Growth Deficiency of a *Xanthomonas oryzae* pv. *oryzae* *fur* Mutant in Rice Leaves Is Rescued by Ascorbic Acid Supplementation

Sujatha Subramoni and Ramesh V. Sonti

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500 007, India

Submitted 29 July 2004. Accepted 11 February 2005.

Xanthomonas oryzae pv. *oryzae* causes bacterial leaf blight, a serious disease of rice. A mutation was isolated in the ferric uptake regulator (*fur*) gene of *X. oryzae* pv. *oryzae* and it was shown to result 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 antioxidant) rescues the growth deficiency of the *fur* mutant in rice leaves. The virulence deficiency of the *X. oryzae* pv. *oryzae* *fur* mutant is proposed to be due, at least in part, to an impaired ability to cope with the oxidative stress conditions that are encountered during infection.

Xanthomonas oryzae pv. *oryzae* causes bacterial leaf blight, a serious disease of rice. Some of the genes required for *X. oryzae* pv. *oryzae* virulence that have been previously described include *gumG* and *gumM*, involved in extracellular polysaccharide (EPS) biosynthesis (Dharmapuri and Sonti 1999; Rajeshwari and Sonti 2000); *rpfC*, involved in regulation of EPS biosynthesis (Tang et al. 1996); *hrp* and *avr*, encoding components of a type III protein secretion system and its effectors and regulators (Bai et al. 2000; Kamdar et al. 1993; 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 (Payne 1993; Weinberg 1984). 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 *X. oryzae* pv. *oryzae* *rpfF* mutants are deficient for virulence and growth under low iron conditions (Chatterjee and Sonti 2002). Exogenous iron supplementation

has been shown to promote in planta growth of the *X. oryzae* pv. *oryzae* *rpfF* mutants. These data suggest that the *rpfF* gene promotes virulence of *X. oryzae* pv. *oryzae* 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 Fe^{3+} with high affinity (Ratledge and Dover 2000). The siderophore- Fe^{3+} 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, and ExbD) of proteins (Postle and Kadner 2003). In *X. campestris* pv. *campestris*, a *tonB* mutant has been reported to be impaired for Fe^{3+} uptake and exhibit increased extracellular siderophore, indicating that TonB plays an important role in Fe^{3+} uptake by this bacterium (Wiggerich et al. 1997). Other methods of iron uptake from heme or host iron-binding proteins and direct Fe^{2+} transport also may be employed by pathogenic bacteria (Ratledge and Dover 2000; Velayudhan et al. 2000). However, excess iron in the cell can be toxic due to the ability of Fe^{2+} 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 Fe^{2+} as corepressor, to specific sequences called Fur boxes that are located in the promoter regions of such genes (Ratledge and Dover 2000). Under conditions of iron starvation, the Fur protein is inactive and the Fur-regulated genes are transcribed. In addition to controlling genes involved in iron metabolism, Fur has been shown to regulate functions involved in varied cellular processes such as defense against acid shock and oxidative stress (Bijlsma et al. 2002; Thompson et al. 2002; Vasil and Ochsner 1999), enzymes involved in energy metabolism (Thompson et al. 2002; Vasil and 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. (Hassett et al. 1996; Vasil and Ochsner 1999). 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 and Gottesman 2002; Wilderman et al. 2004).

Corresponding author: R. V. Sonti; Telephone: +91-40-27192577; Fax: +91-40-27160591; E-mail: sonti@cmb.res.in and rvsonti@yahoo.com

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

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 *X. campestris* pv. *phaseoli*, 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 *Escherichia coli*. The nucleotide sequences of *fur* genes from four xanthomonads have been determined (accession numbers AF146020, AF146021, AF146022, and 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 article, the characteristics of a *fur* mutant of *X. oryzae* pv. *oryzae* are reported.

RESULTS

Gene organization in the *fur* region of *X. oryzae* pv. *oryzae*.

