รายงานวิจัยฉบับสมบูรณ์

โครงการ "การสร้างและวิเคราะห์ทางสรีรวิทยาของแบคทีเรีย *Agrobacterium tumefaciens* ที่สูญเสียยีน *fur* ต่อการตอบสนองต่อสภาวะ oxidative stress"

โดย ดร. รจนา สุขชวลิต

เดือน มิถุนายน พ.ศ. 2549

สัญญาเลขที่ TRG4780004

รายงานวิจัยฉบับสมบูรณ์

โครงการ "การสร้างและวิเคราะห์ทางสรีรวิทยาของแบคทีเรีย *Agrobacterium tumefaciens* ที่สูญเสียยีน *fur* ต่อการตอบสนองต่อสภาวะ oxidative stress"

โดย

ดร. รจนา สุขชวลิต

ห้องปฏิบัติการเทคโนโลยีชีวภาพ

สถาบันวิจัยจุฬาภรณ์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Acknowledgements

I am very grateful to Professor Skorn Mongkolsuk for encouragement and valuable advice. I wish to thank Mrs Worawan Kitphati, Miss Patchara Ngok-ngam, and Miss Sukanya Suwanmaneerat for performing several experiments. Chulabhorn Research Institute is gratefully acknowledged. I would like to thank the Thailand Research Fund for financial support.

บทคัดย่อ

รหัสโครงการ TRG478004

ชื่อโครงการ การสร้างและวิเคราะห์ทางสรีรวิทยาของแบคทีเรีย *Agrobacterium tumefaciens* ที่สูญเสียยีน *fur* ต่อการตอบสนองต่อสภาวะ oxidative stress

ชื่อนักวิจัย ดร. รจนา สุขชวลิต

E-mail addresss rojana@tubtim.cri.or.th

ระยะเวลาโครงการ 2 ปี

แบคทีเรียกลายพันธุ์ *Agrobacterium tumefaciens* ที่สูญเสียยีน *fur* ได้ถูกสร้างขึ้น เพื่อศึกษาบทบาทและความสำคัญของยีน *fur* (ferric uptake regulator) ของแบคทีเรีย *A. tumefaciens* ต่อการตอบสนองต่อสภาวะ oxidative stress และโลหะ ผลการทดสอบความไวของแบคทีเรีย Fur $^-$ mutant ต่อสาร oxidants ได้แก่ H_2O_2, menadione และ *tert*-butyl hydroperoxide พบว่าแบคทีเรีย Fur $^-$ mutant มีความไวเพิ่มขึ้นต่อสารoxidant ทั้งสามชนิด เมื่อเปรียบเทียบกับแบคทีเรียสายพันธุ์ปกติ (wild-type NTL4) และเมื่อทดสอบความไวต่อโลหะได้แก่ $FeCl_3$, $MnCl_2$, $ZnSO_4$ และ $NiCl_3$ พบว่าแบคทีเรีย Fur $^-$ mutant มีความไวเพิ่มขึ้นต่อโลหะ $FeCl_3$, $MnCl_2$ และ $ZnSO_4$ แต่มีความต้านทานเพิ่มขึ้นต่อโลหะ $NiCl_3$ เมื่อเปรียบเทียบกับ wild-type NTL4 เอนไซม์ catalase (KatA) เป็นเอนไซม์ที่แบคทีเรียสร้างขึ้นเพื่อสลาย H_2O_2 ให้เป็นน้ำและออกซิเจนและมีบทบาทสำคัญต่อกลไกของ plant-pathogen interaction เนื่องจาก H_2O_2 เป็นสารพิษที่พืชสร้างขึ้นเพื่อยับยั้งแบคทีเรียที่มารุกราน ผลการวิเคราะห์พบว่าระดับเอนไซม์ catalase ใน Fur $^-$ mutant มีระดับต่ำกว่าในแบคทีเรียสายพันธุ์ปกติ ซึ่งสอดคล้องกับผลการทดสอบความไวต่อ H_2O_2 นอกจากนี้จากการทดสอบโดยวิธี promoter probe-*lacZ* fusion พบว่าการที่เอนไซม์ catalase ใน Fur $^-$ mutant มีระดับลดลงเนื่องมาจากการแสดงออกของยีน *katA* ลดลงในระดับ transcription แม้แบคทีเรีย Fur $^-$ mutant จะมีความไวเพิ่มขึ้นต่อสาร menadione ซึ่งเป็นสารที่ก่อให้เกิดอนุมูลอิสระจำพวก superoxide anion แต่กลับพบว่าไม่มีความเปลี่ยนแปลงของระดับเอนไซม์ superoxide dismutase ซึ่งเป็นเอนไซม์ที่มีหน้าที่ในการทำลายสารอนุมูลอิสระจำพวก superoxide anion ดังนั้นชี้ให้เห็นว่าแบคทีเรีย *A. tumefaciens* มีกลไกอื่นนอกเหนือจากเอนไซม์ superoxide dismutaseในการต้านทานต่อสารอนุมูลอิสระจำพวก superoxide anion และกลไกนี้ถูกควบคุมโดยยีน *fur* ผลการทดสอบการก่อพยาธิสภาพในพืชพบว่าแบคทีเรีย Fur $^-$ mutant มีความสามารถลดลงในการก่อให้เกิด tumor ในพืชใบยาสูบเมื่อเปรียบเทียบกับสายพันธุ์ปกติผลการวิจัยครั้งนี้แสดงให้เห็นถึงความสำคัญของยีน *A. tumefaciens fur* ต่อการตอบสนองต่อสภาวะ oxidative stress และโลหะ ซึ่งมีผลต่อ virulence ของแบคทีเรียชนิดนี้ และการก่อพยาธิสภาพในพืช

คำหลัก: *fur*, metal response, oxidative stress, virulence

Abstract

Project Code: TRG478004

Project Title: Construction and physiological analysis of *Agrobacterium tumefaciens fur* mutant in response to oxidative stress

Investigator: Dr. Rojana Sukchawalit

E-mail addresss rojana@tubtim.cri.or.th

Project Period: 2 years

To determine the roles of *fur* in the response of *A. tumefaciens* to oxidative stress and metal responses, a *fur* mutant (Fur $^-$) was constructed. Sensitivity of Fur $^-$ mutant to oxidants (H_2O_2, MD: menadione, tBOOH: *tert*-butyl hydroperoxide) and metals ($FeCl_3$, $MnCl_2$, $ZnSO_4$, $NiCl_3$) compared to wild-type NTL4 were determined. Fur $^-$ mutant was more sensitive to oxidant compounds, including peroxide (H_2O_2), superoxide (MD), and organic hydroperoxide (tBOOH), than wild-type NTL4. Fur $^-$ mutant showed increased sensitivity to iron, manganese, and zinc whereas an increase in nickel resistance was observed. Catalase encoded by *katA* is known to detoxify H_2O_2. The results showed that Fur $^-$ mutant had lower catalase activity compared to that of wild-type NTL4, which is in accord with the results of H_2O_2 sensitivity. Furthermore, promoter probe-*lacZ* fusion assays showed that decrease in catalase level of Fur $^-$ mutant was due to decreased transcription of *katA*. Although Fur $^-$ mutant showed increased sensitivity to menadione, a superoxide generator, relative to wild-type NTL4, but levels of SOD, an important enzyme in detoxifying superoxide radicals, in both wild-type NTL4 and Fur $^-$ mutant were similar. This suggested that, as yet unidentified, Fur-regulated mechanism(s) are involved in conferring menadione resistance in *A. tumefaciens*. Tumor formation assay on tobacco leaf showed that Fur $^-$ mutant was highly attenuated in tumor-inducing ability compared to those of wild-type NTL4. In this study, *A. tumefaciens fur* has been shown to play an important role in resistance to metals, oxidative stress and its full virulence.

Keywords: *fur*, metal response, oxidative stress, virulence

สารบัญ

หน้า

"The *Agrobacterium tumefaciens* Fur-like protein, an iron and manganese-sensing regulator, is essential for survival under iron-limiting condition, oxidative stress defense, and its full virulence"

การสร้างและวิเคราะห์ทางสรีรวิทยาของแบคทีเรีย *Agrobacterium tumefaciens* ที่สูญเสียยีน *fur* ต่อการตอบสนองต่อสภาวะ oxidative stress

1. บทนำ

แบคทีเรีย *Agrobacterium* เป็นสาเหตุสำคัญของการก่อโรคในพืชใบเลี้ยงคู่ ซึ่งก่อให้เกิดความเสียหายในพืชเศรษฐกิจ เช่น ใบยาสูบ มะเขือเทศ การก่อให้เกิดโรคในพืชได้นั้นเนื่องมาจากแบคทีเรียสามารถที่จะทำลายสารพิษที่พืชสร้างขึ้นเพื่อป้องกันตนเองจากการรุกรานของแบคทีเรีย สารพิษที่พืชสร้างขึ้นคือ Reactive Oxygen Species (ROS) เช่น H_2O_2, superoxides และ organic peroxides [1] แบคทีเรียมีกลไกทำลายล้างสารพิษเหล่านี้ได้ด้วยการผลิตเอนไซม์ catalase (Kat), superoxide dismutase (SOD) และ alkylhydroperoxide reductase (AhpCF) [2] ยีน *fur* (Ferric Uptake Regulator) มีบทบาทสำคัญในการควบคุมการนำเหล็กเข้าเซลแบคทีเรียโดยเป็นตัวยับยั้งการนำเข้าเหล็กในสภาวะที่ภายในเซลมีเหล็กมากเพียงพอแล้ว[3] เหล็กเป็นธาตุที่จำเป็นต่อการเจริญเติบโต แต่หากมีปริมาณมากเกินความจำเป็นก็จะเป็นอันตรายต่อเซลได้เช่นกัน โดยสามารถก่อให้เกิดสารROS [4] ดังนั้นแบคทีเรียจึงต้องมีการควบคุมระดับของเหล็กให้อยู่ปริมาณที่เหมาะสมตลอดเวลา การศึกษาในแบคทีเรีย *Escherichia coli* และ *Burkholderia pseudomallei* พบว่าโปรตีนFur มีหน้าที่ควบคุมการตอบสนองต่อสภาวะ oxidative stress โดยทำหน้าที่เป็นตัวควบคุมการแสดงออกของเอนไซม์SOD และ KatA [5,6] ได้มีรายงานความสำคัญของเอนไซม์ KatA ต่อการก่อโรคของแบคทีเรีย *Agrobacterium* [7,8] ดังนั้นการศึกษาบทบาทของยีน *fur* ต่อการตอบสนองต่อสภาวะ oxidative stress จะเป็นประโยชน์อย่างยิ่งต่อการเข้าใจกลไกของ plant-pathogen interaction

2. วัตถุประสงค์ของโครงการ

2.1 สร้างแบคทีเรียกลายพันธุ์ *Agrobacterium tumefaciens* ที่สูญเสียยีน *fur*

2.1 ทดสอบความต้านทานต่อสาร oxidants และโลหะ ของ *fur* mutant strain เปรียบเทียบกับwild-type strain

2.3 วิเคราะห์ปริมาณเอนไซม์ catalase และ SOD ของ *fur* mutant strain เปรียบเทียบกับwild-type strain

2.4 ศึกษาบทบาทของยีน*fur* ต่อ promoter activity ของยีน *kat*A และ *sod*

2.5 ทดสอบความสามารถในการก่อพยาธิสภาพในพืชของแบคทีเรีย *fur* mutant strain เปรียบเทียบกับ wild-type strain

3 สรุปย่อ

แบคทีเรียกลายพันธุ์ *Agrobacterium tumefaciens* ที่สูญเสียยีน *fur* ได้ถูกสร้างขึ้น เพื่อศึกษาบทบาทและความสำคัญของยีน *fur* (ferric uptake regulator) ของแบคทีเรีย *A. tumefaciens* ต่อการตอบสนองต่อสภาวะ oxidative stress และโลหะ ผลการทดสอบความไวของแบคทีเรีย Fur⁻ mutant ต่อสาร oxidants ได้แก่ H_2O_2, menadione และ *tert*-butyl hydroperoxide พบว่าแบคทีเรีย Fur⁻ mutant มีความไวเพิ่มขึ้นต่อสารoxidant ทั้งสามชนิดเมื่อเปรียบเทียบกับแบคทีเรียสายพันธุ์ปกติ (wild-type NTL4) และเมื่อทดสอบความไวต่อโลหะได้แก่ $FeCl_3$, $MnCl_2$, $ZnSO_4$ และ $NiCl_3$ พบว่าแบคทีเรีย Fur⁻ mutant มีความไวเพิ่มขึ้นต่อโลหะ $FeCl_3$, $MnCl_2$ และ $ZnSO_4$ แต่มีความต้านทานเพิ่มขึ้นต่อโลหะ $NiCl_3$ เมื่อเปรียบเทียบกับ wild-type NTL4 นอกจากนี้ยังพบว่าสามารถทำให้แบคทีเรีย Fur⁻ mutant กลับมามีความไวและต้านทานต่อสารoxidant และโลหะ ในระดับเดียวกับ wild-type NTL4 ได้ โดยการถ่ายทอดพลาสมิดซึ่งมีการแสดงออกของยีน *fur* ปกติเข้าไปในแบคทีเรีย Fur⁻ mutant และเป็นการยืนยันได้ว่าการเปลี่ยนแปลงความไวต่อสารoxidant และโลหะของแบคทีเรีย Fur⁻ mutant ที่แตกต่างไปจากแบคทีเรียสายพันธุ์ปกติเกิดเนื่องมาจากการที่สูญเสียยีน *fur* เอนไซม์ catalase (KatA) เป็นเอนไซม์ที่แบคทีเรียสร้างขึ้นเพื่อสลาย H_2O_2 ให้เป็นน้ำและออกซิเจนและมีบทบาทสำคัญต่อกลไกของ plant-pathogen interaction เนื่องจาก H_2O_2 เป็นสารพิษที่พืชสร้างขึ้นเพื่อยับยั้งแบคทีเรียที่มารุกราน จากการที่พบว่าแบคทีเรีย Fur⁻ mutant มีความไวเพิ่มขึ้นต่อ H_2O_2 จึงได้ทำการวิเคราะห์หาปริมาณเอนไซม์ catalase ใน Fur⁻ mutant เปรียบเทียบกับwild-type NTL4 เพื่อศึกษาว่าการสูญเสียยีน *fur* มีผลต่อระดับเอนไซม์ catalase ของแบคทีเรียหรือไม่ ผลการวิเคราะห์พบว่าระดับเอนไซม์ catalase ใน Fur⁻ mutant มีระดับต่ำกว่าในแบคทีเรียสายพันธุ์ปกติ ซึ่งสอดคล้องกับผลการทดสอบความไวต่อ H_2O_2 นอกจากนี้จากการทดสอบโดยวิธี promoter probe-*lacZ* fusion พบว่าการที่เอนไซม์ catalase ใน Fur⁻ mutant มีระดับลดลงเนื่องมาจากการแสดงออกของยีน *katA* ลดลงในระดับ transcription แม้แบคทีเรีย Fur⁻ mutant จะมีความไวเพิ่มขึ้นต่อสาร menadione ซึ่งเป็นสารที่ก่อให้เกิดอนุมูลอิสระจำพวก superoxide anion แต่กลับพบว่าไม่มีความเปลี่ยนแปลงของระดับเอนไซม์ superoxide dismutase ซึ่งเป็นเอนไซม์ที่มีหน้าที่ในการทำลายสารอนุมูลอิสระจำพวก superoxide anion ดังนั้นชี้ให้เห็นว่าแบคทีเรีย *A. tumefaciens* มีกลไกอื่นนอกเหนือจากเอนไซม์ superoxide dismutaseในการต้านทานต่อสารอนุมูลอิสระจำพวก superoxide anion และกลไกนี้ถูกควบคุมโดยยีน *fur* ผลการทดสอบการก่อพยาธิสภาพในพืชพบว่า แบคทีเรีย Fur⁻ mutant มีความสามารถลดลงในการก่อให้เกิด tumor ในพืชใบยาสูบเมื่อเปรียบเทียบกับสายพันธ์ปกติผลการวิจัยครั้งนี้แสดงให้เห็นถึงความสำคัญของยีน *A. tumefaciens fur* ต่อการตอบสนองต่อสภาวะ oxidative stress และโลหะ ซึ่งมีผลต่อ virulence ของแบคทีเรียชนิดนี้ และการก่อพยาธิสภาพในพืช

4. ผลงานวิจัยและรายละเอียดวิธีวิจัย

4.1 Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed and described in Table 1. *Agrobacterium tumefaciens* and the *fur* mutant (Gm^r: 90 μg ml^{-1}) strains were grown aerobically in LB medium at 28°C with shaking at 150 rpm. *Escherichia coli* strains were grown in aerobically LB medium at 37°C supplemented with 30 μg ml^{-1}gentamicin (Gm), 15 μg ml^{-1} tetracycline (Tet) or 100 μg ml^{-1} ampicillin (Ap) as required.

