Pleiotropic roles of iron-responsive transcriptional regulator Fur in *Burkholderia multivorans*

Satoshi Yuhara, Harunobu Komatsu, Hiroyuki Goto, Yoshiyuki Ohtsubo, Yuji Nagata and Masataka Tsuda

Correspondence

Masataka Tsuda
mtsuda@ige.tohoku.ac.jp

Department of Environmental Life Sciences, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai 980-8577, Japan

The *fur* (ferric uptake regulator) gene of *Burkholderia multivorans* ATCC 17616 was identified by transposon mutagenesis analysis. The *fur* deletion mutant of strain ATCC 17616 (i) constitutively produced siderophores, (ii) was more sensitive to reactive oxygen species (ROS) than the wild-type strain, (iii) showed lower superoxide dismutase and catalase activities than the wild-type strain, (iv) was unable to grow on M9 minimal agar plates containing several substrates that can be used as sole carbon sources by the wild-type strain, and (v) was hypersensitive to nitrite and nitric oxide under microaerobic and aerobic conditions, respectively. These results clearly indicate that the Fur protein in strain ATCC 17616 plays pleiotropic roles in iron homeostasis, removal and/or resistance to ROS and nitrosative stress, and energy metabolism. Furthermore, employment of an *in vivo* Fur titration assay system led to the isolation from the ATCC 17616 genome of 13 Fur-binding DNA regions, and a subsequent electrophoretic mobility-shift assay confirmed the direct binding of Fur protein to all of these DNA regions. Transcriptional analysis of the genes located just downstream of the Fur-binding sites demonstrated that Fur acts as a repressor for these genes. Nine of the 13 regions were presumed to be involved in the acquisition and utilization of iron.

Received 1 December 2007

Revised 27 February 2008

Accepted 5 March 2008

INTRODUCTION

The genus *Burkholderia* belongs to the *Betaproteobacteria* which have been isolated from various environments, including humans, animals, plants, water, rhizosphere and soils (Chiarini *et al.*, 2006). While some strains in this genus are plant pathogens and opportunistic human pathogens (especially in cystic fibrosis patients), other strains have useful properties, e.g. the biocontrol of plant pathogens and the bioremediation of polluted environments (Parke & Gurian-Sherman, 2001). *Burkholderia multivorans* is the second most common species in the so-called *Burkholderia cepacia* complex that is frequently associated with cystic fibrosis (Mahenthiralingam *et al.*, 2005). This species is also prevalent in a diversity of natural environments, probably due to its broad metabolic capacity to use a number of carbon and nitrogen sources (Lessie *et al.*, 1996). *B. multivorans* ATCC 17616 is a soil-

derived strain whose extraordinary metabolic versatility has been well analysed. As is the case in other *Burkholderia* strains, the ATCC 17616 genome has multiple replicons consisting of three chromosomes of 3.4, 2.5 and 0.9 Mb (designated Chr I, Chr II and Chr III, respectively) and a 170 kb plasmid, pTGL1 (Cheng & Lessie, 1994). This strain shows extensive genomic plasticity, exhibiting, for example, frequent and large-scale insertions and deletions (Lessie *et al.*, 1996). Our previous investigations of this strain using Tn5-based transposon mutagenesis have also clarified the unique distribution of housekeeping and functional auxotrophic genes on both Chr I and Chr II (Komatsu *et al.*, 2003; Nagata *et al.*, 2005).

Iron is an essential element for the growth of almost all bacterial species. The solubility of ferric ion is very low in eukaryotic hosts as well as natural environments, and bacteria have evolved various kinds of efficient mechanisms to acquire iron under limiting conditions. However, a high concentration of intracellular iron is very toxic under aerobic conditions, and intracellular ferrous iron, for example, reacts with metabolically produced H₂O₂ to generate a highly toxic and reactive hydroxyl radical by the Fenton reaction (Andrews *et al.*, 2003). Therefore, many bacteria have their own systems to strictly maintain intracellular iron homeostasis, and one of the key regulator proteins for this homeostasis is an iron-responsive

Abbreviations: CAS, Chrome Azurol S; (P)EB, (Poor) Environmental Bacteria (medium); ECF, extracytoplasmic function; EMSA, electrophoretic mobility-shift assay; Fur, ferric uptake regulator; FURTA, Fur titration assay; qRT-PCR, quantitative real-time reverse-transcription PCR; ROS, reactive oxygen species; SOD, superoxide dismutase.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AP009835–AP009838.