A partial sequence (408 bp) of the *X. oryzae* pv. *oryzae* *fur* gene (available as accession no. AF146830) was used to obtain, through inverse polymerase chain reaction (PCR), the sequence of a 1.25-kb genomic region containing the entire *X. oryzae* pv. *oryzae* *fur* gene (411 bp) and flanking sequences. The sequenced region contained one complete open reading frame (ORF1) and two partial ORFs (ORF2 and ORF3) (Fig. 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 *fur* revealed that it exhibits high similarity to the Fur proteins of *X. campestris* pv. *phaseoli* (AAD44804, 98% similarity, 97% identity), *X. campestris* pv. *campestris* (NP636842, 99% similarity, 98% identity), and *Xanthomonas axonopodis* pv. *citri* (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 to *smpA* (small protein A) genes of *X. axonopodis* pv. *citri* (AE011783), *X. campestris* pv. *phaseoli* (AF146829), and *X. campestris* pv. *campestris* (AE012247), respectively. 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 to *recN* (putative DNA repair protein) genes of *X. axonopodis* pv. *citri* (AE011783) and *X. campestris* pv. *campestris* (AE012247), respectively. The gene organization of the *fur* region of *X. oryzae* pv. *oryzae* (Fig. 1) is conserved in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*, except for the presence of an ORF encoding a conserved hypothetical protein of 104 amino acids between *fur* and *recN* genes in *X. axonopodis* pv. *citri* (da Silva et al 2002). A 440-bp intergenic region lies between the *fur* and *recN* genes in *X. oryzae* pv. *oryzae*. The 440-bp region was analyzed using the ORF Finder program as well as FramePlot (Ishikawa and 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 *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* when analyzed by Blast2 (Tatusova and Madden 1999).

Generation of a *X. oryzae* pv. *oryzae* *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 *X. oryzae* pv. *oryzae* (strain BXO43) by homologous recombination (Fig. 1). A *fur*⁺ revertant (designated as BXO1802) was obtained by growing the *fur* mutant in peptone-sucrose (PS) medium without ampicillin and screening for ampicillin-sensitive (Ap^s) colonies that were 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 and FurR1) (Fig. 1, primers 3 and 4) to obtain a product of approximately 408 bp in the revertant as in the wild-type strain.

Fig. 1. Schematic of the arrangement of open reading frames (ORFs) in a 1.248-kb genomic region that encodes the *Xanthomonas oryzae* pv. *oryzae* *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. Arrowhead indicates the orientation of the *lacZ* promoter of the vector.

***X. oryzae* pv. *oryzae* fur mutant produces siderophores in an unregulated manner and is hypersensitive to streptonigrin.**

The chemical assay for siderophore production using chrome azurol sulphate (CAS) was employed (Schwyn and Neilands 1987). BXO43 does not produce siderophores on peptone sucrose agar + CAS (PSA-CAS) plates (Fig. 2). However, under conditions of iron depletion caused by addition of 100 μ M dipyriddy (a ferrous chelator), BXO43 produces siderophores (seen as a yellowish orange halo surrounding the colonies). The *fur* mutant, BXO1801, produced siderophores

Fig. 2. *Xanthomonas oryzae* pv. *oryzae* *fur* mutant produces siderophores in an unregulated manner. The universal chrome azurol-S (CAS) assay for detection of secreted siderophores was performed by growing *X. oryzae* pv. *oryzae* strains for 36 h on **A**, peptone sucrose agar (PSA)-CAS, **B**, PSA-CAS + 100 μ M 2,2'-dipyridyl, and **C**, PSA-CAS + 100 μ M FeSO₄. The halo is indicative of siderophore production. 1-BXO43 = wild type, 2-BXO1801 = *fur1::bla*, and 3-BXO1802 = *fur*⁺ derivative of BXO1801. The *fur* mutant produces siderophores under all growth conditions. The wild-type and *fur*⁺ revertant strains produce siderophores only under conditions of iron starvation.

Fig. 3. *Xanthomonas oryzae* pv. *oryzae* *fur* mutant exhibits hypersensitivity to streptonigrin. *X. oryzae* pv. *oryzae* strains were treated with streptonigrin at 15 μ g ml⁻¹ for 2 h and the viable cell number was estimated by plating bacterial culture before and after treatment. BXO43 = wild-type strain and BXO1801 = *fur1::bla*.

even on PSA-CAS plates. Addition of increasing concentrations of iron in the form of ferrous sulfate (up to 100 μ M) failed to abrogate siderophore production by BXO1801. BXO1802, the *fur*⁺ revertant, behaved 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. Relative intracellular iron was assayed by the streptonigrin sensitivity assay. Streptonigrin is an antibiotic exhibiting iron-dependent catalytic activity resulting in cell death (Ming 2003). BXO1801, when given a 2-h exposure to streptonigrin (15 μ g ml⁻¹), showed an approximately 10- to 100-fold reduction in viability compared with BXO43 (Fig. 3).

BXO1801 is hypersensitive to hydrogen peroxide and exhibits reduced catalase activity.