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
Agrobacterium tumefaciens		
NTL4	Wild-type	[9]
Fur$^-$	*fur* mutant, derivative of NTL4 in which *fur* was disrupted by pKNOCKfur; Gm^r	This study
Escherichia coli		
DH5α	*supE Dlac(f80ZDM15) hsdR recA endA gyrA thi relA*	Laboratory collection
Plasmids		
pGEM-T-easy	a cloning vector; Ap^r	Promega
pKNOCK-Gm	a suicide vector; Gm^r	[10]
pKNOCKfur	pKNOCK-Gm containing a 169 bp *Bam*HI-*Eco*RI fragment of *fur* coding region; Gm^r	This study
pBBR1MCS-4	an expression vector; Ap^r	[11]
pFur	full length *fur* coding region cloned into pBBR1MCS-4; Ap^r	This study
pUFR047*lacZ*	a promoter probe vector; Tet^r	[12]
pP_{katA}	a 330 bp PCR fragment containing *katA* promoter cloned into pUFR047*lacZ*; Tet^r	[13]
pP_{sod}	a 420 bp PCR fragment containing *sod* promoter cloned into pUFR047*lacZ*; Tet^r	Saenkham, P.
pTiC58	Nopaline Ti plasmid, Ap^r, Km^r	Nester, E.W.

4.2 Construction of an *A. tumefaciens fur* mutant

To determine the role of *fur* gene, a *fur* mutant was constructed by insertional inactivation of the *fur* gene on the chromosome by single homologous recombination. The primers BT772-5'TCAGGAATCAGCCGATCATC3' and BT773-5'ATGACCACGCTGTTCTTCAG3' designed from the sequence of a putative *fur* gene, identified from the *A. tumefaciens* C58 genome sequence [14], were used to amplified a 219 bp fragment of *fur* coding region using Taq DNA polymerase and *A. tumefaciens* NTL4 genomic DNA as templates. The PCR product was cloned into pGEM-T-easy vector (Promega) and its nucleotide sequence was confirmed by automated DNA sequencing. Subsequently, the 169 bp *Bam*HI-*Eco*RI fragment of the PCR clone was filled in with the Klenow enzyme and subcloned into pKNOCK-Gm [10], a non-replicative plasmid in *Agrobacterium*, digested with the unique *Sma*I. The resultant plasmid, pKNOCKfur (2.16 Kb), was then transferred to *A. tumefaciens* by conjugation. Recombination of the cloned *fur* fragment in the suicide plasmid with the homologous counterpart on *A. tumefaciens* chromosome resulted in the disruption of the *fur* gene. The *fur* mutant was selected on LB agar containing 25 μg ml^{-1} chloramphenicol and 90 μg ml^{-1} gentamicin. To confirm the *fur* mutant, Southern blot analysis was performed using standard protocol [15]. Chromosomal DNA from *fur* mutant and wild-type *A. tumefaciens* NTL4 were digested with *Sph*I, separated and blotted on a nylon membrane. The blot was hybridized to 169 bp *Bam*HI-*Eco*RI radioactively labeled *fur* probes. As shown in Figure 1, the single hybridizing band of 0.86 Kb was obtained from wild-type NTL4 as expected from the genomic sequence. Whereas, there is an increase of 2.16 Kb in the hybridizing band in the *fur* mutant (Fur $^{-}$) confirming that pKNOCKfur had correctly integrated into *fur*.

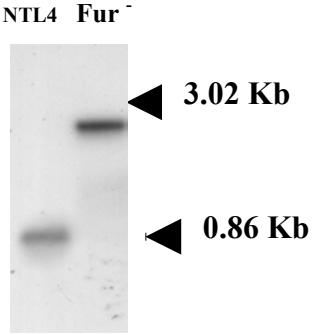


Figure 1. Southern blot analysis of *A. tumefaciens* wild-type NTL4 and Fur $^{-}$ mutant. Numbers to the right indicate the molecular size of positively hybridized bands.

4.3 Determination of oxidant and metal resistance

To investigate the physiological role of *fur* in the *Agrobacterium* oxidative stress and metal responses, sensitivity of Fur⁻ mutant to oxidants (H_2O_2, MD: menadione, tBOOH: *tert*-butyl hydroperoxide) and metals ($FeCl_3$, $MnCl_2$, $ZnSO_4$, $NiCl_3$) compared to wild-type NTL4 were determined. Cells were grown in LB medium with appropriate antibiotics and incubated at 28°C for 24 h with shaking. Stationary phase cells (approximately 10^8) were then resuspended in 1 ml of LB broth. Five 10-fold serial dilutions were done. An aliquot (10 µl) of each dilution was spotted on LB agar containing tested concentrations of oxidant or metal compounds. The plates were incubated at 28°C for 48 h before the growth was recorded. All experiments were performed three times and representative data are shown in Fig. 2 and 3. The results showed that Fur ⁻ mutant was more sensitive to H_2O_2, MD, and tBOOH (Fig. 2B, C, and D respectively) than wild-type NTL4. To confirm that the observed phenotype was due to the inactivation of *fur* gene, complementation assays were performed. The full-length of wild-type *fur* gene was amplified from *A. tumefaciens* NTL4 genomic DNA with primers BT692-5'CCAGAAGACGTGATAGACCT3' and BT693-5'CGGCGTCTCAGCGTTCTTCG3' using Pfu DNA polymerase (Promega). The 438 bp PCR product was cloned into the unique *Sma*I site of an expression vector pBBR1MCS-4 [11], creating the recombinant plasmid, pFur. Its nucleotide sequence was confirmed by automated DNA sequencing. The plasmid pFur was then transferred into wild-type NTL4 and Fur⁻ mutant strains by electroporation. As shown in Fig. 2, expression of the functional *fur* in the Fur ⁻ mutant was able to complement the H_2O_2, MD, and tBOOH sensitive phenotypes. Since the extent of the sensitivity of the complemented strain (Fur ⁻/ pFur) was similar to that of wild-type NTL4. These data confirmed that disruption of *fur* was responsible for the hypersensitive phenotype to oxidant compounds, indicating an important role for *fur* in resistance to oxidative damage.

Fur has been reported to repress iron uptake in many bacteria, sensitivity of Fur ⁻ mutant to metals was also determined (Fig. 3). Fur ⁻ mutant had increased sensitivity to $FeCl_3$, $MnCl_2$, $ZnSO_4$ (Fig. 3B, C, and D respectively) whereas showed higher resistance to $NiCl_3$ (Fig. 3E) compared to those of wild-type NTL4. The increased sensitivity to iron may be due to the excess intracellular iron which resulted from the upregulated influx of iron by the mutant. The altered intracellular iron content might have an effect on differential expression of other metal transports, as a result changes in manganese, zinc and nickel resistance of Fur ⁻ mutant compared to wild-type NTL4. High level expression of

Fur from the expression vector (Fur $^{-}$/ pFur) could complement iron, manganese, and zinc sensitive phenotypes, in addition, conferred increased sensitivity to $NiCl_3$. These data demonstrated an important role of *A. tumefaciens fur* in the metal response.

Figure 2. Determination of oxidant resistance

Stationary phase cells of wild-type NTL4, *fur* mutant (Fur $^{-}$), and *fur* mutant complemented with functional *fur* on the expression vector (Fur $^{-}$/ pFur), were diluted (five 10-fold serial dilutions). An aliquot (10 µl) of each dilution was spotted on LB agar plates containing 450 µM H_2O_2 (B), 500 µM MD: menadione (C) or 600 µM tBOOH: *tert*-butyl hydroperoxide (D). Plates were incubated at 28^{o}C for 48 h.

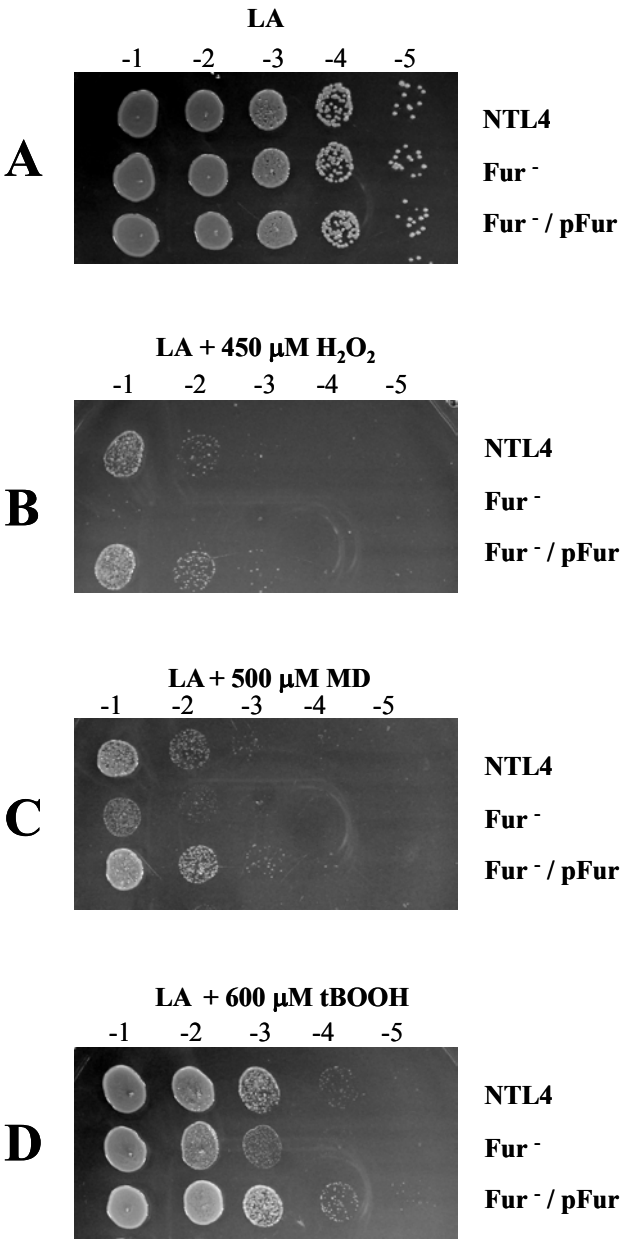
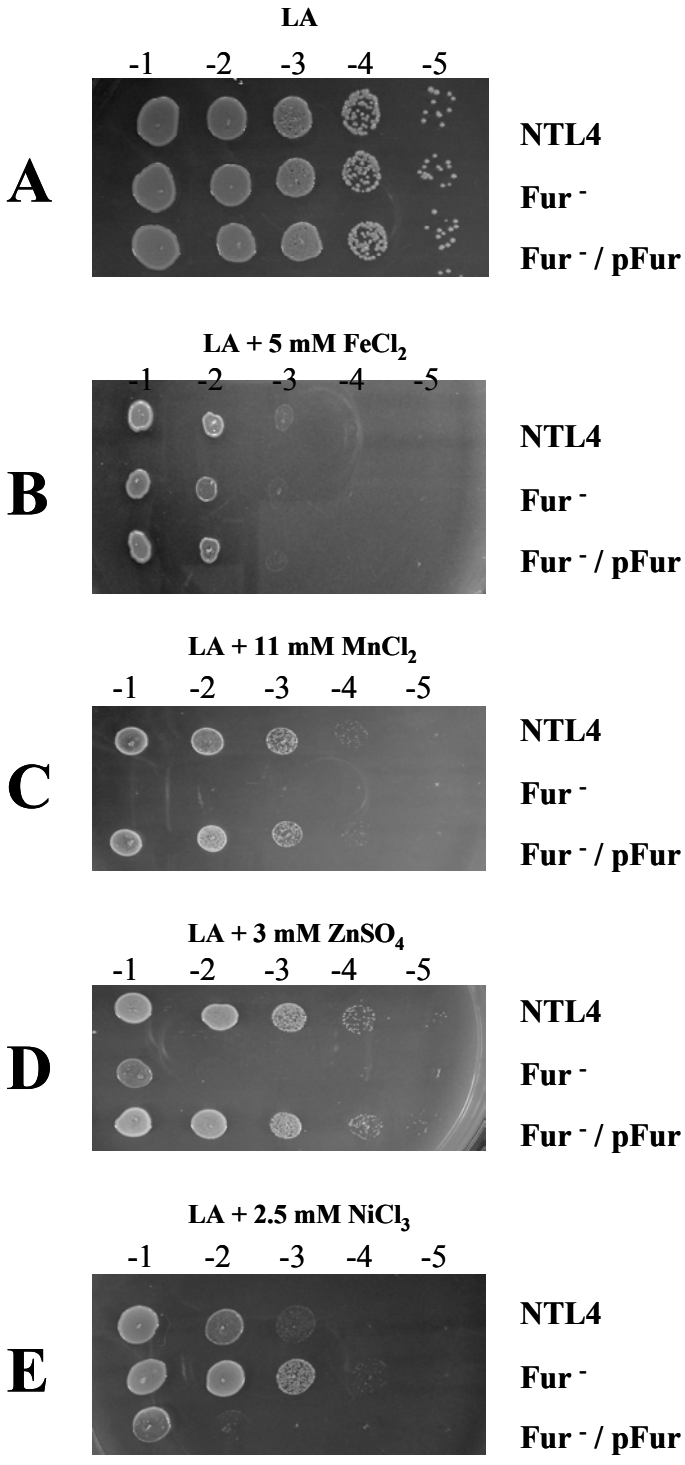


Figure 3. Determination of metal resistance

Stationary phase cells of wild-type NTL4, *fur* mutant (Fur $^-$), and *fur* mutant complemented with functional *fur* on the expression vector (Fur $^-$/ pFur), were diluted (five 10-fold serial dilutions). An aliquot (10 μl) of each dilution was spotted on LB agar plates containing 5 mM $FeCl_3$ (B), 11 mM $MnCl_2$ (C), 3 mM $ZnSO_4$ (D), or 2.5 mM $NiCl_3$ (E). Plates were incubated at 28^{o}C for 48 h.



4.4 Catalase activity assay

Catalase encoded by *katA* is an important enzyme in detoxifying H_2O_2. Due to the fact that the Fur $^-$ mutant was more sensitive to H_2O_2 than wild-type NTL4 suggested that *fur* might be involved in controlling the catalase level. Catalase activity assays were performed to determine the total catalase levels in wild-type NTL4 and Fur $^-$ mutant. Cells were grown on LB agar plates at 28^{o}C for 2 days. The cells were harvested and washed once with 50 mM sodium phosphate buffer pH 7.0 (PB). Bacterial suspensions in PB containing 1 mM PMSF, a protease inhibitor, were lysed by brief sonication followed by centrifugation at 12,000 *g* for 10 min. Clear lysates were used for catalase assay and total protein determination. Protein concentrations were determined using Bradford BIO-RAD protein assay. Catalase activity was monitored by the decomposition of H_2O_2 at A_{240} [16]. One unit of catalase was defined as the amount of enzyme capable of catalyzing the turnover of 1 μmole of substrate per minute under the assay condition. As shown in Fig 4, Fur $^-$ mutant had lower catalase activity about 30% compared to that of wild-type NTL4. These indicate that an increase in sensitivity to H_2O_2 of Fur $^-$ mutant is due to the reduction in catalase activity.

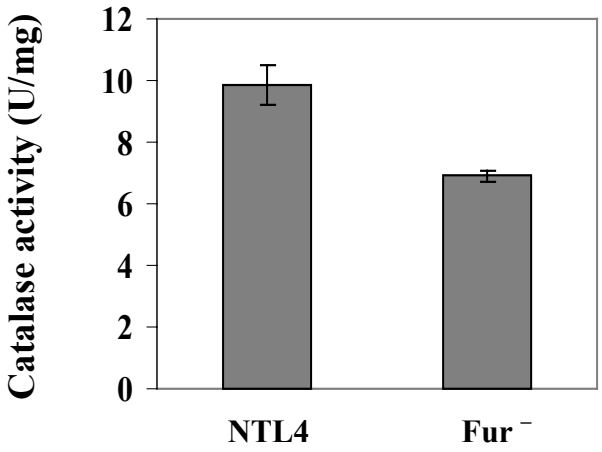


Figure 4. **Catalase activities of wild-type NTL4 and Fur $^-$ mutant.**
Stationary phase cells were harvested from cultures on LB agar plates incubation at 28^{o}C for 2 days. Activity was determined by the method of Beers and Sizer (1952). The results are the means and standard errors of three replicates.

4.5 *katA* promoter activity assay

In order to analyze the expression of *katA* and possible regulation by Fur, the *katA* promoter region was transcriptionally fused to a promoter-less *lacZ* in a low-copy number plasmid vector, pUFR027*lacZ*, creating the recombinant plasmid pP_{katA} [13]. The plasmid, pP_{katA} was transferred into wild-type NTL4 and *fur* mutant strains. Cell lysates were prepared as above. β-galactosidase activity assays were performed as described [17]. The β-galactosidase activity from Fur $^-$ mutant was decreased about 20% compared to those of wild-type NTL4 (Fig. 5). The data confirmed that decrease in catalase level of Fur $^-$ mutant resulted from decreased *katA* expression. This suggests that *katA* expression is positively regulated by Fur, either directly or indirectly.