Two supplementary tables with details of the PCR primer sequences and gene annotation are available with the online version of this paper.

transcriptional regulator, Fur (ferric uptake regulator). The Fur protein binds to specific sequences (Fur boxes), and under iron-rich conditions, usually represses the transcription of genes located downstream of Fur boxes (Escobar *et al.*, 1999). Although many of these genes specify functions for the acquisition and utilization of iron, Fur proteins in pathogenic bacteria have also been shown to function as global regulators for (i) removal of reactive oxygen species (ROS) (Thompson *et al.*, 2002; Touati *et al.*, 1995), (ii) production of virulence factors (Litwin & Calderwood, 1993; Mey *et al.*, 2005), (iii) acid tolerance (Hall & Foster, 1996), (iv) formation of biofilms (Banin *et al.*, 2005), and (v) removal of reactive nitrogen species (Abdul-Tehrani *et al.*, 1999; Mukhopadhyay *et al.*, 2004). Fur is further considered to have essential but unknown function(s) in some bacterial strains because null mutants have not been obtained despite repeated attempts. *Pseudomonas aeruginosa* is one such bacterium, and analysis of a *fur* point mutant in this species has revealed that its wild-type Fur protein most probably regulates the expression of various genes, including those involved in the catabolism of carbon sources, and aerobic and anaerobic respiration (Vasil, 2007).

Fur proteins in the genus *Burkholderia* have been predicted to play pleiotropic roles in the regulation of genes for iron acquisition, intracellular iron storage and release, and

removal of ROS (Loprasert *et al.*, 2000; Lowe *et al.*, 2001; Tuanyok *et al.*, 2005). However, it remains unclear whether Fur is definitely involved in all of these roles. This is because no null mutants of *fur* have been obtained, and in the case of *Burkholderia pseudomallei*, such a mutant has been considered to be lethal for (an) unknown reason(s) (Loprasert *et al.*, 2000). We have indicated in this study that the Fur function in *B. multivorans* ATCC 17616 is not essential for the growth and/or viability of the cells. Subsequent analysis of a *fur* deletion mutant clearly demonstrated that the Fur protein in ATCC 17616 is pleiotropically involved in iron homeostasis, removal of ROS, and carbon and nitrogen metabolism.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were cultivated at 37 °C and *B. multivorans* cells at 30 °C. AnaeroPack-Anaero and AnaeroPack-Microaero systems (Mitsubishi Gas Chemical) were used for cultivation of *B. multivorans* under anaerobic and microaerobic (5% O₂) conditions, respectively. The liquid media used were Luria-Bertani (LB) broth (Ausubel *et al.*, 1991), Environmental Bacteria (EB) broth (l⁻¹: 3.3 g Bacto Tryptone, 1.7 g yeast extract, 5 g NaCl), Poor EB (PEB) broth (l⁻¹: 1 g Bacto Tryptone, 0.5 g yeast extract, 5 g NaCl) and M9 minimal medium with an appropriate carbon source at 0.2% (w/v). Solid media were prepared by the addition of 1.5% (w/v) agar. The production of

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
DH5 α	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 Δ(lac)U169 (ϕ80dlacΔM15)</i>	Ausubel <i>et al.</i> (1991)
BL21(DE3)	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm λ(DE3)</i>	Studier & Moffatt (1986)
H1780	<i>fur fuu::λplacMu</i>	Hantke (1987)
H1717	<i>aroB fhuF::λplacMu</i>	Hantke (1987)
JM109 (λ pir)	<i>λpir</i> lysogen of JM109	Lab collection
<i>B. multivorans</i>		
ATCC 17616	Soil isolate; type strain	ATCC
BT163	Tp ^r ; ATCC 17616 <i>fur::TnMod-RTp'</i>	This study
DF1	Cm ^r ; ATCC 17616 <i>fur</i> deletion mutant	This study
Plasmids		
pTnMod-RTp'	Tp ^r ; TnMod-RTp'-containing plasmid whose transposable region carries the Tp ^r gene and R6KoriV	Dennis & Zylstra (1998)
pEX18Tc	Tc ^r <i>sacB oriT</i> ; suicide vector for gene replacement carrying the pUC18-derived multiple-cloning sites	Hoang <i>et al.</i> (1998)
pACYC184	Cm ^r ; plasmid vector	Chang & Cohen (1978)
pTrc99A	Ap ^r ; expression vector	Amann & Brosius (1985)
pHSG398	Cm ^r ; plasmid vector	Takeshita <i>et al.</i> (1987)
pME6041	Km ^r ; shuttle vector able to replicate in <i>E. coli</i> and <i>B. multivorans</i>	Heeb <i>et al.</i> (2000)
pT7Blue-T	Ap ^r ; T-vector	Novagen
pColdIV	Ap ^r ; cold-shock expression vector under the control of the <i>cspA</i> promoter and <i>lacI</i> ^q	Qing <i>et al.</i> (2004)
pFur	Ap ^r ; pT7Blue-T containing the ATCC 17616 <i>fur</i> gene	This study
pDF1	Tc ^r Cm ^r ; pEX18Tc containing 5'-flanking region of <i>fur</i> -Cm ^r gene-3'-flanking region of <i>fur</i>	This study
pCF1	Km ^r ; pME6041 derivative with the <i>KpnI-PstI fur</i> -containing fragment from pFur	This study
pEF1	Ap ^r ; pTrc99A derivative with the <i>KpnI-HindIII fur</i> -containing fragment from pFur	This study
pEF2	Ap ^r ; pColdIV containing the ATCC 17616 <i>fur</i> gene	This study