Another feature of defects in iron regulation is concomitant changes in sensitivity to reactive oxygen species (ROS). 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 (discussed below). BXO43 and BXO1802 exhibited a 10-fold reduction in cell number, whereas BXO1801 showed an almost 1,000-fold reduction in viability

Fig. 4. *Xanthomonas oryzae* pv. *oryzae* *fur* mutant is hypersensitive to hydrogen peroxide. *X. oryzae* pv. *oryzae* 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*, and BXO1802 = *fur*⁺ derivative of BXO1801.

Fig. 5. *Xanthomonas oryzae* pv. *oryzae* *fur* mutant exhibits reduced catalase activity. The *X. oryzae* pv. *oryzae* 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. 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.

after H₂O₂ treatment (Fig. 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 because *X. oryzae* pv. *oryzae* 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 approximately 18

units/mg of protein, which was approximately threefold more than BXO1801 (5.5 units/mg of protein) (Fig. 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. Compared with BXO43 and BXO1802 (*fur*⁺ revertant), the lesion lengths on rice leaves caused by the BXO1801 (*fur* mutant) strain were reduced significantly (approximately 15% of *fur*⁺) 7 days after inoculation (DAI) (Fig. 6). Interestingly, lesions were found to be approximately 50% of the *fur*⁺ strains at 14 DAI. BXO1801 reverted to *fur*⁺ at a frequency of 1:10⁴ *fur*⁺:*fur*⁻ cells (data not shown). To determine whether the increase in lesion length was caused by a selection for *fur*⁺ revertants in planta, bacteria were isolated from leaves inoculated with BXO1801. At 7 DAI, the frequency was 1:10² to 10³ *fur*⁺:*fur*⁻ cells; whereas, at 14 DAI, it changed to 1:1 to 10 *fur*⁺:*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.

Growth curves were measured for BXO43 and BXO1801 in PS medium. BXO1801 exhibited a severe growth deficiency compared with BXO43 (Fig. 7). The relative proportion of *fur*⁺ revertants to *fur*⁻ cells was estimated, and a strong selection for *fur*⁺ revertants was observed. The ratio of *fur*⁺:*fur*⁻ was 1 × 10⁻² cells at the time of inoculation and approximately 1 × 10⁴ after 48 h in PS medium. Between 36 and 48 h after inoculation, a nearly 100-fold reduction in the total number of *fur*⁻ cells in the BXO1801 culture was observed. The BXO1802 strain (*fur*⁺ revertant) behaved similarly to BXO43 in these assays (data not shown).

Fig. 6. *Xanthomonas oryzae* pv. *oryzae fur* mutant is virulence deficient. Virulence assays were done by wound inoculation of leaves of the susceptible rice cv. Taichung Native-1 (TN-1). Lesion lengths were measured 7 and 14 days after inoculation. Mean and standard deviation of 15 replicate measurements of lesion lengths are given. BXO43 = wild-type strain, BXO1801 = *fur*⁻:*bla*, and BXO1802 = *fur*⁺ derivative of BXO1801. Similar results were obtained in independent experiments.

Fig. 7. *Xanthomonas oryzae* pv. *oryzae fur* mutant exhibits a slow growth phenotype in peptone-sucrose (PS) medium which is partially rescued by ascorbic acid supplementation. *X. oryzae* pv. *oryzae* strains were grown in PS at 28°C with or without ascorbic acid (500 μM) and cell numbers were estimated by plating bacterial cultures at intervals. BXO43 PS and BXO43 AA = wild type without or with ascorbic acid, respectively; BXO1801 PS and BXO1801 AA = *fur*⁻:*bla* mutant without or with ascorbic acid, respectively; and BXO1801 revertants PS and BXO1801 revertants AA = *fur*⁺ revertants obtained in the BXO1801 culture without or with ascorbic acid, respectively.

In planta growth defect of the *X. oryzae* pv. *oryzae fur* mutant is rescued by ascorbic acid supplementation.