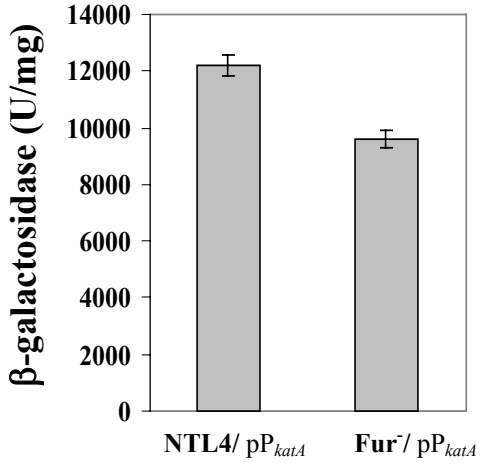


Figure 5. **Expression of *A. tumefaciens katA–lacZ* fusion in wild-type NTL4 and Fur $^-$ mutant.** β-galactosidase activities were measured from wild-type NTL4 or Fur $^-$ mutant containing the *katA–lacZ* fusion plasmid pP_{katA}. Values are means and standard errors of three replicates.

4.6 SOD activity assay

Superoxide dismutase (SOD) encoded by *sod* is an important enzyme in detoxifying superoxide radicals. Due to the fact that the Fur $^{-}$ mutant was more sensitive to menadione (a superoxide generator) than wild-type NTL4 suggested that *fur* might be involved in controlling the SOD level. SOD activity assays were performed to determine the total SOD levels in wild-type NTL4 and Fur $^{-}$ mutant. Cells were grown on LB agar plates at 28^{o}C for 2 days. The cells were harvested and washed once with 50 mM sodium phosphate buffer pH 7.0 (PB). Bacterial suspensions in PB containing 1 mM PMSF, a protease inhibitor, were lysed by brief sonication followed by centrifugation at 12,000 *g* for 10 min. Clear lysates were used for SOD assay and total protein determination. Protein concentrations were determined using Bradford BIO-RAD protein assay. To visualize SOD activity on gel, non-denaturating electrophoresis was performed and stained with nitroblue tetrazolium/riboflavin photochemical stain [18]. Xanthine-xanthine oxidase coupled reduction of cytochrome c was used to monitor SOD activity. One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50% [19]. The results showed that Fur $^{-}$ mutant produced level of SOD that was similar to those of wild-type NTL4 as judged by SOD activity gel staining (Fig. 6A) and total SOD activity assay (Fig. 6B). This indicated that increased-menadione sensitivity of Fur $^{-}$ mutant was not involved in the levels of SOD.

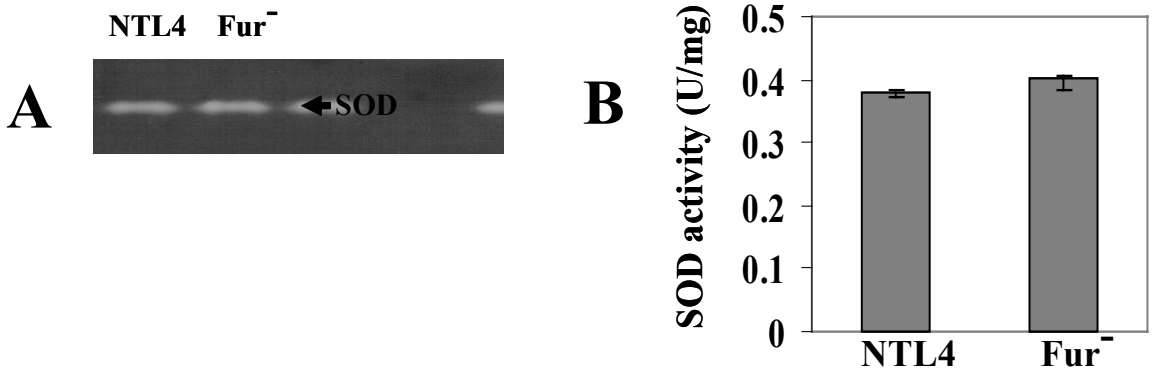


Figure 6. SOD activities of wild-type NTL4 and Fur $^{-}$ mutant.

Cell lysates were prepared from stationary phase cells cultured on LB agar plates, incubation at 28^{o}C for 2 days. (A) SOD activity staining was performed by the method of Clare *et al* [18]. (B) Total SOD activity was determined by the method of McCord and Fridovich [19]. The results are the means and standard errors of three replicates.

4.7 *sod* promoter activity assay

In order to analyze the expression of *sod* and possible regulation by Fur, the *sod* promoter region was transcriptionally fused to a promoter-less *lacZ* in a low-copy number plasmid vector, pUFR027*lacZ*, creating the recombinant plasmid pP_{sod}. The plasmid, pP_{Sod} was transferred into wild-type NTL4 and Fur $^-$ mutant strains. Bacteria grown overnight in LB medium were sub-cultured into fresh LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were induced with 250 μM menadione. The cultures were then allowed to grow for an additional 30 min and were harvested. Cell lysates were prepared as above. β-galactosidase activity assays were performed as described [9]. As shown in Fig. 7, no significant difference in β-galactosidase activities was observed between uninduced Fur $^-$ mutant and wild-type NTL4 cells. Furthermore, the expression of *sod-lacZ* was induced approximately 10-fold after menadione exposure in both Fur $^-$ mutant and wild-type NTL4. These data together with SOD activity staining and total SOD activity assay showed that levels of SOD were not affected by inactivation of *fur*. The fact that Fur $^-$ mutant was more sensitive to menadione than wild-type NTL4 suggested that, as yet unidentified, Fur-regulated mechanism(s) are involved in conferring menadione resistance in *A. tumefaciens*.

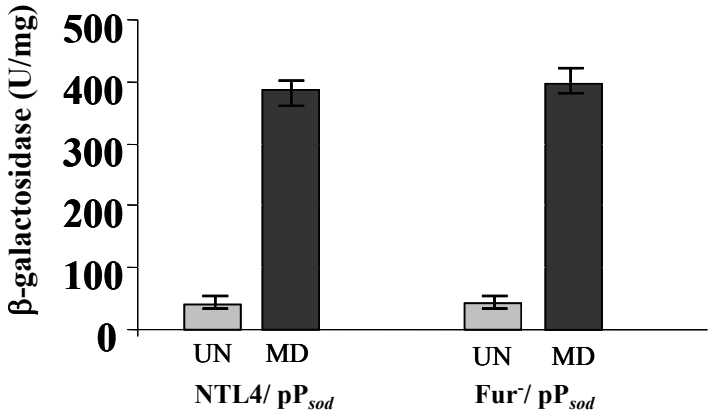


Figure 7. Expression of *A. tumefaciens sod–lacZ* fusion in wild-type NTL4 and Fur $^-$ mutant. β-galactosidase activities were measured from wild-type NTL4 or Fur $^-$ mutant containing the *sod–lacZ* fusion plasmid pP_{sod}. Cell lysates were prepared from uninduced cells (UN) and cells induced with 250 μM menadione (MD). Values are means and standard errors of three replicates.

4.8 Tumor formation assay

A. tumefaciens induces the formation of crown gall tumors by transferring a piece of DNA, the T-DNA, from the tumor-inducing (Ti) plasmid into host plant cells. To determine whether *fur* gene is important in tumor formation or not, *A. tumefaciens* wild-type NTL4 as well as Fur $^-$ mutant strains containing Ti plasmid were used to infect tobacco (*Nicotiana tabacum*) leaf according to the method of Banta *et al* [20]. Cells were grown on LB agar plates at 28^{o}C for 2 days. The cells were washed with MS medium [21] and the cell concentration was adjusted to an OD_{600} of 0.01 with MS medium. The cells were cocultivated with ~0.5 cm square leaf of tobacco at room temperature for 10 min. The infected tobacco leaf pieces (n=30 for each bacterial strain) were transferred onto MS agar plates containing 300 μM acetosyringone and incubated at 28^{o}C in the dark for 2 days. The tobacco leaf pieces were then transferred onto MS agar plates containing 200 μg/ml timentin and incubated at 28^{o}C in the dark. The tumors on each leaf square were observed after 14 days. As shown in Fig. 8, Fur $^-$ mutant (Fur$^-$/pTi) was significantly less virulence than the wild-type strain (NTL4/pTi). The tumors that did form on tobacco leaf squares infected with *A. tumefaciens fur* mutant strain were smaller than those formed by wild-type NTL4. Furthermore, the attenuated virulence of Fur $^-$ mutant could be complemented by the expression of functional Fur from a plasmid vector (pFur) since the tumor-inducing ability of Fur$^-$/pTi/pFur was completely restored to those of wild-type NTL4/pTi. These data confirmed that inactivation of *fur* was responsible for the attenuated virulence and *fur* is important in tumor formation.

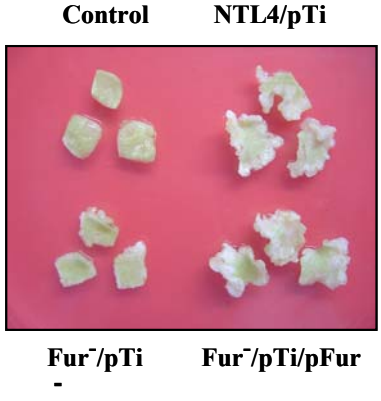


Figure 8. Tumor formation assay. *A. tumefaciens* wild-type NTL4 as well as Fur$^-$ mutant strains containing Ti plasmid, (NTL4/pTi and Fur$^-$/pTi respectively) were used to infect tobacco (*Nicotiana tabacum*) leaf according to the method of Banta *et al* [20]. Fur$^-$ mutant was complemented by expression of functional Fur from a plasmid vector (pFur), Fur$^-$/pTi/pFur. Control: tobacco leaf without infection.

4.9 Summary

To determine the roles of *fur* in the response of *A. tumefaciens* to oxidative stress and metal responses, a *fur* mutant (Fur $^-$) was constructed. Sensitivity of Fur $^-$ mutant to oxidants (H_2O_2, MD: menadione, tBOOH: *tert*-butyl hydroperoxide) and metals ($FeCl_3$, $MnCl_2$, $ZnSO_4$, $NiCl_3$) compared to wild-type NTL4 were determined. Fur $^-$ mutant was more sensitive to oxidant compounds, including peroxide (H_2O_2), superoxide (MD), and organic hydroperoxide (tBOOH), than wild-type NTL4. Fur $^-$ mutant showed increased sensitivity to iron, manganese, and zinc whereas an increase in nickel resistance was observed. In addition, Fur $^-$ phenotype could be complemented by the expression of functional Fur from a plasmid vector, confirming that disruption of *fur* was responsible for the Fur $^-$ phenotype. These data indicate that *A. tumefaciens fur* plays an important role in oxidative stress and metal responses. Catalase encoded by *katA* is known to detoxify H_2O_2.

Due to the fact that Fur $^-$ mutant was hypersensitive to H_2O_2, catalase activities of wild-type NTL4 and Fur $^-$ mutant were measured to determine whether Fur affected the catalase activity. The results showed that Fur $^-$ mutant had lower catalase activity compared to that of wild-type NTL4, which is in accord with the results of H_2O_2 sensitivity. Furthermore, promoter probe-*lacZ* fusion assays showed that decrease in catalase level of Fur $^-$ mutant was due to decreased transcription of *katA*. This suggests the positive regulation of *katA* expression by Fur. Fur $^-$ mutant showed increased sensitivity to menadione, a superoxide generator, relative to wild-type NTL4. However, the data from SOD activity staining, total SOD activity assay and *sod-lacZ* fusion assay showed that levels of SOD, an important enzyme in detoxifying superoxide radicals, in both wild-type NTL4 and Fur $^-$ mutant were similar. This suggested that, as yet unidentified, Fur-regulated mechanism(s) are involved in conferring menadione resistance in *A. tumefaciens*. Tumor formation assay on tobacco leaf showed that Fur $^-$ mutant was highly attenuated in tumor-inducing ability compared to those of wild-type NTL4. In this study, *A. tumefaciens fur* has been shown to play an important role in resistance to metals, oxidative stress and its full virulence.

References

[1] Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. Annu Rev Phytopathol. 33:299-321.

[2] Storz G, Imlay JA (1999) Oxidative stress. Curr Opin Microbiol. 2:188-194.

[3] Bagg A, Neilands JB (1987) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry. 26:5471-5477.

[4] Farr SB, Kogoma T (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium.* Microbiol Rev. 55:561-585.

[5] Niederhoffer EC, Naranjo CM, Bradley KL, Fee JA (1990) Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. J Bacteriol. 172:1930-1938.

[6] Loprasert S, Sallabhan R, Whangsuk W, Mongkolsuk S (2000) Characterization and mutagenesis of *fur* gene from *Burkholderia pseudomallei*. 254:129-137.

[7] Xu XQ, Li LP, Pan SQ. (2001) Feedback regulation of an *Agrobacterium* catalase gene *katA* involved in *Agrobacterium*-plant interaction. Mol Microbiol. 42:645-657.

[8] Xu XQ, Pan SQ. (2000) An *Agrobacterium* catalase is a virulence factor involved in tumorigenesis. Mol Microbiol. 35:407-414.

[9] Luo, Z.Q., Clemente, T.E., and Farrand, S.K. (2001) Construction of a derivative of *Agrobacterium tumefaciens* C58 that does not mutate to tetracycline resistance. Mol Plant Microbe Interact. 14:98-103.

[10] Alexeyev, M.F. (1999) The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. Biotechniques. 26:824-826, 828.

[11] Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A. II, and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene.166:175-176.

[12] DeFeyter, R., Kado, C.I., and Gabriel, D.W. (1990) Small, stable shuttle vectors for use in *Xanthomonas*. Gene.88:65-72.

[13] Nakjarung, K., Mongkolsuk, S., and Vattanaviboon, P. (2003) The *oxyR* from *Agrobacterium tumefaciens*: evaluation of its role in the regulation of catalase and peroxide responses. Biochem Biophys Res Commun. 304:41-47.

[14] Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Qurollo, B., Goldman, B.S., Cao, Y., Askenazi, M., Halling, C., Mullin, L., Houmiel, K., Gordon, J.,

Vaudin, M., Iartchouk, O., Epp, A., Liu, F., Wollam, C., Allinger, M., Doughty, D., Scott ,C., Lappas, C., Markelz, B., Flanagan, C., Crowell, C., Gurson, J., Lomo, C., Sea, C., Strub, G., Cielo, C., and Slater, S. (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science. 294:2323-2328.

[15] Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

[16] Beers, R.F., and Sizer, I.W. (1952) A spectrophotometric method for measuring the break down of hydrogen peroxide by catalase. J. Biol Chem. 195:133-140.

[17] Miller, J.H. A short course in bacterial genetics, Cold Spring Habor Laboratory, Cold Spring Harbor, NY, 1992.

[18] Clare DA, Duong MN, Darr D, Archibald F, Fridovich I. (1984) Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal Biochem. 140:532-537.

[19] McCord JM, Fridovich I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem. 244:6049-6055.

[20] Banta LM, Joerger RD, Howitz VR, Campbell AM, Binns AN. (1994) Glu-255 outside the predicted ChvE binding site in VirA is crucial for sugar enhancement of acetosyringone perception by *Agrobacterium tumefaciens*. J Bacteriol. 176:3242-3249.

[21] Murashige T, Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497.

Manuscript

The *Agrobacterium tumefaciens* Fur-like protein, an iron and manganese-sensing regulator, is essential for survival under iron-limiting condition, oxidative stress defense, and its full virulence.

Worawan Kitphati,[1,2] Patchara Ngok-ngam,[1,3] Sukanya Suwanmaneerat,[1]
Rojana Sukchawalit,[1] * and Skorn Mongkolsuk[1,3] *

[1]Laboratory of Biotechnology, Chulabhorn Research Institute, Lak Si, Bangkok 10210, Thailand

[2]Post Graduate Training and Research Program in Environmental Science, Technology and Management, Asia Institute of Technology, Pathumthani 12120, Thailand

[3]Department of Biotechnology, Faculty of Sciences, Mahidol University, Bangkok 10400, Thailand

*Corresponding author. Tel.: +66 (2) 5740622 ext. 3816; Fax: +66 (2) 5742027

E-mail addresses: skorn@cri.or.th (Skorn Mongkolsuk)

rojana@cri.or.th (Rojana Sukchawalit)

ABSTRACT

Agrobacterium tumefaciens is a soil-born plant pathogen that causes crown gall tumor disease. Iron is an essential metal for bacterial survival and plays an important role in the plant-pathogen interaction. However, iron can be toxic in excess due to its ability to generate highly deleterious free radicals via the Fenton reaction. Therefore, intracellular iron concentrations need to be carefully adjusted by controlling iron transport, storage and consumption. In many bacteria, this regulation is mediated in part by the repressor Fur (ferric uptake regulator) protein to help maintain the balance between acquiring enough iron to grow and avoiding iron toxicity. In order to determine the functional role of the *A. tumefaciens* Fur-like protein (Fur_{At}), a chromosomal *fur* mutant (NTLfur) was constructed by single recombination. Fur_{At} was not the iron response regulator of the siderophore biosynthesis and transport genes. *A. tumefaciens sitA*, the periplasmic binding protein of an ABC-type iron and manganese transport system, was strongly repressed by Mn^{2+} and a lesser extent by Fe^{2+} and this regulation was Fur dependent. Fur_{At} showed a positive regulatory role under iron-limiting condition. The NTLfur mutant was more sensitive to an iron chelator, 2,2'-dipyridyl than wild-type NTL4, suggesting that NTLfur may be iron-deficient. Further evidence supporting this, NTLfur mutant showed increased resistance to an iron-activated antibiotic, streptonigrin, compared with wild-type NTL4. Moreover, overproduction of Fur conferred additional tolerance to 2,2'-dipyridyl in wild-type NTL4 and rendered cells became more sensitive to streptonigrin. These data demonstrated a role for Fur_{At} in regulating intracellular iron concentrations. Fur_{At} was shown to play a role in oxidative resistance. The NTLfur mutant was hypersensitive to hydrogen peroxide and had reduced catalase activity. The virulence assay showed that the NTLfur mutant had reduced ability to cause tumor on tobacco leaves compared to those of wild-type NTL4.