siderophores was monitored on Chrome Azurol S (CAS) agar prepared by adding 10 ml CAS reagent (Schwyn & Neilands, 1987) to 90 ml PEB agar. For iron-rich and iron-limiting conditions, FeCl_3 and 2,2-dipyridyl were added to the medium at final concentrations of 30 and 100 μM , respectively. When needed, IPTG and X-Gal were used at final concentrations of 0.5 mM and 40 μM , respectively. Antibiotics were added to media at the following concentrations: (for *E. coli*) 100 μg ampicillin (Ap) ml^{-1} , 100 μg chloramphenicol (Cm) ml^{-1} , 50 μg kanamycin (Km) ml^{-1} , 10 μg tetracycline (Tc) ml^{-1} , 200 μg trimethoprim (Tp) ml^{-1} ; (for *B. multivorans*) 100 μg Cm ml^{-1} , 100 μg Km ml^{-1} , 50 μg Tc ml^{-1} , 100 μg Tp ml^{-1} .

Carbon assimilation tests. Cells grown to late exponential phase in PEB broth were washed three times with M9 minimal solution and diluted approximately 5×10^1 to 5×10^8 c.f.u. ml^{-1} using the same solution. A 5 μl aliquot of cell suspension was spotted onto EB and M9 minimal agar plates containing an appropriate carbon source. The number of c.f.u. was assessed after incubation at 30 °C for 4 days.

The oxidation of 95 different carbon compounds by *B. multivorans* cells was also examined using a Biolog GN2 microtitre-plate system. The cells grown to the late exponential phase in PEB broth were collected, washed three times with M9 minimal solution, and diluted in the same solution to an OD_{660} of 0.25. A 150 μl aliquot of cell suspension was dispersed into each well of the microtitre plate. When the carbon compound in the well is a substrate of cellular respiration that generates electrons, the colourless tetrazolium dye is reduced, giving rise to a purple colour. The purple coloration of each well was examined after incubation at 30 °C for 24 h.

Sensitivity of *B. multivorans* cells to H_2O_2 , paraquat and nitrogen compounds. The sensitivity of cells to two ROSs, H_2O_2 and superoxide, was examined as follows. A late-exponential-phase culture was spread onto EB plates onto which were placed disks supplemented with 10 μl 30% H_2O_2 (v/v) or 100 μM paraquat (a superoxide generator). The plates were incubated at 30 °C for 36 h, and the diameters of the growth inhibition zones surrounding the disks were measured. To assess the sensitivity to nitrate, nitrite or acidified nitrite [a nitric oxide (NO) producer], late-exponential-phase cells were diluted to approximately 5×10^3 – 5×10^8 c.f.u. ml^{-1} in fresh EB broth. A 10 μl aliquot of cell suspension was spotted onto an EB agar plate containing 10 mM potassium nitrate or 5 mM sodium nitrite. The sensitivity to the two nitrogen compounds was assessed by measuring the number of c.f.u. after incubation at 30 °C for 48 h under aerobic, anaerobic and microaerobic conditions. Since the growth of neither strain ATCC 17616 nor its *fur* deletion mutant (DF1) was detected on agar plates supplemented with acidified nitrite, their growth rates were measured using liquid media. Cells grown to late exponential phase (approx. 1×10^9 c.f.u. ml^{-1}) were diluted 100-fold in fresh EB broth with or without 2 mM acidified nitrite, cultivated at 30 °C, and the OD_{660} was monitored by using a Bio-Photorecorder TVS062CA (Advantec). Because the ATCC 17616 cells grew very slowly in the presence of nitrite under anaerobic conditions, the microanaerobic condition was employed to examine the sensitivity of *B. multivorans* cells to nitrite.

Native PAGE and staining for superoxide dismutase (SOD) and catalase activities. Mid-exponential-phase cells were disrupted by sonication with a Branson Sonifier for 1 min, and their lysates were recovered by centrifugation at 13 000 g for 10 min. The resulting supernatants were subjected to electrophoresis in 10% (w/v) native polyacrylamide gel. Total protein concentrations were estimated by using a Bradford colorimetric assay system (Bio-Rad). SOD and catalase activities were examined as described by Lefebvre & Valvano (2001).