Detached leaf inoculation experiments were carried out to determine whether the in planta growth defect of BXO1801 could be alleviated by supplementation with ascorbic acid, an antioxidant. Survival of bacteria in rice leaves was monitored by determining the viable cell number of the re-isolated bacteria (Fig. 8). The cell numbers of wild-type and *fur* mutant were similar (approximately 10^3 - to 10^4 CFU ml⁻¹) without or with 500 μ M ascorbic acid supplementation, 1 h after inoculation, indicating equal efficiency in leaf entry. After 48 h, wild-type cell numbers increased by 100- to 1,000-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 approximately 10^6 CFU ml⁻¹ (almost wild-type level) in ascorbic acid-supplemented medium. Thus, a significant increase (approximately 100-fold) was found in the cell numbers of the *fur* mutant upon ascorbic acid supplementation. This experiment demonstrated that, at least in the first 48 h of leaf infection, the growth defect of the *X. oryzae* pv. *oryzae fur* mutant may be corrected by providing ascorbic acid, an antioxidant.

The effect of ascorbic acid supplementation on growth of BXO43 (*fur*⁺) and BXO1801 (*fur*⁻) in PS medium also was assessed. Ascorbic acid had a slight inhibitory effect on growth of BXO43 (Fig. 7). Ascorbic acid improved the growth of BXO1801 because the numbers of cells of BXO1801 were higher in PS and ascorbic acid compared with PS alone (Fig. 7). The reduction in the number of *fur*⁻ mutant cells between 36 and 48 h was less severe in PS and ascorbic acid compared with PS alone. However, even with ascorbic acid supplementation, BXO1801 did not grow as well as the *fur*⁺ strain in PS medium and ascorbic acid only partially alleviated the slow growth phenotype of BXO1801 in PS medium.

DISCUSSION

The regulation of iron uptake is critical for vital cellular functions in an aerobic environment, and this function is carried out by the Fur protein in many bacteria. In *R. leguminosarum*,

Fig. 8. In planta growth deficiency of a *Xanthomonas oryzae* pv. *oryzae fur* mutant is rescued by ascorbic acid supplementation. *X. oryzae* pv. *oryzae* strains were inoculated on leaves dipped in sterile water containing 0.1% glucose without or with ascorbic acid (500 μ M). Bacteria were re-isolated by crushing 1 cm² of the cut end of the leaf and viable cell numbers were estimated after 1 and 48 h. Mean $\pm$ standard deviation of cell numbers obtained from three different leaves are indicated for each strain. BXO43-glc and BXO43-aa = wild type without or with ascorbic acid, respectively; BXO1801-glc and BXO1801-aa = *fur*⁻ mutant without or with ascorbic acid, respectively. Similar results were obtained in independent experiments.

many of the iron-regulated genes do not exhibit Fur-dependent expression and have been shown to be regulated by another protein, RirA (Todd et al. 2002; Wexler et al. 2003). Therefore, iron-dependent expression need not be regulated by Fur in all bacteria. The *fur* mutant of *X. oryzae* pv. *oryzae* 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 *X. oryzae* pv. *oryzae* 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 *X. oryzae* pv. *oryzae* 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* (Carson et al. 1996; Prince et al. 1993; Thomas and Sparling 1996). Although an *X. oryzae* pv. *oryzae 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 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 dimerization activities, respectively (Stojiljkovic and Hantke 1995). The plasmid insertion in the *X. oryzae* pv. *oryzae fur* gene resulted in a truncated *fur* allele in which the first 370 bp of the 411-bp *fur* gene were 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 dimerization activity. A dominant negative *fur* allele of *Bradyrhizobium japonicum* previously has been 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 *X. oryzae* pv. *oryzae fur* mutant).

Similar to *E. coli*, the *X. oryzae* pv. *oryzae fur* mutant is hypersensitive to hydrogen peroxide (Touati et al. 1995) which may be due to reduced catalase activity. In *P. 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 H₂O₂ hypersensitivity. The mechanism by which Fur protein affects catalase expression in *X. oryzae* pv. *oryzae* is not known (Masse and Gottesman 2002; Wilderman et al. 2004).

What might be the reason for reduced pathogenicity of the *X. oryzae* pv. *oryzae fur* mutant? Recent reports suggest that 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 antioxidant 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 antioxidant (Bielski 1982; Horemans et al. 2000). The in planta growth defect of the *X. oryzae* pv. *oryzae* *fur* mutant can be rescued by supplementing with ascorbic acid. This observation suggests that the reduced virulence of the *X. oryzae* pv. *oryzae* *fur* mutant may be due, at least in part, 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 *X. oryzae* pv. *oryzae*. 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) compared with oxidative stress from within the cell (as would be experienced during growth in PS medium). Alternatively, enhanced oxidative stress may be only partially responsible for the slow growth phenotype of the *fur* mutant in PS medium. More studies on the *fur* regulon of *X. oryzae* pv. *oryzae* are needed to understand how Fur controls iron uptake, response to oxidative stress, and growth in laboratory conditions and within the plant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media.