INTRODUCTION

Iron is required for the growth of most living organisms due to its important in many biological processes such as metabolic electron transport chains, the tricarboxylic acid (TCA) cycle, DNA biosynthesis, regulation of gene expression, and protection against oxidative stress. At neutral or alkaline pH in the presence of oxygen, iron exists in the insoluble oxidized ferric (Fe^{3+}) form and thus is not readily bioavailable. Bacteria have evolved various iron-sequestering mechanisms to acquire iron from the environment. One of the effective approaches is to secrete a high-affinity ferric ion chelator called siderophore (Andrews, Robinson et al. 2003). The iron-siderophore complexes are subsequently taken up into the cell by specific membrane transporters. Despite of the indispensability of iron, it can be toxic in excess due to its ability to catalyze the production of highly deleterious hydroxyl radicals via the Fenton reaction (Imlay, Chin et al. 1988). The generated reactive hydroxyl radicals can damage DNA, protein, and lipids. Therefore, iron availability in the cell must be carefully controlled to ensure survival. To achieve this, iron transport, storage and consumption are co-modulated to help maintain the balance between acquiring enough iron to grow and avoiding iron toxicity. In many bacteria, this regulation is mediated in part by the Fur (ferric uptake regulator) protein (Andrews, Robinson et al. 2003). Fur functions as a transcriptional repressor of iron uptake systems and iron-regulated genes under the iron sufficient condition. The molecular basis of iron regulation by Fur has been most extensively studied in *Escherichia coli* providing the classic model of Fur regulation (Andrews, Robinson et al. 2003). Binding to its co-repressor ferrous ion (Fe^{2+}), the Fe^{2+}-Fur complex binds to the conserved sequence, known as Fur box (GATAATGATAATCATTATC), which located in the promoter regions of iron uptake genes leading to repression of iron uptake systems under the iron sufficient condition. In the absence of cofactor Fe^{2+}, Fur no longer binds to the regulated promoters, leads to derepression for iron uptake systems under the iron deficient

condition. Several studies had been shown that Fur regulatory function was not limited to the control of iron metabolism genes. Fur has also been reported to regulate genes involved in acid tolerance (Hall and Foster 1996; Bijlsma, Waidner et al. 2002), production of toxins (Calderwood and Mekalanos 1987; Barton, Johnson et al. 1996), virulence factors (Goldberg, Boyko et al. 1991; Litwin and Calderwood 1993; Mey, Wyckoff et al. 2005), and defense against oxidative stress (Touati, Jacques et al. 1995; Horsburgh, Ingham et al. 2001). The wide range of genes controlled by Fur indicates that Fur serves as a global regulator, therefore, defects in Fur could have serious consequences for bacterial survival. Supporting this, construction of *fur* null mutants using standard techniques such as gene replacement or gene disruption was not possible in many organisms including *Pseudomonas aeruginosa* (Prince, Cox et al. 1993; Hassett, Sokol et al. 1996), *Vibrio anguillarum* (Tolmasky, Wertheimer et al. 1994), *Neisseria gonorrhoeae* (Thomas and Sparling 1996), and *Burkholderia pseudomallei* (Loprasert, Sallabhan et al. 2000). A complete loss of functional *fur* genes was lethal for these organisms, however, an alternative manganese selection method (Hantke 1987) was used to isolate manganese-resistant *fur* mutant strains that contain point mutations in *fur* genes. Based on the assumption that manganese mimics iron by binding to the Fur protein and causes repression of iron uptake systems. Therefore, bacteria with wild-type Fur starve for iron, whereas manganese-resistant *fur* mutants producing altered Fur protein fail to repress iron uptake systems and survive in the presence of high concentrations of manganese. Possibly, mutated Fur proteins cause deregulation of iron uptake but still maintain partial function in the regulation of a critical process necessary for bacterial survival, consequently allowing for the selection of *fur* mutants.

In the case of regulating iron uptake systems, Fur is active only in the presence of iron by the repression mechanism mentioned above, however, Fur could mediate targeted-gene repression in its apo form as observed in *Helicobacter pylori*. Fur protein from *H.*

pylori has been shown to bind *pfr* (ferritin) (Waidner, Greiner et al. 2002) and *sodB* (Fe-superoxide dismutase) (Ernst, Homuth et al. 2005) promoters in its iron-free form. Fur was originally identified as a repressor, subsequently, Fur has also been reported to be an activator. This positively regulation by Fur observed in *E. coli* is an indirect via Fur-mediated repression of a small RNA molecule, RhyB, which encodes a small regulatory RNA that blocks gene expression by binding to and degrading target mRNAs (Masse and Gottesman 2002; Masse, Escorcia et al. 2003). Genes positively regulated by Fur via RyhB include *acnA* (aconitase), *bfr* (bacterioferritin), *fumA* (fumarase), *ftn* (ferritin), *sdhCDAB* (succinate dehydrogenase), and *sodB*. Unlike in *E. coli*, Fur mediated positive regulation by direct activation of gene transcription was found in *Neisseria meningitidis* (Delany, Rappuoli et al. 2004). In the presence of iron, *N. meningitidis* Fur protein was demonstrated to bind the promoter region of *pan1*(nitrite reductase), *norB* (nitric oxide reductase), and *nuoA* (NADH dehydrogenase) genes resulting in the activation of transcription.

A. tumefaciens is a Gram-negative, member of α-proteobacteria, soil-born plant pathogen that causes crown gall tumor disease on dicotyledonous plants. The infection process involves attachment of the bacteria to the wounded plant cells and subsequent transfer a segment of DNA of its tumor inducing (Ti) plasmid into plant cells (Ziemienowicz 2001). Two regions of the Ti plasmid are required for pathogenesis, the transferred (T)-DNA and the virulence (*vir*) region. The *vir* genes are induced in response to acidic pH (approximately 4.8 to 5.5) and phenolic compounds, such as acetosyringone (AS), that are released by wounded plant cells. The *vir* genes are involved in surface interaction between bacteria and plant cells, and transferring of T-DNA into the plant nucleus. The T-DNA is then integrated into the plant chromosome. Expression of the integrated T-DNA results in tumor formation by promoting growth hormone biosynthesis of the transformed plant cells. Although the role of *vir* genes located in the Ti plasmid is

primarily important for interaction of *Agrobacterium* with its host, additional chromosomal genes are also required for full virulence of *Agrobacterium*. Mutations in *chvA*, *chvB* (Douglas, Staneloni et al. 1985), *att* (Matthysse 1987), and *pscA* (Thomashow, Karlinsey et al. 1987) led to an inability of the bacteria to attach to plant cells. Mutations in *chvD* (Winans, Kerstetter et al. 1988), *chvE* (Cangelosi, Ankenbauer et al. 1990), *chvG*, *chvI* (Charles and Nester 1993), *ivr* (Metts, West et al. 1991), and *miaA* (Gray, Wang et al. 1992), *pckA* (Liu, Wood et al. 2005), and citrate synthase genes (Suksomtip, Liu et al. 2005) cause defects in expression of *vir* genes on the Ti plasmid. Other chromosomal genes affecting *Agrobacterium* virulence include *acvB* (Wirawan, Kang et al. 1993), *lon* (Su, Stephens et al. 2006), and a gene involved with the oxidative defense, *katA* (Xu and Pan 2000). Plants induce oxidative stress in infecting bacteria by generating ROS such as; H_2O_2, superoxides, and organic peroxides, to inhibit microbial invasion (Baker and Orlandi 1995). Another environmental stress encountered by microbes during infection is severe iron restriction. Plants possess mechanisms that cause invading pathogen to be deprived of iron, for instance, by constitutively producing an iron-sequestering compound called tannin (Scalbert 1991; Neema, Laulhere et al. 1993; Mila, Scalbert et al. 1996). Therefore, the microbial abilities of iron sequestering as well as defending against oxidative stress would indeed determine its pathogenesis on plant host. The iron-sensing regulator *fur* genes from plant pathogens have been shown to play a critical role during the plant-pathogen interaction. Reduced virulence associated with mutations in *fur* genes has been reported in *Erwinia chrysanthemi* (Franza, Sauvage et al. 1999) and *Xanthomonas oryzae* pv. *oryzae* (Subramoni and Ramesh 2005).

Analysis of the *A. tumefaciens* genome revealed three *fur* homologues, named *fur*, *irr* (iron response regulator) and *zur* (zinc uptake regulator) (Wood, Setubal et al. 2001). The *A. tumefaciens fur*-like gene (Atu0354), fur_{At}, is flanked upstream by a gene encoding a putative acethyltransferase (Atu0353) and downstream by *plsC* gene encoding a glycerol

phosphate acetyltransferase (Atu0355). The *fur*$_{At}$ is 429 bp and encodes a protein of 142 amino acid residues with a deduced molecular weight of 16.7 kDa. The amino acid sequence of the *A. tumefaciens* Fur (Fur_{At}) protein shows highest identity to Fur from the closely relatives *Rhizobium leguminosarum* (84%), *Bradyrhizobium japonicum* (68%), and *Sinorhizobium meliloti* (45%). Fur_{At} has moderate levels of identity to Fur from *E. coli* (37%) and *Pseudomonas aeruginosa* (32%). The physiological function of Fur_{At} has not been defined. Characterization of Fur_{At} would help to understand iron regulation in *Agrobacterium* as well as its impacts on bacterial survival during plant-pathogen interactions. Interestingly, it emerges that the metal specificity and function of Fur-like proteins from members of α-proteobacteria are diverse from the model γ-proteobacterium *E. coli*. Fur is best known as an iron-sensing regulator, however, there were reports a different role of Fur-like proteins from *R. leguminosarum* (Fur_{Rl}) and *S. meliloti* (Fur_{Sm}). Disruption of *fur* gene had no effect on the expression of several genes that are involved in iron acquisition (Wexler, Todd et al. 2003; Chao, Becker et al. 2004; Platero, Peixoto et al. 2004). Alternatively, Fur_{Rl} and Fur_{Sm} physiologically function in response to manganese by repressing transcription of the *sitABCD* operon, which encodes a Mn^{2+} uptake system, under manganese replete condition (Chao, Becker et al. 2004; Diaz-Mireles, Wexler et al. 2004; Platero, Peixoto et al. 2004). *S. meliloti sitABCD* operon was strongly repressed by Mn^{2+} and moderately by Fe^{2+}. The Mn^{2+}-mediated repression of *sitABCD* operon was Fur-dependent, whereas, Fe^{2+}-mediated repression was partially affected by Fur (Chao, Becker et al. 2004). Interestingly, *R. leguminosarum sitABCD* operon was repressed by manganese only but not iron (Diaz-Mireles, Wexler et al. 2004). Based on that finding, Fur_{Rl} was renamed Mur (manganese uptake regulator) (Diaz-Mireles, Wexler et al. 2004). Furthermore, Mur_{Rl} has been shown to recognize DNA sequences within the *sitABCD* promoter region which are different from the conventional *E. coli* Fur box, named Mur-responsive sequences (MRS1 TGCAATT-N_7-AATTGCA and MRS2 TGCAAAT-N_7-

AATCGCA) (Diaz-Mireles, Wexler et al. 2005). The MRS-like motif, TGCAAAT-N_7-AGTTTGC, was also found in the *S. meliloti sitABCD* promoter region (Chao, Becker et al. 2004). Mur_{Rl} and Fur_{Sm} respond to manganese and recognize a different DNA sequence from that of typical Fur, which in turn affect the range of their regulated-genes, and do not serve as a major regulator of iron-responsive genes. The protein named RirA (rhizobial iron regulator) showing no sequence homology with Fur proteins, first identified in *R. leguminosarum*, has been shown to replace typical Fur function in regulation of iron-responsive genes for controlling iron homeostasis in those two rhizobial species (Todd, Wexler et al. 2002; Chao, Buhrmester et al. 2005). The RirA protein belongs to the Rrf2 family of putative transcription regulators. Other members of the Rrf2 family include: Rrf2 of *Desulfovibrio vulgaris*, which regulates cytochrome synthesis by repressing *hmc* operon (Keon, Fu et al. 1997), IscR of *E. coli*, is a repressor of *iscRSUA* operon which involved in Fe-S cluster formation (Schwartz, Giel et al. 2001), and NsrR of *Nitrosomonas europaea*, is a nitrite (NO_2^-)-sensitive transcription repressor of nitrite reductase *nirK* gene (Beaumont, Lens et al. 2004). Recently, another member of this protein family was identified in *E. coli* named YjeB (NsrR), which is a nitric oxide (NO)-sensitive transcription repressor of the nitrosative stress response genes (*ytfE*, *hmpA* and *ygbA*) (Bodenmiller and Spiro 2006). The close RirA homologues appear to be confined to some of α-proteobacteria, the rhizobia *Mesorhizobium* and *Sinorhizobium,* the human pathogen *Bartonella*, the animal pathogen *Brucella*, and the phytopathogen *Agrobacterium*. The RirA-like protein does not exist in *B. japonicum*, a member of α-proteobacteria, and its regulation of iron metabolism is, at least in part, still modulated by Fur-like protein (Fur_{Bj}) in cooperation with another regulator called Irr (iron response regulator) through controlling heme biosynthesis pathway (Hamza, Chauhan et al. 1998; Hamza, Qi et al. 2000). The *B. japonicum* Irr protein repressed *hemB*, the gene encoding the enzyme δ-

aminolevulinic acid dehydratase that catalyses the second step in heme biosynthesis, under iron restricted condition to prevent the accumulation of toxic protoporphyrin intermediates from exceeding iron availability. The presence of iron causes repression of *irr* transcription via Fur (Hamza, Qi et al. 2000; Friedman and O'Brian 2004) and degradation of Irr protein by oxidation in an heme-dependent mechanism (Qi and O'Brian 2002; Yang, Panek et al. 2006). These both transcriptionally and post-translationally control lead to derepression of heme biosynthesis. Another gene, *hemA* encoding δ-aminolevulinic acid synthase in the first step of heme biosynthesis pathway, has been shown to be controlled by iron depending on Fur, however Irr was not involved in this regulation (Hamza, Qi et al. 2000). The promoter region of the *hemA* gene contains a putative Fur box (GATAATCTGCTGAATTGTG), which matches at 10 of 19 positions to the *E. coli* Fur box (LeVier and Guerinot 1996), however, There is no sequence similarity to conventional Fur box in the promoter region of the *irr* gene. Instead, Fur_{Bj} has been shown to bind three imperfect direct repeat sequences, TGCGAGAAACTTGCATCTGCATC, within the *irr* promoter (Friedman and O'Brian 2003). These reflected the flexibility of Fur_{Bj} to recognize different DNA binding sequences in its different regulated genes.

In this study, the physiological function of *A. tumefaciens fur* gene was investigated. The important roles of fur_{At} for metal and oxidative stress responses are demonstrated. Fur contributes significantly to the virulence of *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial growth conditions. Bacterial strains and plasmids are listed in Table 1. *A. tumefaciens* strains were grown aerobically in Luria-Bertani (LB) medium at 28^{o}C with shaking at 150 rpm supplemented with 100 μg ml^{-1} ampicillin (Ap), 25 μg ml^{-1} chloramphenicol (Cm), 90 μg ml^{-1}gentamicin (Gm), or 10 μg ml^{-1} tetracycline (Tc) as

required. *E. coli* strains were grown in aerobically LB medium at 37^{o}C supplemented with 100 μg ml^{-1} Ap, 30 μg ml^{-1} Gm, or 15 μg ml^{-1} Tc as required.

Molecular techniques. Unless otherwise stated, general molecular techniques were performed by using standard procedures (Sambrook, Fritsch et al. 1989). Plasmid purification was performed by using the QIAprep kit (Qiagen). DNA was sequenced by using a BigDye terminator cycle sequencing kit (PE Biosystems) on an ABI 310 automated DNA sequencer (Applied Biosystems). Plasmids were transformed into *A. tumefaciens* strains by electroporation (Cangelosi, Best et al. 1991).

