isolated bacteria (Figure 2.8). The cell numbers of wild-type and fur mutant were similar (~10^3-10^4 cfu ml^-1), without or with 500 μM ascorbic acid supplementation, 1 h after inoculation, indicating equal efficiency in leaf entry. After 48 h, wild-type cell numbers increased by 100 - 1000 fold in either glucose alone or glucose and ascorbic acid. On the other hand, cell numbers of the fur mutant either were reduced or remained constant after 48 h in leaves with glucose alone. The cell numbers increased to ~10^6 cfu ml^-1 (almost wild-type level) in ascorbic acid supplemented medium. Thus, a significant increase (~100-fold) was found in the cell numbers of the fur mutant upon ascorbic acid supplementation. This experiment demonstrates that, at least in the first 48 h of leaf infection, the growth defect of the Xoo fur mutant may be corrected by providing ascorbic acid, an anti-oxidant.

The effect of ascorbic acid supplementation on growth of BXO43 (fur+) and BXO1801 (fur) in PS medium was also assessed. Ascorbic acid has a
slight inhibitory effect on growth of BXO43 (Figure 2.7). Ascorbic acid improves the growth of BXO1801 as the numbers of cells of BXO1801 are higher in PS and ascorbic acid as compared to PS alone (Figure 2.7). The reduction in the number of fur mutant cells between 36 - 48 h is less severe in PS and ascorbic acid as compared to PS alone. However, even with ascorbic acid supplementation, BXO1801 does not grow as well as the fur+ strain in PS medium and ascorbic acid only partially alleviates the slow growth phenotype of BXO1801 in PS medium.

2.5 Discussion

Iron uptake in a regulated manner is critical for vital cellular functions in an aerobic environment, and this function is carried out by the Fur protein in many bacteria. In Rhizobium leguminosarum, many of the iron-regulated genes do not exhibit Fur dependent expression and have been shown to be regulated by another protein, RirA (Wexler et al. 2003; Todd et al. 2002). Therefore, iron dependent expression need not be regulated by Fur in all bacteria. The fur mutant of Xoo exhibits deregulated siderophore production, a slow growth phenotype on rich medium, sensitivity to oxidative stress and virulence deficiency. The constitutive secretion of siderophores by the fur mutant of Xoo is indicative of deregulated iron uptake machinery. The identity of the genes that are involved in iron uptake or in the synthesis of siderophore in Xoo is not known and additional evidence for fur regulated iron uptake may be obtained only after identification of these genes.

Previous reports have indicated a failure to construct null mutations in the fur gene of Pseudomonas aeruginosa, Haemophilus ducreyi and Neisseria gonorrhoea (Prince et al. 1993; Carson et al. 1996; Thomas & Sparling 1996). Although an Xoo fur mutant was obtained, it grew poorly and reverted back to wild-type in the absence of antibiotic selection in rich medium, indicating that a functional fur gene provides selective advantages. Complementation of the fur mutant with a cosmid containing the fur gene and the flanking genomic region resulted only in partial restoration of the wild-type phenotype for both
Figure 2.7 The Xoo fur mutant exhibits a slow growth phenotype in PS medium which is partially rescued by ascorbic acid supplementation. Xoo strains were grown in PS at 28°C with or without ascorbic acid (500μM) and cell numbers were estimated by plating bacterial cultures at intervals. BXO43 PS & BXO43 AA = wild-type, without or with ascorbic acid; BXO1801 PS & BXO1801 AA = fur1::bla mutant, without or with ascorbic acid; BXO1801 revertants PS & BXO1801 revertants AA = fur<sup>+</sup> revertants obtained in the BXO1801 culture without or with ascorbic acid respectively.
Figure 2.8 In planta growth deficiency of an Xoo fur mutant is rescued by ascorbic acid supplementation. Xoo strains were inoculated on leaves dipped in sterile water containing 0.1% glucose without or with ascorbic acid (500 μM). Bacteria were re-isolated by crushing 1 sq. cm of the cut end of the leaf and viable cell numbers were estimated after 1 h and 48 h (see materials and methods). Mean +/- S.D of cell numbers obtained from 3 different leaves are indicated for each strain.
BXO43-glc & BXO43-aa = wild-type, without or with ascorbic acid; BXO1801-glc & BXO1801-aa = furl::bla, without or with ascorbic acid respectively. Similar results were obtained in independent experiments.
siderophore production and growth (data not shown). Introduction of the cosmid clone improved growth of the fur mutant on PSA medium (as judged by colony size) and resulted in lesser production of siderophore (as judged by halo size on PSA-CAS plates). However, this strain did not grow as well and also produced more siderophore than the wild type strain. The N- and C-terminal portions of the Fur protein are reported to have DNA binding and dimerisation activities, respectively (Stojiljkovic & Hantke 1995). The plasmid insertion in the Xoo fur gene results in a truncated fur allele in which the first 370 bp of the 411 bp fur gene are intact. This might result in the production of a truncated Fur protein that could interfere with functioning of the wild type protein; possibly because it might possess some residual DNA binding or dimerisation activity. A dominant negative fur allele of Bradyrhizobium japonicum has been previously isolated as a spontaneous manganese resistant mutant and shown to have multiple point mutations within the fur coding sequence (Benson et al. 2004). It is interesting to note that several of these mutations result in amino acid substitutions in the extreme C-terminal region of the protein (the equivalent region is missing in the Xoo fur mutant).

Similar to E.coli, the Xoo fur mutant is hypersensitive to hydrogen peroxide (Touati et al. 1995), which may be due to reduced catalase activity. In Pseudomonas aeruginosa, fur mutants exhibit reduced catalase activity (Hassett et al. 1996). The presence of excess free intracellular iron, due to deregulation of iron uptake, leads to increased generation of toxic hydroxyl radicals (Touati 2000). Therefore, the excess oxidative stress, along with reduced catalase activity, might contribute to H$_2$O$_2$ hypersensitivity. The mechanism by which Fur protein affects catalase expression in Xoo is not known (Masse & Gottesman 2002; Wilderman et al. 2004).

What might be the reason for reduced pathogenicity of the Xoo fur mutant? Recent reports suggest that reactive oxygen species (ROS) are produced in compatible interactions (Venisse et al. 2003). Successful neutralization of this oxidative stress would be critical for in planta survival. Therefore, a fur mutant that is compromised in anti-oxidant functions would be at a tremendous
disadvantage, once the plant defense reactions are induced. The removal of ROS should promote survival of the fur mutant in planta. Ascorbic acid is known to scavenge hydroxyl radicals, thus acting as an anti-oxidant (Bielski 1982; Horemans et al. 2000). The in planta growth defect of the Xoo fur mutant can be rescued by supplementing with ascorbic acid. This observation suggests that the reduced virulence of the Xoo fur mutant may be, at least in part, due to an inability to survive oxidative stress during infection.

Ascorbic acid supplementation does not fully alleviate the slow growth phenotype of BXO1801 in PS medium. It is possible that ascorbic acid is not taken up very efficiently by cells of Xoo. In such a situation, ascorbic acid supplementation might provide better protection to the fur mutant against oxidative stress emanating from an external source (as would be experienced during growth in the leaf) as compared to oxidative stress from within the cell (as would be experienced during growth in PS medium). Alternatively, enhanced oxidative stress may only be partially responsible for the slow growth phenotype of the fur mutant in PS medium. More studies on the fur regulon of Xoo are needed to understand how Fur controls iron uptake, response to oxidative stress, growth in laboratory conditions and within the plant.

2.6 Literature Cited


Thomas, C. E., & Sparling, P. F. (1996). Isolation and analysis of a *fur* mutant of