The *X. oryzae* pv. *oryzae* strains used were BXO43 (*rif*-2, rifampicin resistant; laboratory wild-type strain), BXO1801 (*fur1::bla rif*-2, Ap^r), and BXO1802 (*fur*⁺ derivative of BXO1801, Ap^s). These strains were grown at 28°C in 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, U.S.A.). *E. coli* cells were cultured at 37°C in Luria Bertani medium (Miller 1992). The concentrations of antibiotics used were rifampicin (Rf) at 50 µg ml⁻¹, ampicillin (Ap) at 100 µg ml⁻¹, cycloheximide (Cy) at 80 µg ml⁻¹, and cephalixin (Cp) at 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 stock solution at 1 mg ml⁻¹ and the concentration used was 15 µg ml⁻¹.

Generation of a *fur* mutant of *X. oryzae* pv. *oryzae*.

An internal fragment of the *X. oryzae* pv. *oryzae* *fur* gene was amplified using primers FurF1 (5'-GCAGAAGACATC-TATCGCCAG-3') and FurR (5'-GCTCTTCGAGTTCATAGC CG-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. Approximately 500 ng of the plasmid was introduced by electroporation into electrocompetent cells of BXO43 (wild-type strain). The transformants were grown in PS for 3 h and then plated on PSA containing Ap. Integration of the plasmid into the genome of Ap^r colonies was confirmed by PCR using flanking primers FurF (5'-ATGGAA ACCACGACCTGC-3') and FurR1 (5'-GCGCGGACGCTTC TTGC-3') in combination with vector-specific primers M13F and T7. Sequencing of the PCR products was done to confirm integration into the *fur* gene.

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, U.S.A.) as per the supplier's instructions. Inverse PCR to obtain the DNA sequences flanking the *X. oryzae* pv. *oryzae* *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 a 50-µl volume to circularize the DNA fragments. Different aliquots of the ligated product were taken for inverse PCR using the FR1 primer (5'-CGCTGCAGCGCCA-GATTGCC-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, U.S.A.). 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 and Hotta 1999).

Siderophore production assay.

CAS containing siderophore indicator plates was prepared as described by Schwyn and Neilands (1987). *X. oryzae* pv. *oryzae* grows very poorly on minimal medium; therefore, the siderophore indicator plates were made in PS instead of MM9 medium. *X. oryzae* pv. *oryzae* 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 sulfate (100 µM) when required. The colonies were scored for the orange halo phenotype after 36 h.

Sensitivity to hydrogen peroxide and streptonigrin.

X. oryzae pv. *oryzae* 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 then were pelleted and washed once with sterile distilled water before plating to count cell number. The logarithmic value of viable cells per milliliter of culture is indicated as mean ± standard deviation (SD).

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 the wild type. Into 30 ml of 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 7,000 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 a 1-min interval during which the probe was removed from the sample. Tubes containing the samples were always kept on ice. Debris was pelleted by centrifugation at 13,000 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 and 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 was 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 cata-

lase was calculated as follows: $1,000 \times \Delta A_{240} \text{ min}^{-1} / 43.6 \times \text{milligram of protein ml}^{-1}$ of reaction mixture (Visick and Clarke 1997).

Virulence assays on rice plants.

Virulence assays were performed on approximately 40-day-old rice plants of the highly susceptible rice cv. Taichung Native (TN-1) in the greenhouse. Inoculation was done by clipping leaf tips with scissors dipped in bacterial culture (10^8 CFU/ml resuspended in sterile DDW) (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 also were grown in PS + 2,2'-dipyridyl. Inoculated plants were incubated in the greenhouse and lesion lengths were taken 7 and 14 DAI. 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 $\pm$ SD. Similar results were obtained in independent experiments.

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) for 1 min and washing three times 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. The *fur*⁺ revertants were identified by their larger colony size, and these colonies then were patched on PSA-CAS plates for halo phenotype (no halo was detected because these colonies would behave like the 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 also were examined and, as expected, found to have characteristics of the *fur* mutant strain.

Detached leaf inoculation experiment with exogenous ascorbic acid supplementation.