***A. tumefaciens fur* mutant construction and analysis.** The *fur* mutant was constructed by insertional inactivation of the *fur* gene on the chromosome by single homologous recombination. The primers BT772-5'TCAGGAATCAGCCGATCATC3' and BT773-5'ATGACCACGCTGTTCTTCAG3' designed from the sequence of a putative *fur* gene (Atu0354), identified from the *A. tumefaciens* C58 genome sequence (Wood, Setubal et al. 2001), were used to amplified a 219 bp fragment of *fur* coding region using Taq DNA polymerase and *A. tumefaciens* NTL4 genomic DNA as templates. The PCR product was cloned into pGEM-T-easy vector (Promega) and its nucleotide sequence was confirmed by automated DNA sequencing. Subsequently, the 169 bp *Bam*HI-*Eco*RI fragment of the PCR clone was filled in with the Klenow enzyme and subcloned into pKNOCK-Gm (Alexeyev 1999), a non-replicative plasmid in *Agrobacterium*, digested with the unique *Sma*I. The resultant plasmid, pKNOCKfur (2.16 Kb), was then transferred to *A. tumefaciens* by conjugation. Recombination of the cloned *fur* fragment in the suicide plasmid with the homologous counterpart on *A. tumefaciens* chromosome resulted in the disruption of the *fur* gene. The *fur* mutant was selected on LB agar containing 25 μg ml^{-1} chloramphenicol and 90 μg ml^{-1} gentamicin. To verify the *fur* mutant, Southern blot

analysis was performed using standard protocol (Sambrook, Fritsch et al. 1989). Chromosomal DNA from *fur* mutant and wild-type *A. tumefaciens* NTL4 were digested with *Sph*I, separated and blotted on a nylon membrane. The blot was hybridized to 169 bp *Bam*HI-*Eco*RI radioactively labeled *fur* probes. Probes were radioactively labeled using a random priming kit (Amersham Phamacia Biotech) and [α-^{32}P]dCTP. The single hybridizing band of 0.86 Kb was obtained from wild-type NTL4 as expected from the genomic sequence. Whereas, a hybridizing band of 3.02 Kb was detected in the *fur* mutant confirming that pKNOCKfur had correctly integrated into the *fur* gene. The *fur* mutant was named NTLfur.

Construction of *fur* full length. The full-length of wild-type *fur* gene was amplified from *A. tumefaciens* NTL4 genomic DNA with primers BT692-5'CCAGAAGACGTGATAGACCT3' and BT693-5'CGGCGTCTCAGCGTTCTTCG3' using Pfu DNA polymerase (Promega). The 438 bp PCR product was cloned into the unique *Sma*I site of an expression vector pBBR1MCS-4 (Kovach, Elzer et al. 1995), creating the recombinant plasmid, pFur. Cloned DNA region was confirmed by automated DNA sequencing.

Construction of *fhuA-lacZ* fusion. The putative *fhuA* promoter region was amplified amplified from *A. tumefaciens* NTL4 genomic DNA with primers BT1095-5'CGTAGCTCGAATGTATCCGC3' and BT1096-5'CGCGACATAACCTTTCACCG3' using Pfu DNA polymerase (Promega). The 414 bp PCR product was cloned into the unique *Hin*dIII site (end gap fill with Klenow enzyme) of the promoter probe vector, pUFR027*lacZ*, a derivative of pUFR027 (DeFeyter, Kado et al. 1990). The resultant recombinant plasmid was named p*fhuA-lacZ* and was transferred into wild-type NTL4 and NTLfur mutant. Bacteria grown overnight in LB medium were sub-cultured into fresh

LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were treated with 50 μM of $FeCl_3$, or or 200 μM Dipy for 30 min. Cells were harvested and used for β-galactosidase activity assay.

Reverse transcriptase PCR (RT-PCR) analysis of *sitABCD* transcripts. Bacteria grown overnight in LB medium were sub-cultured into fresh LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were treated with 50 μM of $FeCl_3$, or 50 μM of $MnCl_2$ for 15 min. Total RNA was extracted from untreated and treated cells by using RNeay Mini Kit (Qiagen) and were treated with DNase I using DNA-*free*™ Kit (Ambion), according to the manufacturer's protocols. Reverse transcription (converts mRNA to cDNA before PCR) was accomplished using SuperScript™ II Reverse Transcriptase (Invitrogen) with reverse primer BT662 for *sitA* or BT1422 for 16S rRNA. Reverse transcribed RNA sample (2 μg) from each condition was used in the PCR reaction. Control reactions, where reverse transcriptase was omitted, were run in parallel to ensure there was no DNA contamination. Positive controls were performed with genomic DNA. Gene-specific primers for *sitA* (BT661-5'TGATGTGACGGTGAGCGATG3' and BT662-5'GGCGCCTTCGCTCGTTACCA3' to generate the 280 bp PCR product) and 16S rRNA (BT1421-5'GAATCTACCCATCTCTGCGG3' and BT1422-5'AAGGCCTTCATCACTCACGC3' to generate the 280 bp PCR product) were used for separate PCR reactions using Taq PCR Master Mix Kit (Qiagen). PCR reactions were carried out with an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. RT-PCR products were visualized through gel electrophoresis on a 2% agarose gel and ethidium bromide staining.

Sensitivity to 2,2'-dipyridyl. Overnight cultures grown in LB medium were washed once with fresh LB medium. Cells were diluted to an OD_{600} of 0.01 in 5 ml of LB medium. Iron-limiting condition was achieved by adding 300 μM of the iron chelator, 2,2'-dipyridyl (Dipy, Sigma). Growth was monitored by measuring OD_{600} after incubation at 28°C with shaking for 24 h.

Sensitivity to streptonigrin. Overnight cultures were streaked on LB agar plates containing 300 μM of Dipy and incubation at 28°C for 2 days. Cells were washed once with fresh LB medium. Cells (10^4) were treated with streptonigrin (SNG) at a concentration of 100 μg ml^{-1} in LB medium. SNG was prepared in dimethyl sulfoxide as a stock solution at 10 mg ml^{-1}. Control cells (untreated) received equivalent amounts of dimethyl sulfoxide. The cells were incubated at 28°C with shaking for 24 h and were diluted (10-fold serial dilutions). An aliquot (10 μl) of each dilution was spotted on LB agar plates and incubated at 28°C for 2 days. Each strain was performed in duplicate and the experiment was repeated twice.

Siderophore analysis. Siderophore production was analysed using chrome azural S (CAS) agar plates (Schwyn and Neilands 1987). Solid CAS medium was made by adding 10 ml CAS stock (Schwyn and Neilands 1987) to 100 ml YEM medium (Vincent 1970) containing 1.5% agar. Overnight cultures grown in LB medium (5 μl at OD_{600} of 0.1) were spotted onto YEM+CAS plates containing 50 μM $FeCl_3$ or 200 μM Dipy, and incubated at 28°C for 2 days. Production of siderophore is indicated by the presence of orange halo zone around the bacteria. This occurs because iron is removed from the original blue CAS-

Fe(III) complex contained in the plate by siderophores produced by bacteria resulting in a change in color of the dye.

Sensitivity to hydrogen peroxide. Cells grown on LB agar plates at 28°C for 2 days were washed and adjusted to an OD_{600} of 0.01 in LB medium. Ten-fold serial dilutions were made. An aliquot (10 μl) of each dilution was spotted on LB agar plates containing 400 μM H_2O_2 and incubated at 28°C for 48 h. Cells spotted on LB agar plate were used as a control.

Enzyme activity assays. Crude bacterial lysates were prepared by using bacterial suspensions in 50 mM sodium phosphate buffer pH 7.0 containing 1 mM PMSF, a protease inhibitor. Cell suspensions were lysed by brief sonication followed by centrifugation at 12,000 *g* for 10 min. Clear lysates were used for total protein determination, β-Galactosidase assay, and catalase activity assay. Protein concentrations were determined using Bradford BIO-RAD protein assay. β-Galactosidase assay was done as described previously and presented in Miller units (Miller). Catalase activity was monitored by the decomposition of H_2O_2 at the absorbance 240 nm (Beers and Sizer 1952). One unit of catalase was defined as the amount of enzyme capable of catalyzing the turnover of 1 μ mole of substrate per minute under the assay condition.

Tumor formation assays. *A. tumefaciens* strains containing pTiC58 plasmid were used to infect tobacco (*Nicotiana tabacum*) leaves according to the method described previously (Liu, Wood et al. 2005). Cells were grown on LB agar plates at 28°C for 2 days. The cells were washed with hormone-free MS liquid medium (Murashige and Skoog 1962) and the cell concentration was adjusted to an OD_{600} of 0.01 in 20 ml of hormone-free MS medium.

The cell suspensions were cocultivated with ~0.5 cm square leaf of tobacco (30 leaf squares for each bacterial strain) at room temperature for 10 min. Tobacco leaf pieces incubated in hormone-free MS medium without bacterial cells were used as a negative control. The tobacco leaf pieces were transferred onto hormone-free MS agar plates containing 300 μM acetosyringone and incubated at 28°C in the dark for 2 days. The tobacco leaf pieces were then transferred onto hormone-free MS agar plates containing 200 μg ml^{-1} timentin and incubated at 28°C in the dark. The tumors on each leaf piece were observed after 14 days. The experiments were repeated twice.

RESULTS

Fur is essential for *A. tumefaciens* survival under the iron-limiting condition. In order to determine the functional role of the *fur* gene in *A. tumefaciens*, a chromosomal *fur* mutant (NTLfur) was constructed by single recombination and was confirmed by Southern blot analysis (data not shown and see Material and Methods). First, we investigated the potential biological effect of the *fur* mutation in response to iron, the wild-type NTL4 and NTLfur mutant strains were grown under iron-sufficient (LB) and iron-deficient (LB+300 μM Dipy) conditions (Fig. 1A). In LB medium, NTL4 and NTLfur mutant strains harboring plasmid vector pBBR1MCS-4 (NTL4/pBBR and NTLfur/pBBR) or plasmid expression of functional Fur (NTL4/pFur and NTLfur/pFur) showed no differences in growth since all strains exhibited similar optical density at 600 nm. In the presence of an iron chelator 2,2'-dipyridyl (300 μM Dipy), the growth of all tested strains was strongly inhibited. However, the *fur* mutant showed more sensitivity to Dipy compared with wild-type as judged by the OD_{600} obtained from NTLfur/pBBR was lower than NTL/pBBR. The Dipy-sensitive phenotype of NTLfur could be complemented by expression of the functional Fur on the expression vector, as observed in NTLfur/pFur. Moreover,

overproduction of Fur conferred additional tolerance to Dipy since the OD_{600} obtained from strains NTL4/pFur and NTLfur/pFur were higher than NTL/pBBR. This suggested a positive regulatory role for Fur_{At} under the iron-limiting condition.

The NTLfur mutant strain is iron-deficient compared to the wild-type NTL4. The hypersensitivity to Dipy of NTLfur mutant led us to ask whether NTLfur is in fact iron-deficient compared to the wild-type NTL4. Sterptonigrin (SNG) has been used to assess free iron levels in bacterial cells (Wilson, Bertrand et al. 1998; Elgrably-Weiss, Park et al. 2002; Subramoni and Ramesh 2005). SNG is an aminoquinone that is capable of cyclic reduction and oxidation inside bacteria to produce superoxide and hydroxyl radicals which damage DNA (Gregory and Fridovich 1973; Hassett, Britigan et al. 1987). The availability of intracellular iron is an important factor in the action of SNG. The increased sensitivity to SNG was shown to correlate with the increase in the levels of intracellular free iron (White and Yeowell 1982; Yeowell and White 1982). Relative intracellular iron levels in NTL4 and NTLfur mutant were assessed by SNG sensitivity assays. Cells grown under the iron-deficient condition (LB agar plates containing 300 μM Dipy) for 2 days were used for SNG sensitivity assays (see Materials and Methods). The results in Fig. 1B show that untreated cells of all tested strains exhibited similar viability. The NTLfur/pBBR mutant had greater tolerance to 100 μg ml^{-1} SNG than wild-type NTL4/pBBR. These data provide the evidence that the *fur* mutant has lower levels of intracellular free iron compared with wild-type. Another possible explanation for increased resistance to SNG could be due to the overproduction of superoxide dismutase (SOD) (Gregory and Fridovich 1973). However, SOD activity assays showed that NTL4/pBBR and NTLfur/pBBR had similar levels of SOD activity (data not shown). Complementation of the mutant by overproduction of functional Fur on a plasmid pFur, NTLfur/pFur, could restore SNG sensitivity to the same level of wild-type NTL4/pBBR. These results suggested that Fur_{At}

has a positive regulatory role in elevating the levels of intracellular iron. Further evidence supporting this, overproduction of functional Fur in the wild-type background, NTL4/pFur, enhanced sensitivity to SNG compared with NTL4/pBBR.

Fur is not the iron-responsive regulator of the siderophore biosynthesis and transport genes in *A. tumefaciens*. Siderophores are ferric ion chelating molecules synthesized and secreted by bacteria growing under low iron stress. In many bacteria, synthesis of siderophores is negatively regulated by iron and the Fur protein. One of the common phenotypes in *fur* mutants is the loss of iron-mediated regulation of siderophore synthesis resulting in overproduction or constitutively secreting siderophores (Hassett, Sokol et al. 1996; Loprasert, Sallabhan et al. 2000; Horsburgh, Ingham et al. 2001; Thompson, Beliaev et al. 2002; Subramoni and Ramesh 2005). To determine whether the fur_{At} mutation affects siderophore biosynthesis, production of siderophores by the NTLfur mutant was compared with those by the wild-type NTL4 on YEM+CAS plates under iron-replete (50 μM $FeCl_3$) and -deplete (200 μM Dipy) conditions. As shown in Fig. 2A, wild-type NTL4 and NTLfur mutant did not produce siderophore under iron-replete condition. Both strains only produced siderophores under iron-limiting condition (Fig. 2B) as indicated by the halo zones. In addition, the sizes of the halo zones surround both strains were similar. This evidenced that iron-mediated regulation of siderophore synthesis was maintained in the NTLfur mutant as normal as in the wild-type NTL4, therefore, *fur* is not involved in iron regulation of siderophore production in *A. tumefaciens*.

Ferri-siderophore complexes are too large to permeate the outer membranes (OMs) of Gram-negative bacteria. Ferri-siderophore complexes are taken up via specific OM receptors. Bacteria often possess multiple OM receptors, which are specific for different siderophores. Siderophores are classified by their iron-binding ligand types (catechols, hydroxamates, or α-hydroxycarboxylates) (Winkelmann 2002). In *E. coli*, at least three

OM siderophore receptors have been reported, including FepA (for enterochelin), FecA (for ferric citrate), and FhuA (for ferric hydroxamate). OM siderophore receptors are generally induced by iron starvation and negatively regulated by Fur under the iron-replete condition (Andrews, Robinson et al. 2003). To investigate a potential role of Fur_{At} in iron regulation of the OM siderophore receptor, expression of *fhuA* from the *fhuA-lacZ* transcriptional fusion plasmid (pP*fhuA-lacZ*) was monitored in wild-type and *fur* mutant backgrounds. β-galactosidase activities were measured from wild-type NTL4 and NTLfur mutant containing pP*fhuA-lacZ* grown under iron-replete (LB+50 μM $FeCl_3$) and iron-deplete (LB+200 μM Dipy) conditions (Fig. 2C). Levels of β-galactosidase activities were higher in cells grown under the iron-deplete condition. The *fhuA* expression was reduced under the iron-replete condition both in wild-type NTL4/pP*fhuA-lacZ* and mutant NTLfur/pP*fhuA-lacZ* backgrounds. The iron-controlled expression of *fhuA* in wild-type and *fur* mutant were similar, indicating that iron-regulated *fhuA* expression was not mediated by Fur_{At}.

Fur_{At} is the repressor of *sitA*. It has been reported that, Fur_{Rl} and Fur_{Sm} physiologically function in response to manganese by repressing transcription of the *sitABCD* operon, which encodes a Mn^{2+} uptake system, under manganese replete condition (Chao, Becker et al. 2004; Diaz-Mireles, Wexler et al. 2004; Platero, Peixoto et al. 2004). *sitA* encodes the periplasmic binding protein, followed by *sitB*, which encodes an ATPase. *sitC* and *sitD* encode the membrane-located transporters. The *sitABC/D* operon was originally reported as being involved in iron uptake (Zhou, Hardt et al. 1999; Janakiraman and Slauch 2000), but subsequent studies have defined this system as a manganese transporter and proposed to rename this operon as *mntABC/D* (Platero, Peixoto et al. 2004). Analysis of the *A. tumefaciens* genome revealed a putative *sitABCD* operon (Atu4471, Atu4470, Atu4469 and

Atu4468 respectively). To determine the role of Fur_{At} on the expression of *sitABCD* operon, reverse transcriptase PCR (RT-PCR) was used to analyze the mRNA expression of the *sitA* in wild-type NTL4 and NTLfur mutant grown in the absence or presence of metal (Fig. 3). The housekeeping gene 16S rRNA was used as an internal control in RT-PCR, and similar levels of PCR products (Fig. 3B) confirmed that the RNA quantities used as a template in all RT reaction were equal. Unlike the 16S rRNA product, the amount of amplicon corresponding to the *sitA* gene differed depending on the RNA samples Fig. 3A). As shown in Fig. 3A, expression of *sitA* in wild-type NTL4 was detected in untreated cells and was completely repressed by the addition of manganese. Although, addition of iron greatly reduced, but did not abolish the expression of *sitA*. This similar to what observed in *S. meliloti* (Chao, Becker et al. 2004; Platero, Peixoto et al. 2004). Regulation of *sitA* expression by iron and manganese was lost in the NTLfur mutant, resulting in constitutively expression of *sitA* in the presence of iron or manganese. These data indicated that Fur is involved in metal-dependent repression of *sitA*.