Leaves of 40-day-old greenhouse-grown rice plants of the susceptible rice cv. 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 and Sonti 2002), with or without 500 μ M ascorbic acid (Sigma-Aldrich, St. Louis). The leaves were maintained for 24 h on a laboratory bench top prior to inoculation with different strains of *X. oryzae* pv. *oryzae* by leaf-clip method (Kauffman et al. 1973). At desired time intervals, the infected leaves were surface sterilized as mentioned above. Leaf material (1 cm²) from the point of inoculation was taken, crushed using a mortar and pestle in 1 ml of sterile DDW, and dilution plated on PSA media containing Rf + Cp + Cy. After 3 to 4 days, colonies were counted and mean $\pm$ SD of the logarithmic value of cell number obtained from three different leaves was calculated. Similar results were obtained in independent experiments.

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 500 μ M ascorbic acid, and grown at 28°C and 200 rpm. To determine the cell numbers per milliliter 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 also was done on Ap-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 $\pm$ SD of logarithmic value of cell number per milliliter at different intervals. An estimation of number of *fur*⁺ revertants in the BXO1801 culture was obtained by subtracting the cell numbers obtained on Ap-containing medium from that on PSA medium. A representative number of the Ap^r and Ap^s cells were checked for the siderophore overproduction phenotype. As expected, the Ap^r colonies exhibited excess siderophore secretion whereas the Ap^s colonies were similar to the wild-type strain.

ACKNOWLEDGMENTS

S. Subramoni was supported by a fellowship from the Council of Scientific and Industrial Research (CSIR), Government of India. We thank M. Sultana for primer synthesis and N. Nagesh for DNA sequencing.

LITERATURE CITED

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Bai, J., Choi, S. H., Ponciano, G., Leung, H., and Leach, J. E. 2000. *Xanthomonas oryzae* pv. *oryzae* avirulence genes contribute differently and specifically to pathogen aggressiveness. *Mol. Plant-Microbe Interact.* 13:1322-1329.
- Barton, H. A., Johnson, Z., Cox, C. D., Vasil, A. I., and Vasil, M. L. 1996. Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. *Mol. Microbiol.* 21:1001-1017.
- Beers, R. F., Jr., and Sizer, I. W. 1951. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 196:133-140.
- Benson, H. P., LeVier, K., and Gueriot, M. L. 2004. A dominant-negative *fur* mutation in *Bradyrhizobium japonicum*. *J. Bacteriol.* 186:1409-1414.
- Bielski, B. H. J. 1982. Page 81 in: Ascorbic Acid: Chemistry, Metabolism and Uses. P. A. Seib and B. M. Tolbert, eds. Advances in Chemistry Series 200, American Chemical Society, Washington, D.C.
- Bijlsma, J. J., Waidner, B., Vliet, A. H., Hughes, N. J., Hag, S., Bereswill, S., Kelly, D. J., Vandenbroucke-Grauls, C. M., Kist, M., and Kusters, J. G. 2002. The *Helicobacter pylori* homologue of the ferric uptake regulator is involved in acid resistance. *Infect. Immun.* 70:606-611.
- Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Carson, S. D., Thomas, C. E., and Elkins, C. 1996. Cloning and sequencing of a *Haemophilus ducreyi* *fur* homolog. *Gene* 176:125-129.
- Chamnongpol, S., Mongkolsuk, S., Vattanaviboon, P., and Fuangthong, M. 1995. Unusual growth phase and oxygen tension regulation of oxidative stress protection enzymes, catalase and superoxide dismutase, in the phytopathogen *Xanthomonas oryzae* pv. *oryzae*. *Appl. Environ. Microbiol.* 61:393-396.
- Chatterjee, S., Sankaranarayanan, R., and Sonti, R.V. 2003. PhyA, a secreted protein of *Xanthomonas oryzae* pv. *oryzae*, is required for optimum virulence and growth on phytic acid as a sole phosphate source. *Mol. Plant-Microbe Interact.* 16:973-982.
- Chatterjee, S., and Sonti, R.V. 2002. *rpjF* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. *Mol. Plant-Microbe Interact.* 15:463-471.
- da Silva, A. C., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M., do Amaral, A. M., Bertolini, M. C., Camargo, L. E., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M., Coutinho, L. L., Cursino-Santos, J. R., El-Dorri, H., Faria, J. B., Ferreira, A. J., Ferreira, R. C., Ferro, M. I., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G., Lemos, M. V., Locali, E. C., Machado, M. A., Madeira, A. M., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A., Silva, C., de Souza, R. F., Spinola, L. A., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I., Trindade dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C., and

- Kitajima, J. P. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* 417:459-463.
- Dharmapuri, S., and Sonti, R. V. 1999. A transposon insertion in the *gumG* homologue of *Xanthomonas oryzae* pv. *oryzae* causes loss of extracellular polysaccharide production and virulence. *FEMS (Fed. Eur. Microbiol. Soc.) Lett.* 179:53-59.
- Franza, T., Sauvage, C., and Expert, D. 1999. Iron regulation and pathogenicity in *Erwinia chrysanthemi* 3937: role of the Fur repressor protein. *Mol. Plant-Microbe Interact.* 12:119-128.
- Grifantini, R., Sebastian, S., Frigimelica, E., Draghi, M., Bartolini, E., Muzzi, A., Rappuoli, R., Grandi, G., and Genco, C. A. 2003. Identification of iron-activated and -repressed Fur-dependent genes by transcriptome analysis of *Neisseria meningitidis* group B. *Proc. Natl. Acad. Sci. U.S.A.* 100:9542-9547.
- Hassett, D. J., Sokol, P. A., Howell, M. L., Ma, J. F., Schweizer, H. T., Ochsner, U., and Vasil, M. L. 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. *J. Bacteriol.* 178:3996-4003.
- Horemans, N., Foyer, C. H., and Asard, H. 2000. Transport and action of ascorbate at the plant plasma membrane. *Trends Plant Sci.* 5: 263-267.
- Ishikawa, J., and Hotta, K. 1999. FramePlot: a new implementation of the frame analysis for predicting protein-coding regions in bacterial DNA with a high G + C content. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 174:251-253.
- Kamdar, H. V., Kamoun, S., and Kado, C. I. 1993. Restoration of pathogenicity of avirulent *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* pathovars by reciprocal complementation with *hrpXo* and *hrpXc* genes and identification of HrpX function by sequence analyses. *J. Bacteriol.* 175:2017-2025.
- Kauffman, H. E., Reddy, A. P. K., Hsieh, S. P. Y., and Merca, S. D. 1973. An improved technique for evaluation of resistance of rice varieties to *Xanthomonas oryzae* pv. *oryzae*. *Plant Dis. Rep.* 57:537-541.
- Loprasert, S., Sallabhan, R., Atichartpongkul, S., and Mongkolsuk, S. 1999. Characterization of a ferric uptake regulator (*fur*) gene from *Xanthomonas campestris* pv. *phaseoli* with unusual primary structure, genome organization, and expression patterns. *Gene* 239:251-258.
- Masse, E., and Gottesman, S. 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 99:4620-4625.
- Mila, I., Scalbert, A., and Expert, D. 1996. Iron withholding by plant polyphenols and resistance to pathogens and rots. *Phytochemistry* 42:1551-1555.
- Miller, J. H. 1992. *A Short Course in Bacterial Genetics: A Laboratory Manual for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Ming, L. J. 2003. Structure and function of "metalloantibiotics". *Med. Res. Rev.* 23:697-762.
- Neema, C., Lalluhere, J. P., and Expert, D. 1993. Iron deficiency induced by chrysobactin in saintpaulia leaves inoculated with *Erwinia chrysanthemi*. *Plant Physiol.* 102:967-973.
- Payne, S. M. 1993. Iron acquisition in microbial pathogenesis. *Trends Microbiol.* 1:66-69.
- Postle, K., and Kadner, R. J. 2003. Touch and go: tying TonB to transport. *Mol. Microbiol.* 49:869-882.
- Prince, R. W., Cox, C. D., and Vasil, M. L. 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. *J. Bacteriol.* 175:2589-2598.
- Rajeshwari, R., and Sonti, R. V. 2000. Stationary-phase variation due to transposition of novel insertion elements in *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* 182:4797-4802.
- Ratledge, C., and Dover, L. G. 2000. Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* 54:881-941.
- Ray, S. K., Rajeshwari, R., Sharma, Y., and Sonti, R. V. 2002. A high molecular-weight outer membrane protein of *Xanthomonas oryzae* pv. *oryzae* exhibits similarity to non-fimbrial adhesions of animal pathogenic bacteria and is required for optimum virulence. *Mol. Microbiol.* 46:637-647.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
- Schwyn, B., and Neilands, J. B. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160:47-56.
- Stojiljkovic, I., and Hantke, K. 1995. Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). *Mol. Gen. Genet.* 247: 199-205.