The NTLfur mutant is hypersensitive to hydrogen peroxide and exhibits reduced catalase activity. Plants induce oxidative stress by generating ROS such as; H_2O_2, superoxides, and organic peroxides, to inhibit microbial invasion (Baker and Orlandi 1995). To determine the roles of Fur in the response of *A. tumefaciens* to oxidative stress, the sensitivity of wild-type and *fur* mutant to H_2O_2 was determined. Cells grown on LB agar plates at 28^{o}C for 2 days were used for H_2O_2 sensitivity assays. The NTLfur/pBBR was more sensitive to 400 μM H_2O_2 than NTL4/pBBR (Fig. 4A). Expression of the functional Fur in the *fur* mutant was able to complement the H_2O_2 hypersensitive phenotype, since the extent of the sensitivity of the complemented strain, NTLfur/pFur was similar to that of wild-type NTL4/pBBR. These data confirmed that disruption of *fur* was responsible for the H_2O_2 hypersensitive phenotype of NTLfur, indicating an important role

for Fur_{At} in response to oxidative damage. However, overproduction of Fur did not confer additional resistance to H_2O_2 as demonstrated by that NTL/pFur showed similar levels of H_2O_2 resistance compared to those of NTL/pBBR.

Catalase is an important enzyme in detoxifying H_2O_2. Due to the fact that the NTLfur mutant was more sensitive to H_2O_2 than wild-type NTL4, catalase activity assay was performed in order to know whether hypersensitivity to H_2O_2 was associated with catalase levels. As shown in Fig 4B, the NTLfur mutant had lower total catalase activity about 30% compared to that of wild-type NTL4. These indicated that an increase in sensitivity to H_2O_2 of NTLfur mutant was due to the reduction in catalase activity. In *A. tumefaciens*, catalase encoded by *katA* is the major enzyme responsible for H_2O_2 resistance (Prapagdee, Eiamphungporn et al. 2004). In order to analyze the expression of *katA* and possible regulation by Fur, pP*katA-lacZ* was transferred into wild-type NTL4 and NTLfur mutant strains. Cells were grown on LB agar plates at 28°C for 2 days and the β-galactosidase activity was measured. The β-galactosidase activity obtained from NTLfur/pP*katA-lacZ* was decreased about 20% compared to those of wild-type NTL4/pP*katA-lacZ* (Fig. 4C). The data confirmed that decrease in the catalase levels of NTLfur mutant resulted from decreased *katA* expression. This suggested that *katA* expression is positively regulated by Fur, either directly or indirectly.

The NTLfur mutant has reduced virulence. In many pathogenic bacteria, *fur* mutants have shown a reduction in virulence (Horsburgh, Ingham et al. 2001; Palyada, Threadgill et al. 2004; Rea, Gahan et al. 2004). Among plant pathogens, reduced virulence has been associated with mutations in the *fur* gene of *Erwinia chrysanthemi* (Franza, Sauvage et al. 1999) and *Xanthomonas oryzae* pv. *oryzae* (Subramoni and Ramesh 2005). *A. tumefaciens* induces the formation of crown gall tumors by transferring a piece of DNA, the T-DNA, from the tumor-inducing (Ti) plasmid into host plant cells. To determine whether *fur* gene

is important in *A. tumefaciens* virulence, tumor formation on tobacco leaves were assessed. Tobacco leaf pieces were infected with wild-type NTL4 or NTLfur mutant containing pTiC58 (pTi) plasmid as described in the Material and Methods. *A. tumefaciens* strains lacking pTi plasmid were not able to cause tumor on tobacco leaves (data not shown). Fig. 5 shows that *fur* mutant (NTLfur/pTi) was significantly less virulence than the wild-type strain (NTL4/pTi). The tumors that formed on tobacco leaf pieces infected with *A. tumefaciens* NTLfur mutant strain were not visible or smaller than those formed by wild-type NTL4. Furthermore, the attenuated virulence of NTLfur mutant could be complemented by the expression of functional Fur from a plasmid pFur since the tumor-inducing ability of NTLfur/pTi/pFur was completely restored to that of wild-type NTL4/pTi (Fig. 5). Whereas NTLfur mutant containing the plasmid vector pBBR1MCS-4 (NTLfur/pTi/pBBR1MCS-4) was unable to complement the reduced virulence phenotype (data not shown). These results confirmed that inactivation of *fur* was responsible for the attenuated virulence and *A. tumefaciens fur* is important in its pathogenesis on plant host.

DISCUSSION

The Fur_{At} protein has a high degree of similarity to many Fur proteins (Fig. 6), with the most closely related proteins fromα-proteobacteria including *R. leguminosarum* (84%), *B. japonicum* (68%) and *S. meliloti* (45%). Fur_{At} has moderate levels of identity to Fur from *E. coli* (37%) and *Pseudomonas aeruginosa* (32%). The crystal structure of the *P. aeruginosa* Fur (Fur_{Pa}) protein revealed two metal-binding sites in the C-terminal end of the protein (Pohl, Haller et al. 2003). The putative regulatory Fe-sensing site (S1) consisted of amino acid residues H86, D88, E107, and H124. The second binding site (S2) was a structural Zn-binding site involved with H32, E80, H89, and E100. All these eight amino acids are highly conserved among Fur proteins from many bacteria, including *A. tumefaciens* (Fig. 6). Alteration of some of these conserved residues could lead to

inactivation of Fur activity as observed in *P. aeruginosa*, *Vibrio cholerae*, *Salmonella typhimurium* (Lam, Litwin et al. 1994; Hall and Foster 1996; Lewin, Doughty et al. 2002). By contrast, substitution each of all four conserved amino acids corresponding to the Fe-sensing site S1 or the structural Zn-binding site S2 in Fur_{Bj}, had no effect on iron-dependent DNA binding and transcriptional repression from *irr* promoter (Friedman and O'Brian 2004). Therefore, the conserved metal-binding S1 and S2 sites, proposed from the structure of Fur_{Pa} are not required for FurBj activity. This suggested that the mechanisms for metal-mediated activation of Fur in the α-proteobacteria are different from those in the γ-proteobacteria, such as *P. aeruginosa* and *E. coli*. Fur proteins from different bacteria might be functionally diverse even though the two metal-binding sites, proposed from the structure of *P. aeruginosa* Fur, are very highly conserved. The motif GLATVYRVLTQF (residues 50-61) in the N-terminal of *P. aeruginosa* Fur protein was predicted as a putative DNA-binding motif and was conserved in many bacteria (Pohl, Haller et al. 2003). In *E. coli*, the corresponding G50 has been shown to be required for DNA-binding activity (Coy 1995). In Fur_{At}, Fur_{Bj}, and Mur_{Rl} the corresponding putative DNA-binding motif is SISTVYRTVKLF, in which the G50 is substituted by S. Another conserved residue A10 in the N-terminal of Fur_{Pa} also contributed to DNA interaction (Barton, Johnson et al. 1996). This corresponding A10 is replaced by R in Fur_{At} and Mur_{Rl}, or T in Fur_{Bj}. Despite of some amino acid changes in the putative DNA-binding motif, Fur_{Bj} and Mur_{Rl} were able to bind to *E. coli* Fur boxes. Fur_{Bj} had ability to repress the *fiu-lacZ* transcription in an *E. coli fur* mutant strain H1780 (Hamza, Hassett et al. 1999). Mur_{Rl} partially repressed *bfd-lacZ* transcription in an *E. coli fur* mutant strain JRG2653 and this repression was Fe-dependent, not Mn (Diaz-Mireles, Wexler et al. 2004). There were evidences that rhizobia Fur-like proteins can recognize regulatory sequences that do not resemble classical Fur boxes and response to metal other than Fe. The Fur DNA binding domain in α-

proteobacteria may differ from that in γ-proteobacterium. These differences may contribute the differences in DNA recognition sequences other than conventional Fur box (Friedman and O'Brian 2003; Diaz-Mireles, Wexler et al. 2005). Alteration of some residues in DNA binding domain together with the report that Mur_{Rl} protein was capable of binding other metals (Bellini and Hemmings 2006) may provide the flexibility of α-proteobacteria Fur-like proteins to bind to regulated genes and sense other metal(s) in addition to iron. It is becoming increasingly clear that in α-proteobacteria rhizobia, Fur-like proteins can recognize regulatory sequences that do not resemble classical Fur boxes and response to manganese, and are not served as a global regulator of iron-responsive genes. This also seems to be the case of *A. tumefaciens*. This study showed that Fur_{At} controls a putative manganese transport system, *sitABCD* operon, and is not the regulator of siderophore biosynthesis and transport genes. However Fur_{At} has been shown to play a positive role under iron limiting condition as yet unidentified mechanism(s). The protein RirA has been shown to replace typical Fur function in regulation of iron-responsive genes for controlling iron homeostasis in rhizobia (Todd, Wexler et al. 2002; Chao, Buhrmester et al. 2005). A *rirA* homolog is also present in the *A. tumefaciens* genome, although its function has not been studied.

The NTLfur mutant showed highly attenuated in virulence on tobacco leaves (Fig. 5). In some organisms, Fur plays a role in resistance to low pH. It is the fact that wounded plant cell is acidic environment. It was possible that the reduced virulence of NTLfur mutant might be a result of decrease in acid tolerance. We therefore examined the ability of NTLfur mutant to grow under acid condition. The growth rate of NTLfur mutant in the IB medium pH 5.5, which was shown to resemble the bacterial growth condition in plant tissues (Li, Li et al. 1999), and in the MS medium pH 5.5 (Murashige and Skoog 1962) was compared to that of the wild-type NTL4 by measuring optical density at 600 nm. The

NTLfur mutant was not affected in growth at low pH since the OD_{600} of bacterial cultures at 28°C were similar to those of wild-type in both media (data not shown). Thus, the *fur*$_{At}$ gene is not involved in acid resistance and the reduced virulence of NTLfur mutant was not due to an inability to cope with acidic plant wound environment during infection. *A. tumefaciens fur* gene showed a positive regulatory role in response to iron starvation and oxidative stress. Inactivation of the *fur*$_{At}$ caused cells become more sensitive to iron-limiting condition (Fig. 1A) and H_2O_2 (Fig. 4). The hypersensitivity to the low iron condition and H_2O_2 reflect an important role of *fur*$_{At}$ for survival and infection since the availability of iron in plant environment is limited (Scalbert 1991; Neema, Laulhere et al. 1993; Mila, Scalbert et al. 1996) and oxidative stress is an initial plant defense (Baker and Orlandi 1995). The reduced catalase activity in NTLfur mutant, together with evidence that catalase was essential for *A. tumefaciens* virulence (Xu and Pan 2000), suggested that the virulence deficiency of the NTLfur mutant was likely due, at least in part, to an impaired ability to cope with the iron restricted and oxidative stress conditions that are major plant-environment stresses encountered during infection.

Acknowledgements

This work was supported by a Research Scholar Grant TRG4780004 from the Thailand Research Fund to R. Sukchawalit and by a Research Team Strengthening Grant from the BIOTEC and a Senior Research Scholar Grant RTA4580010 from the Thailand Research Fund to S. Mongkolsuk.

REFERENCES

Alexeyev, M. F. (1999). "The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria." Biotechniques **26**: 824-826, 828.

Andrews, S. C., A. K. Robinson, et al. (2003). "Bacterial iron homeostasis." FEMS Microbiol. Rev. **27**: 215-237.

Baker, C. J. and E. W. Orlandi (1995). "Active oxygen in plant pathogenesis." Annu. Rev. Phytopathol. **33**: 299-321.

Barton, H. A., Z. Johnson, et al. (1996). "Ferric uptake regulator mutants of Pseudomonas aeruginosa with distinct alterationsin the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments." Mol. Microbiol. **21**: 1001-1017.

Beaumont, H. J., S. I. Lens, et al. (2004). "Expression of nitrite reductase in Nitrosomonas europaea involves NsrR, a novel nitrite-sensitive transcription repressor." Mol. Microbiol. **54**: 148-158.

Beers, R. F. and I. W. Sizer (1952). "A spectrophotometric method for measuring the break down of hydrogen peroxide by catalase." J. Biol. Chem. **195**: 133-140.

Bellini, P. and A. M. Hemmings (2006). "In vitro characterization of a bacterial manganese uptake regulator of the fur superfamily." Biochemistry **28**: 2686-2698.

Bijlsma, J. J., B. Waidner, et al. (2002). "The Helicobacter pylori homologue of the ferric uptake regulator is involved in acid resistance." Infect. Immun. **70**: 606-611.

Bodenmiller, D. M. and S. Spiro (2006). "The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator." J. Bacteriol. **188**: 874-881.

Calderwood, S. B. and J. J. Mekalanos (1987). "Iron regulation of Shiga-like toxin expression in Escherichia coli is mediated by the fur locus." J. Bacteriol. **169**: 4759- 4764.

Cangelosi, G. A., R. G. Ankenbauer, et al. (1990). "Sugars induce the Agrobacterium virulence genes through a periplasmic binding protein and a transmembrane signal protein." Proc. Natl. Acad. Sci. U S A. **87**: 6708-6712.

Cangelosi, G. A., E. A. Best, et al. (1991). "Genetic analysis of Agrobacterium." Methods Enzymol. **204**: 384-397.

Chao, T. C., A. Becker, et al. (2004). "The Sinorhizobium meliloti fur gene regulates, with dependence on Mn(II), transcription of the sitABCD operon, encoding a metal-type transporter." J. Bacteriol. **186**: 3609-3620.

Chao, T. C., J. Buhrmester, et al. (2005). "Role of the regulatory gene rirA in the transcriptional response of Sinorhizobium meliloti to iron limitation." Appl. Environ. Microbiol. **71**: 5969-5982.

Charles, T. C. and E. W. Nester (1993). "A chromosomally encoded two-component sensory transduction system is required for virulence of Agrobacterium tumefaciens." J. Bacteriol. **175**: 6614-6625.

Coy, M. (1995). "The interaction of the ferric uptake regulator protein with DNA." Biochem. Biophys. Res. **212**: 784-792.

DeFeyter, R., C. I. Kado, et al. (1990). "Small, stable shuttle vectors for use in Xanthomonas." Gene **88**: 65-72.

Delany, I., R. Rappuoli, et al. (2004). "Fur functions as an activator and as a repressor of putative virulence genes in Neisseria meningitidis." Mol. Microbiol. **52**: 1081-1090.

Diaz-Mireles, E., M. Wexler, et al. (2004). "The Fur-like protein Mur of Rhizobium leguminosarum is a Mn(2+)-responsive transcriptional regulator." Microbiology **150**: 1447-1456.

Diaz-Mireles, E., M. Wexler, et al. (2005). "The manganese-responsive repressor Mur of Rhizobium leguminosarum is a member of the Fur-superfamily that recognizes an unusual operator sequence." Microbiology **151**: 4071-4078.

Douglas, C. J., R. J. Staneloni, et al. (1985). "Identification and genetic analysis of an Agrobacterium tumefaciens chromosomal virulence region." J. Bacteriol. **161**: 850-860.

Elgrably-Weiss, M., S. Park, et al. (2002). "A Salmonella enterica serovar typhimurium hemA mutant is highly susceptible to oxidative DNA damage." J. Bacteriol. **184**: 3774-3784.

Ernst, F. D., G. Homuth, et al. (2005). "Iron-responsive regulation of the Helicobacter pylori iron-cofactored superoxide dismutase SodB is mediated by Fur." J. Bacteriol. **187**: 3687-3692.

Franza, T., C. Sauvage, et al. (1999). "Iron regulation and pathogenicity in Erwinia chrysanthemi 3937: role of the Fur repressor protein." Mol. Plant Microbe. Interact. **12**: 119-128.

Friedman, Y. E. and M. R. O'Brian (2003). "A novel DNA-binding site for the ferric uptake regulator (Fur) protein from Bradyrhizobium japonicum." J. Biol. Chem. **278**: 38395-38401.

Friedman, Y. E. and M. R. O'Brian (2004). "The ferric uptake regulator (Fur) protein from Bradyrhizobium japonicum is an iron-responsive transcriptional repressor in vitro." J. Biol. Chem. **279**: 32100-32105.

Goldberg, M. B., S. A. Boyko, et al. (1991). "Positive transcriptional regulation of an iron-regulated virulence gene in Vibrio cholerae." Proc. Natl .Acad. Sci. U S A. **88**: 1125-1129.

Gray, J., J. Wang, et al. (1992). "Mutation of the miaA gene of Agrobacterium tumefaciens results in reduced vir gene expression." J. Bacteriol. **174**: 1086-1098.

Gregory, E. M. and I. Fridovich (1973). "Oxygen toxicity and the superoxide dismutase." J. Bacteriol. **114**: 1193-1197.

Hall, H. K. and J. W. Foster (1996). "The role of fur in the acid tolerance response of Salmonella typhimurium is physiologically and genetically separable from its role in iron acquisition." J. Bacteriol. **178**: 5683-5691.

Hamza, I., S. Chauhan, et al. (1998). "The bacterial irr protein is required for coordination of heme biosynthesis with iron availability." J. Biol. Chem. **273**: 21669-21674.

Hamza, I., R. Hassett, et al. (1999). "Identification of a functional fur gene in Bradyrhizobium japonicum." J. Bacteriol. **181**: 5843-5846.

Hamza, I., Z. Qi, et al. (2000). "Fur-independent regulation of iron metabolism by Irr in Bradyrhizobium japonicum." Microbiology **146**: 669-676.

Hantke, K. (1987). "Selection procedure for deregulated iron transport mutants (fur) in Escherichia coli K 12: fur not only affects iron metabolism." Mol. Gen. Genet. **210**: 135-139.

Hassett, D. J., B. E. Britigan, et al. (1987). "Bacteria form intracellular free radicals in response to paraquat and streptonigrin. Demonstration of the potency of hydroxyl radical." J. Biol. Chem. **262**: 13404-13408.

Hassett, D. J., P. A. Sokol, et al. (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.

Horsburgh, M. J., E. Ingham, et al. (2001). "In Staphylococcus aureus, Fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis." J. Bacteriol. **183**: 468-475.

Imlay, J. A., S. M. Chin, et al. (1988). "Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro." Science **240**: 640-642.

Janakiraman, A. and J. M. Slauch (2000). "The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of Salmonella typhimurium." Mol. Microbiol. **35**: 1146-1155.
Keon, R. G., R. Fu, et al. (1997). "Deletion of two downstream genes alters expression of the hmc operon of Desulfovibrio vulgaris subsp. vulgaris Hildenborough." Arch. Microbiol. **167**: 376-383.
Kovach, M. E., P. H. Elzer, et al. (1995). "Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes." Gene **166**: 175-176.
Lam, M. S., C. M. Litwin, et al. (1994). "Vibrio cholerae fur mutations associated with loss of repressor activity: implications for the structural-functional relationships of fur." J. Bacteriol. **176**: 5108-5115.
LeVier, K. and M. L. Guerinot (1996). "The Bradyrhizobium japonicum fegA gene encodes an iron-regulated outer membrane protein with similarity to hydroxamate-type siderophore receptors." J. Bacteriol. **178**: 7265-7275.
Lewin, A. C., P. A. Doughty, et al. (2002). "The ferric uptake regulator of Pseudomonas aeruginosa has no essential cysteine residues and does not contain a structural zinc ion." Microbiology **148**: 2449-2456.
Li, L., Y. Li, et al. (1999). "GFP-aided confocal laser scanning microscopy can monitor Agrobacterium tumefaciens cell morphology and gene expression associated with infection." FEMS Microbiol. Lett. **179**: 141-146.
Litwin, C. M. and S. B. Calderwood (1993). "Role of iron in regulation of virulence genes." Clin. Microbiol. Rev. **6**: 137-149.
Liu, P., D. W. Wood, et al. (2005). "Phosphoenolpyruvate carboxykinase is an acid-induced, chromosomally encoded virulence factor in Agrobacterium tumefaciens." J. Bacteriol. **187**: 6039-6045.
Loprasert, S., R. Sallabhan, et al. (2000). "Characterization and mutagenesis of fur gene from Burkholderia pseudomallei." Gene **254**: 129-137.
Masse, E., F. E. Escorcia, et al. (2003). "Coupled degradation of a small regulatory RNA and its mRNA targets in Escherichia coli." Genes Dev. **17**: 2374-2383.
Masse, E. and S. Gottesman (2002). "A small RNA regulates the expression of genes involved in iron metabolism in Escherichia coli." Proc. Natl. Acad. Sci. USA. **99**: 4620-4625.
Matthysse, A. G. (1987). "Characterization of nonattaching mutants of Agrobacterium tumefaciens." J. Bacteriol. **169**: 313-323.
Metts, J., J. West, et al. (1991). "Characterization of three Agrobacterium tumefaciens avirulent mutants with chromosomal mutations that affect induction of vir genes." J. Bacteriol. **173**: 1080-1087.
Mey, A. R., E. E. Wyckoff, et al. (2005). "Iron and fur regulation in Vibrio cholerae and the role of fur in virulence." Infect. Immun. **73**: 8167-8178.
Mila, I., A. Scalbert, et al. (1996). "Iron withholding by plant polyphenols and resistance to pathogens and rots." Phytochemistry **42**: 1551-1555.
Miller, J. H. "A short course in bacterial genetics, Cold Spring Habor Laboratory, Cold Spring Harbor, NY, 1992."
Murashige, T. and F. Skoog (1962). "A revised medium for rapid
growth and bioassay with tobacco tissue culture." Physiol. Plant **15**: 473-496.
Neema, C., J. P. Laulhere, et al. (1993). "Iron Deficiency Induced by Chrysobactin in Saintpaulia Leaves Inoculated with Erwinia chrysanthemi." Plant Physiol. **102**: 967-973.
Palyada, K., D. Threadgill, et al. (2004). "Iron acquisition and regulation in Campylobacter jejuni." J. Bacteriol. **186**: 4714-4729.

Platero, R., L. Peixoto, et al. (2004). "Fur is involved in manganese-dependent regulation of mntA (sitA) expression in Sinorhizobium meliloti." Appl. Environ. Microbiol. **70**(4349-4355).

Pohl, E. P., J. C. Haller, et al. (2003). "Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator." Mol. Microbiol. **47**: 903-915.

Prapagdee, B., W. Eiamphungporn, et al. (2004). "Analysis of growth phase regulated KatA and CatE and their physiological roles in determining hydrogen peroxide resistance in Agrobacterium tumefaciens." FEMS Microbiol. Lett. **237**: 219-226.

Prince, R. W., C. D. Cox, et al. (1993). "Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the Pseudomonas aeruginosa fur gene." J. Bacteriol. **175**: 2589-2598.

Qi, Z. and M. R. O'Brian (2002). "Interaction between the bacterial iron response regulator and ferrochelatase mediates genetic control of heme biosynthesis." Mol. Cell **9**: 155-162.

Rea, R. B., C. G. Gahan, et al. (2004). "Disruption of putative regulatory loci in Listeria monocytogenes demonstrates a significant role for Fur and PerR in virulence." Infect. Immun. **72**: 717-727.

Sambrook, J., E. F. Fritsch, et al. (1989). "Molecular cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York."

Scalbert, A. (1991). "Antimicrobial properties of tannins." Phytochemistry **30**: 3875-3883.

Schwartz, C. J., J. L. Giel, et al. (2001). "IscR, an Fe-S cluster-containing transcription factor, represses expression of Escherichia coli genes encoding Fe-S cluster assembly proteins." Proc. Natl. Acad. Sci. U S A: 14895-14900.

Schwyn, B. and J. B. Neilands (1987). "Universal chemical assay for the detection and determination of siderophores." Anal. Biochem. **160**: 47-56.

Su, S., B. B. Stephens, et al. (2006). "Lon protease of the alpha-proteobacterium Agrobacterium tumefaciens is required for normal growth, cellular morphology and full virulence." Microbiology **152**: 1197-1207.

Subramoni, S. and V. S. Ramesh (2005). "Growth deficiency of a Xanthomonas oryzae pv. oryzae fur mutant in rice leaves is rescued by ascorbic acid supplementation." Mol. Plant Microbe Interact. **18**: 644-651.

Suksomtip, M., P. Liu, et al. (2005). "Citrate synthase mutants of Agrobacterium are attenuated in virulence and display reduced vir gene induction." J. Bacteriol. **187**: 4844-4852.

Thomas, C. E. and P. F. Sparling (1996). "Isolation and analysis of a fur mutant of Neisseria gonorrhoeae." J. Bacteriol. **178**: 4224-4232.

Thomashow, M. F., J. E. Karlinsey, et al. (1987). "Identification of a new virulence locus in Agrobacterium tumefaciens that affects polysaccharide composition and plant cell attachment." J. Bacteriol. **169**: 3209-3216.

Thompson, D. K., A. S. Beliaev, et al. (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., M. Wexler, et al. (2002). "RirA, an iron-responsive regulator in the symbiotic bacterium Rhizobium leguminosarum." Microbiology **148**: 4059-4071.

Tolmasky, M. E., A. M. Wertheimer, et al. (1994). "Characterization of the Vibrio anguillarum fur gene: role in regulation of expression of the FatA outer membrane protein and catechols." J. Bacteriol. **176**: 213-220.

Touati, D., M. Jacques, et al. (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.
Vincent, J. M. (1970). "A Manual for the Practical Study of Root Nodule Bacteria." Oxford, UK; Blackwell Scientific Publications.
Waidner, B., S. Greiner, et al. (2002). "Essential role of ferritin Pfr in Helicobacter pylori iron metabolism and gastric colonization." Infect. Immun. **70**: 3923-3929.
Wexler, M., J. D. Todd, et al. (2003). "Fur is not the global regulator of iron uptake genes in Rhizobium leguminosarum." Microbiology **149**: 1357-1365.
White, J. R. and H. N. Yeowell (1982). "Iron enhances the bactericidal action of streptonigrin." Biochem. Biophys. Res. Commun. **106**: 407-411.
Wilson, T. J., N. Bertrand, et al. (1998). "The rpfA gene of Xanthomonas campestris pathovar campestris, which is involved in the regulation of pathogenicity factor production, encodes an aconitase." Mol. Microbiol. **28**: 961-970.
Winans, S. C., R. A. Kerstetter, et al. (1988). "Transcriptional regulation of the virA and virG genes of Agrobacterium tumefaciens." J. Bacteriol. **170**: 4047-4054.
Winkelmann, G. (2002). "Microbial siderophore-mediated transport." Biochem. Soc. Trans. **30**: 691-696.
Wirawan, I. G., H. W. Kang, et al. (1993). "Isolation and characterization of a new chromosomal virulence gene of Agrobacterium tumefaciens." J. Bacteriol. **175**: 3208-3212.
Wood, D. W., J. C. Setubal, et al. (2001). "The genome of the natural genetic engineer Agrobacterium tumefaciens C58." Science **294**: 2317-2323.
Xu, X. Q. and S. Q. Pan (2000). "An Agrobacterium catalase is a virulence factor involved in tumorigenesis." Mol. Microbiol. **35**: 407-414.
Yang, J., H. R. Panek, et al. (2006). "Oxidative stress promotes degradation of the Irr protein to regulate haem biosynthesis in Bradyrhizobium japonicum." Mol. Microbiol. **60**: 209-218.
Yeowell, H. N. and J. White (1982). "Iron requirement in the bactericidal mechanism of streptonigrin." Antimicrob. Agents. Chemother. **22**: 961-968.
Zhou, D., W. D. Hardt, et al. (1999). "Salmonella typhimurium encodes a putative iron transport system within the centisome 63pathogenicity island." Infect. Immun. **67**: 1974-1981.
Ziemienowicz, A. (2001). "Odyssey of agrobacterium T-DNA." Acta Biochim. Pol. **48**: 623-635.

TABLE 1. Bacterial strains, plasmids and used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
A. tumefaciens		
NTL4	Wild-type	Luo 2001
NTLfur	*fur* mutant, derivative of NTL4 in which *fur* was disrupted by pKNOCKfur, Gm^r	This study
E. coli		
DH5α	*supE Dlac(f80ZDM15) hsdR recA endA gyrA thi relA*	Laboratory collection
Plasmids		
pGEM-T-easy	a cloning vector, Ap^r	Promega
pKNOCK-Gm	a suicide vector, Gm^r	Alexeyev 1999
pKNOCKfur	pKNOCK-Gm containing a 169 bp *Bam*HI-*Eco*RI fragment of *fur* coding region, Gm^r	This study
pBBR1MCS-4	an expression vector, Ap^r	Kovach 1995
pFur	full length *fur* coding region cloned into pBBR1MCS-4, Ap^r	This study
pUFR047*lacZ*	a promoter probe vector, Tet^r	DeFeyter 1990
p*fhuA-lacZ*	a 414 bp PCR fragment containing *fhuA* promoter cloned into pUFR047*lacZ*,Tet^r	This study
p*katA-lacZ*	a 330 bp PCR fragment containing *katA* promoter cloned into pUFR047*lacZ*; Tet^r	Nakjarung 2003

Figure legend

FIG. 1. The NTLfur mutant exhibits hypersensitivity to the iron-limiting condition and is iron-deficient compared with wild-type NTL4. (A) Sensitivity to 2,2'-dipyridyl (Dipy). NTL4/pBBR and NTLfur/pBBR strains are wild-type and *fur* mutant, respectively, containing the plasmid vector pBBR1MCS-4. NTL4/pFur and NTLfur/pFur strains are wild-type and *fur* mutant, respectively, expressing functional Fur on the plasmid pFur. Cells were grown under iron-sufficient (LB) and iron-limiting (LB+300 μM Dipy) conditions. Growth was monitored by measuring OD_{600} after incubation at 28°C with shaking for 24 h. Values presented are means and the error bars indicate standard deviations of three replicates. (B) Sensitivity to streptonigrin. Intracellular iron levels in the wild-type and mutant strains were assessed by sensitivity to the iron-activated antibiotic, streptonigrin (SNG). Cells grown on LB agar plates containing 300 μM Dipy for 2 days were washed and resuspended in LB medium. Cells (10^4) were treated with 100 μg ml^{-1} SNG (added as a solution in dimethyl sulfoxide) in LB medium. Control cells (untreated) received equivalent amounts of dimethyl sulfoxiide. After incubation at 28°C for 24 h, cell cultures were diluted (10-fold serial dilutions). An aliquot (10 μl) of each dilution was spotted on LB agar plates and incubated at 28°C for 2 days. Each strain was performed in duplicate and the experiment was repeated twice.

FIG. 2. Effect of the *fur* mutation on siderophore production and on expression of *fhuA-lacZ* fusion. Analysis of siderophore production was performed using siderophore indicator CAS agar plates. Wild-type NTL4 and NTLfur mutant strains were spotted onto YEM+CAS agar plates containing 50 μM $FeCl_3$ (A) or 200 μM Dipy (B), and incubated at 28°C for 2 days. A halo zone surrounding bacteria indicates the production of siderophores. (C) Expression of *fhuA–lacZ* fusion in wild-type and *fur* mutant backgrounds. β-galactosidase activities were measured from wild-type NTL4 and NTLfur mutant containing the *fhuA–lacZ* fusion plasmid

pP*fhuA-lacZ* grown in LB medium with 50 μM $FeCl_3$ (Fe) or 200 μM Dipy (Dippy). Values are means and standard errors of three replicates.

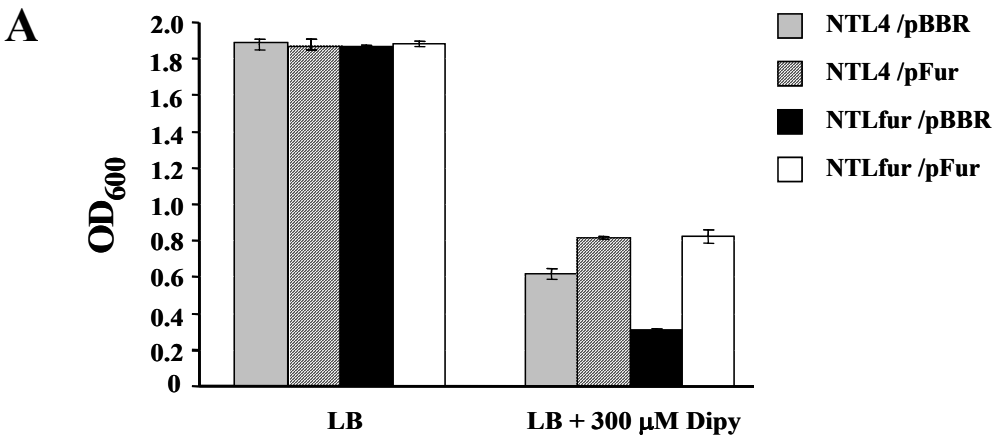
FIG. 3. Effect of the *fur* mutation on *sitA* expression. Exponential phase cells from wild-type NTL4 and NTLfur mutant were untreated (Un) or treated with 50μM $FeCl_3$ (Fe), and 50μM $MnCl_2$ (Mn) for 15 min. Reverse transcriptase PCR (RT-PCR) was used to analyze the mRNA expression of the *sitA* genes. (A) *sitA* amplicon. (B) 16S rRNA amplicon. P: positive control.