- Tang, J.-L., Feng, J.-X., Li, Q.-Q., Wen, H.-X., Zhou, D.-L., Wilson, T. J. G., Dow, J. M., Ma, Q.-S., and Daniels, M. J. 1996. Cloning and characterization of the *rpfC* gene of *Xanthomonas oryzae* pv. *oryzae*: Involvement in exopolysaccharide production and virulence to rice. *Mol. Plant-Microbe Interact.* 9:664-666.
- Tatusova, T. A., and Madden, T. L. 1999. Blast 2 sequences—A new tool for comparing protein and nucleotide sequences. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 174:247-250.
- Thomas, C. E., and Sparling, P. F. 1996. Isolation and analysis of a *fur* mutant of *Neisseria gonorrhoeae*. *J. Bacteriol.* 178:4224-4232.
- Thompson, D. K., Beliaev, A. S., Giometti, C. S., Tollaksen, S. L., Khare, T., Lies, D. P., Nealson, K. H., Lim, H., Yates, J., III., Brandt, C. C., Tiedje, J. M., and Zhou, J. 2002. Transcriptional and proteomic analysis of a ferric uptake regulator (*fur*) mutant of *Shewanella oneidensis*: possible involvement of *fur* in energy metabolism, transcriptional regulation, and oxidative stress. *Appl. Environ. Microbiol.* 68:881-892.
- Todd, J. D., Wexler, M., Sawers, G., Yeoman, K. H., Poole, P. S., and Johnston, A. W. 2002. RirA, an iron-responsive regulator in the symbiotic bacterium *Rhizobium leguminosarum*. *Microbiology* 148:4059-4071.
- Touati, D. 2000. Iron and oxidative stress in bacteria. *Arch. Biochem. Biophys.* 373:1-6.
- Touati, D., Jacques, M., Tardat, B., Bouchard, L., and Despied, S. 1995. Lethal oxidative damage and mutagenesis are generated by iron in delta *fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. *J. Bacteriol.* 177:2305-2314.
- Tsuchiya, K., Mew, T. W., and Wakimoto, S. 1982. Bacteriological and pathological characteristics of wild-type and induced mutants of *Xanthomonas campestris* pv. *oryzae*. *Phytopathology* 72:43-46.
- Vasil, M. L., and Ochsner, U. A. 1999. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol. Microbiol.* 34:399-413.
- Velayudhan, J., Hughes, N. J., McColm, A. A., Bagshaw, J., Clayton, C. L., Andrews, S. C., and Kelly, D. J. 2000. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol. Microbiol.* 37:274-286.
- Venisse, J. S., Barny, M. A., Paulin, J. P., and Brisset, M. N. 2003. Involvement of three pathogenicity factors of *Erwinia amylovora* in the oxidative stress associated with compatible interaction in pear. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 537:198-202.
- Visick, J. E., and Clarke, S. 1997. RpoS- and OxyR-independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of *rpoS* mutations in common laboratory strains. *J. Bacteriol.* 179:4158-4163.
- Weinberg, E. D. 1984. Iron withholding: a defense against infection and neoplasia. *Physiol. Rev.* 64:65-102.
- Wexler, M., Todd, J. D., Kolade, O., Bellini, D., Hemmings, A. M., Sawers, G., and Johnston, A. W. 2003. Fur is not the global regulator of iron uptake genes in *Rhizobium leguminosarum*. *Microbiology* 149:1357-1365.
- Wiggerich, H. G., Klauke, B., Koplin, R., Priefer, U. B., and Puhler, A. 1997. Unusual structure of the *tonB-exb* DNA region of *Xanthomonas campestris* pv. *campestris*: *tonB*, *exbB*, and *exbD1* are essential for ferric iron uptake, but *exbD2* is not. *J. Bacteriol.* 179:7103-7110.
- Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P. C., Gottesman, S., Ochsner, U. A., and Vasil, M. L. 2004. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc. Natl. Acad. Sci. U.S.A.* 101:9792-9797.
- Yang, B., Zhu, W., Johnson, L. B., and White, F. F. 2000. The virulence factor AvrXa7 of *Xanthomonas oryzae* pv. *oryzae* is a type III secretion pathway-dependent nuclear-localized double-stranded DNA-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 97:9807-9812.
- Zhu, W., MaGbanua, M. M., and White, F. F. 2000. Identification of two novel *hrp*-associated genes in the *hrp* gene cluster of *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* 182:1844-1853.

AUTHOR-RECOMMENDED INTERNET RESOURCES

National Centre for Biotechnology Information website:
www.ncbi.nlm.nih.gov
 National Institutes of Health Frame Plot 2.3.2 website: www.nih.gov/jp