FIG. 4. The NTLfur mutant is hypersensitive to hydrogen peroxide and exhibits reduced catalase activity. (A) Sensitivity to H_2O_2. Cells grown on LB agar plates at 28°C for 2 days were adjusted to an OD_{600} at 0.01in LB medium and were 10-fold serial diluted. An aliquot (10 μl) of each dilution was spotted on LB agar plates containing 400 μM H_2O_2 and incubated at 28°C for 48 h. Control: cells were spotted on LB agar plate (LA). NTL4/pBBR and NTLfur/pBBR strains are wild-type and *fur* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTL4/pFur and NTLfur/pFur strains are wild-type and *fur* mutant, respectively, expressing functional Fur on the plasmid pFur. Each strain was performed in duplicate and the experiment was repeated twice. (B) Catalase activity assay. Cells were harvested after grown on LB agar plates at 28°C for 2 days. Catalase activity was determined by the method of Beers and Sizer (1952). The results are the means and standard errors of three replicates. (C) Expression of *katA–lacZ* fusion in wild-type and *fur* mutant backgrounds. β-galactosidase activities were measured from wild-type NTL4 and NTLfur mutant containing the *katA–lacZ* fusion plasmid pP*katA-lacZ* grown on LB agar plates at 28°C for 2 days. Values are means and standard errors of three replicates.

FIG. 5. Virulence assay. Tumor formation was determined on tobacco leaf squares infected with *A. tumefaciens* wild-type NTL4 or NTLfur mutant containing pTiC58 (pTi) plasmid, (NTL4/pTi and NTLfur/pTi respectively). NTLfur mutant was complemented by expression of functional Fur from a plasmid pFur (NTLfur/pTi/pFur). Control: tobacco leaf squares without infection. Representative leaf pieces (from n=30) are shown.

FIG. 6. Primary sequence alignment generated using CLUSTALW (Pearson 1990) of selected proteins belonging to the Fur superfamily. Sequences shown are those from *A. tumefaciens* (Fur_{At}), *R. leguminosarum* (Mur_{Rl}), *B. japonicum* (Fur_{Bj}), *S. meliloti* (Fur_{Sm}), *E. coli* (Fur_{Ec}), and *P. aeruginosa* (Fur_{Pa}). The putative regulatory Fe-sensing site residues (S1) are indicated with circles, and the structural Zn-binding site residues (S2) are indicated with triangles. Residues involved in the DNA binding are shaded in gray.

FIG. 1


A
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD600
LB
LB + 300 μM Dipy
NTL4 /pBBR
NTL4 /pFur
NTLfur /pBBR
NTLfur /pFur

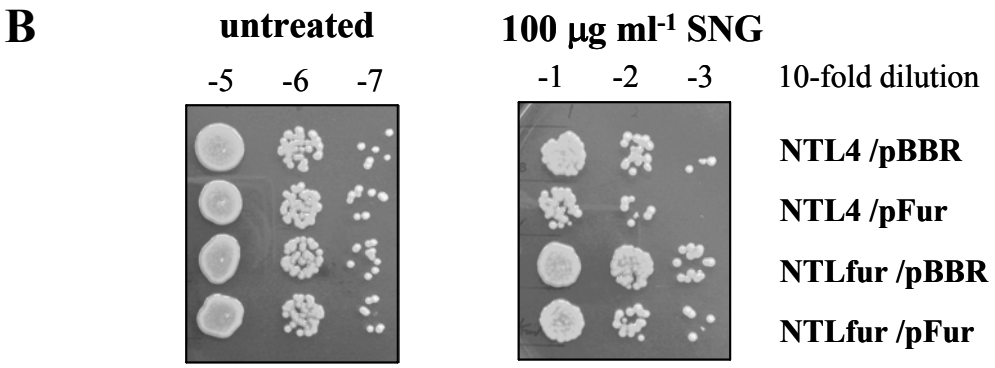

B
untreated
100 μg ml-1 SNG
-5
-6
-7
-1
-2
-3
10-fold dilution
NTL4 /pBBR
NTL4 /pFur
NTLfur /pBBR
NTLfur /pFur

FIG. 2

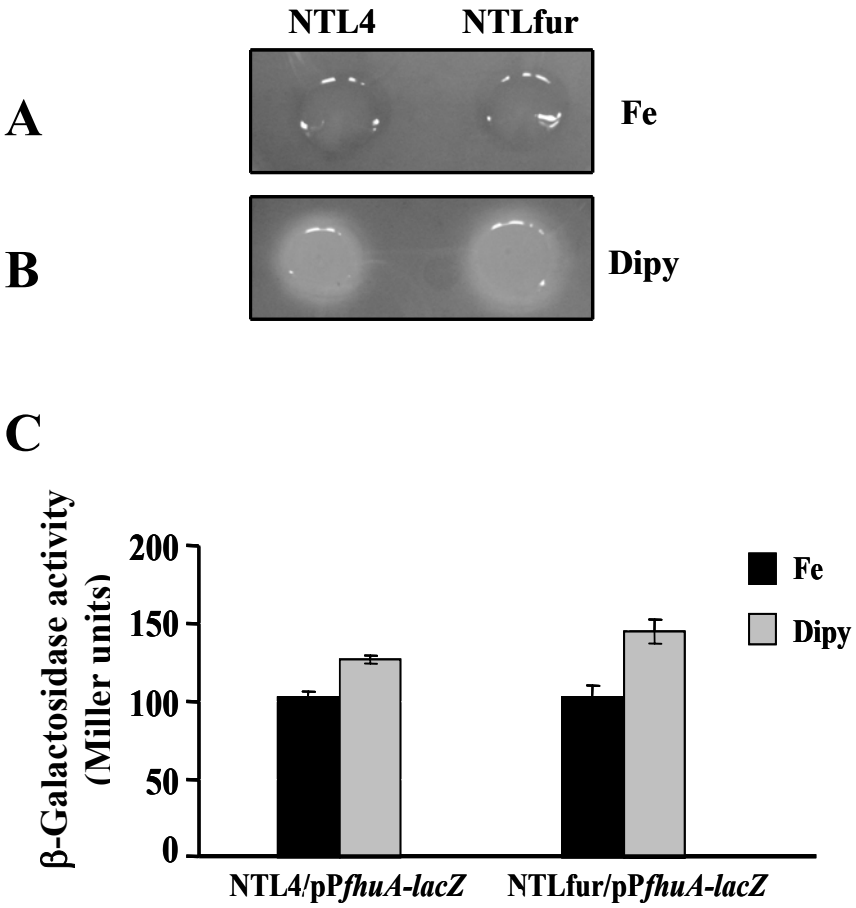

NTL4
NTLfur
A
Fe
B
Dipy
C
β-Galactosidase activity (Miller units)
200
150
100
50
0
Fe
Dipy
NTL4/pP*fhuA-lacZ*
NTLfur/pP*fhuA-lacZ*

FIG. 3

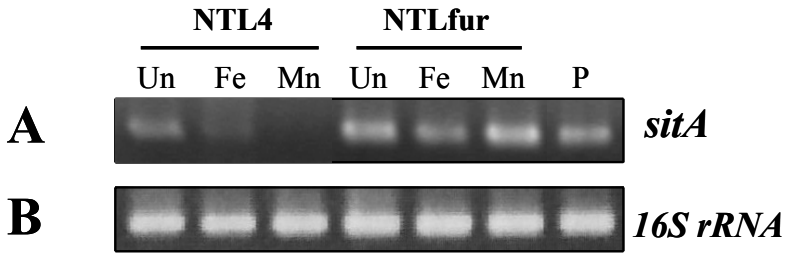


FIG. 4

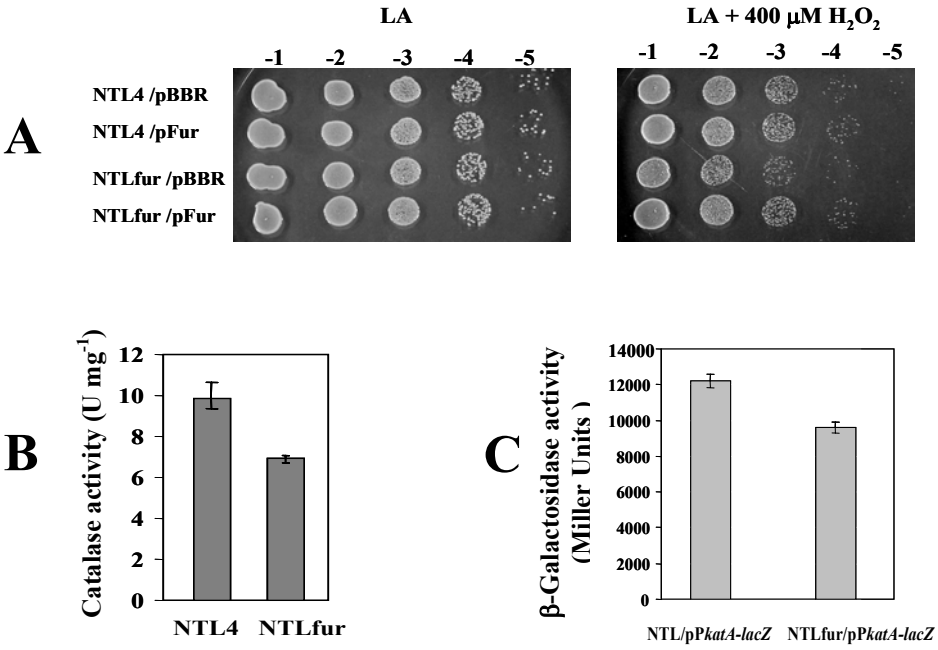

A
LA
LA + 400 μM H_2O_2
-1 -2 -3 -4 -5
NTL4 /pBBR
NTL4 /pFur
NTLfur /pBBR
NTLfur /pFur
B
Catalase activity (U mg^{-1})
0 2 4 6 8 10 12
NTL4 NTLfur
C
β-Galactosidase activity (Miller Units)
0 2000 4000 6000 8000 10000 12000 14000
NTL/pP*katA-lacZ* NTLfur/pP*katA-lacZ*

FIG. 5

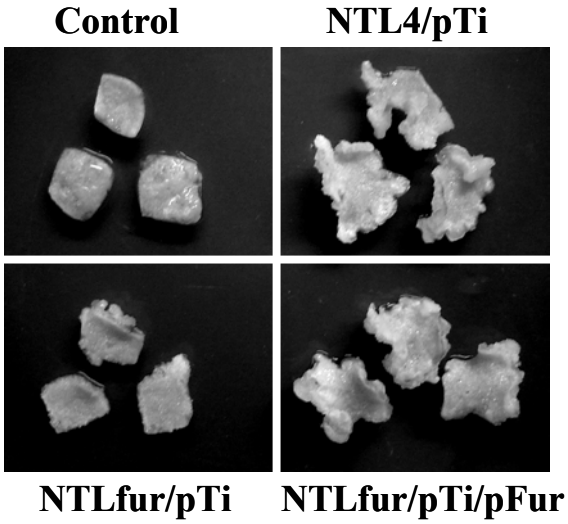
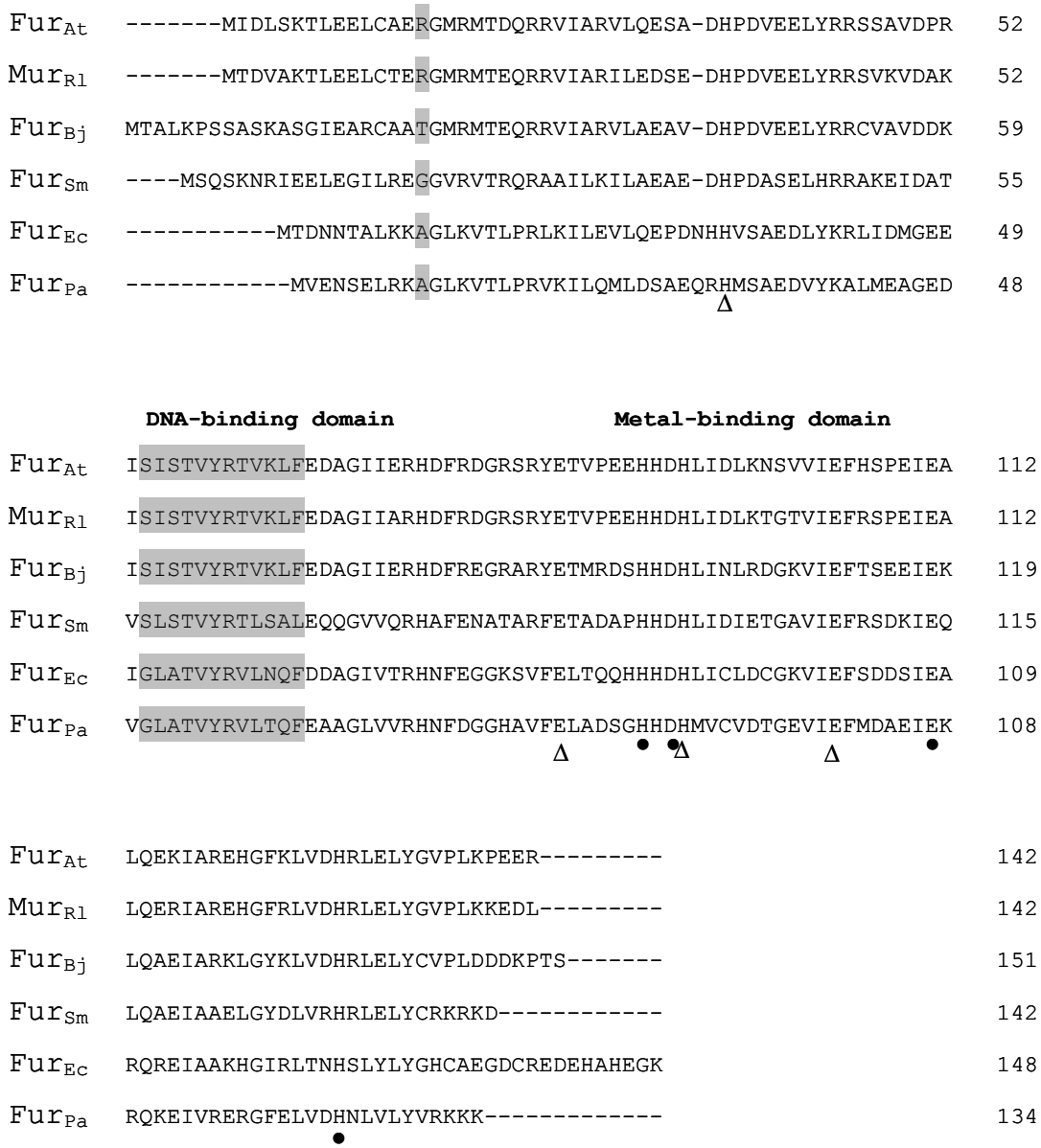


FIG.6


Fur At -------MIDLSKTLEELCAERGMRMTDQRRVIARVLQESA-DHPDVEELYRRSSAVDPR 52
Mur Rl -------MTDVAKTLEELCTERGMRMTEQRRVIARILEDSE-DHPDVEELYRRSVKVDAK 52
Fur Bj MTALKPSSASKASGIEARCAATGMRMTEQRRVIARVLAEAV-DHPDVEELYRRCVAVDDK 59
Fur Sm ----MSQSKNRIEELEGILREGGVRVTRQRAAILKILAEAE-DHPDASELHRRAKEIDAT 55
Fur Ec -----------MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEE 49
Fur Pa ------------MVENSELRKAGLKVTLPRVKILQMLDSAEQRHMSAEDVYKALMEAGED 48
DNA-binding domain
Metal-binding domain
Fur At ISISTVYRTVKLFEDAGIIERHDFRDGRSRYETVPEEHHDHLIDLKNSVVIEFHSPEIEA 112
Mur Rl ISISTVYRTVKLFEDAGIIARHDFRDGRSRYETVPEEHHDHLIDLKTGTVIEFRSPEIEA 112
Fur Bj ISISTVYRTVKLFEDAGIIERHDFREGRARYETMRDSHHDHLINLRDGKVIEFTSEEIEK 119
Fur Sm VSLSTVYRTLSALEQQGVVQRHAFENATARFETADAPHHDHLIDIETGAVIEFRSDKIEQ 115
Fur Ec IGLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLICLDCGKVIEFSDDSIEA 109
Fur Pa VGLATVYRVLTQFEAAGLVVRHNFDGGHAVFELADSGHHDHMVCVDTGEVIEFMDAEIEK 108
Fur At LQEKIAREHGFKLVDHRLELYGVPLKPEER--------- 142
Mur Rl LQERIAREHGFRLVDHRLELYGVPLKKEDL--------- 142
Fur Bj LQAEIARKLGYKLVDHRLELYCVPLDDDKPTS------- 151
Fur Sm LQAEIAAELGYDLVRHRLELYCRKRKD---------- 142
Fur Ec RQREIAAKHGIRLTNHSLYLYGHCAEGDCREDEHAHEGK 148
Fur Pa RQKEIVRERGFELVDHNLVLYVRKKK---------- 134