Basic DNA and RNA manipulations, construction of plasmids, and allelic exchange mutagenesis. Established protocols were

used for the preparation of genomic and plasmid DNA, DNA digestion with restriction endonucleases, ligation, standard agarose gel electrophoresis, DNA sequencing, and transformation of *E. coli* and *B. multivorans* cells (Komatsu *et al.*, 2003; Sambrook & Russell, 2001). PCR was performed with *ExTaq* DNA polymerase (Takara) or KOD -Plus- DNA polymerase (Toyobo). To measure the mRNA level of each gene in *B. multivorans* cells, an Isogen kit (Nippon Gene) was used to isolate the total RNA fraction from mid-exponential-phase cells in liquid culture. Reverse transcription was carried out using an RNA PCR kit (AMV) version 3 (Takara). The cDNA was used to carry out quantitative real-time reverse-transcription PCR (qRT-PCR) using an Opticon 2 system (Bio-Rad) and SYBR Premix *ExTaq* (Takara) in a reaction volume of 10 μl . The transcript level of the *B. multivorans dnaA* gene was used as the internal standard.

A pHS398-based library of the ATCC 17616 genome was constructed by partial digestion of the genomic DNA with *Sau3AI*, ligation of the restricted fragments with *Bam*HI-treated pHS398 DNA and subsequent transformation of DH5 α . The library consisted of approximately 9×10^4 clones, and the mean size of inserts was 460 bp.

Cloning of the *TnMod*-RTp'-containing genomic region from BT163 was carried out by digestion of its genomic DNA with *Eco*RI or *Sph*I (which is absent from *TnMod*-RTp'), self-ligation and transformation of JM109(λ pir) to select the Tp^r clones. The wild-type *fur*-containing fragment of strain ATCC 17616 was amplified by PCR using the primer set FURF and FURR (see supplementary Table S1, available with the online version of this paper) and cloned into the pT7Blue T-vector (Novagen). The resulting plasmid (pFur) was digested by *Kpn*I and *Pst*I, and the DNA fragment containing *fur* was recloned into pME6041 to obtain pCF1. The wild-type *fur*-containing fragment of strain ATCC 17616 was also amplified by PCR using another primer set, FUR-F-BAMHI and FUR-R-HINDIII-NDEI (see supplementary Table S1), and cloned into the *Nde*I–*Bam*HI sites of pColdIV to construct pEF2.

To remove the *fur* gene from the ATCC 17616 genome, the three primer sets shown in supplementary Table S1 were used to amplify the following three fragments by PCR: the *fur* upstream region flanked by *Eco*RI and *Bam*HI sites at the 5' and 3' ends, respectively; the Cm^r gene fragment from pACYC184 flanked by *Bam*HI sites; and the *fur* downstream region flanked by *Bam*HI and *Hind*III sites at the 5' and 3' ends, respectively. The upstream and downstream fragments were sequentially cloned into the *Eco*RI–*Bam*HI and *Bam*HI–*Hind*III sites, respectively, of pEX18Tc, and the Cm^r gene fragment was finally inserted into the *Bam*HI site of the pEX18Tc derivative. The resulting plasmid (pDF1) was introduced into strain ATCC 17616 by electroporation to select the transformants that showed resistance to both Cm and 5% (w/v) sucrose. The expected double-cross-over-mediated homologous recombination in the transformants was confirmed by PCR. One of these transformants completely lacked the *fur* gene and was designated DF1.

Measurement of LacZ activities. LacZ activities were measured as described previously (Ohtsubo *et al.*, 2006).

Isolation Fur-binding sequences from the *B. multivorans* genome. The isolation of Fur-binding DNA sequences was carried out according to the Fur titration assay (FURTA) system developed by Stojiljkovic *et al.* (1994). The pHS398-based genomic library from ATCC 17616 was introduced into *E. coli* H1717 to select the Cm^r transformants on MacConkey lactose agar plates containing 30 μM FeCl_3 . Colonies exhibiting red coloration after 24 h incubation at 37 °C were purified on the same selective plates, and the plasmids residing in such colonies were predicted to contain the *B. multivorans* DNA sequences that bind the *E. coli* Fur protein with high affinities.

Purification of Fur protein and electrophoretic mobility-shift assay (EMSA) of the Fur-binding DNA sequence. *E. coli* BL21(DE3) cells carrying pEF2 were cultivated in LB at 37 °C to an OD₆₆₀ of 0.6. The culture was kept at 15 °C for 30 min, and IPTG was added to a final concentration of 1 mM. After cultivation at 15 °C for 24 h, the cells were harvested by centrifugation and suspended in sonication buffer [50 mM Tris/HCl (pH 7.5), 200 mM KCl, 5 mM MgCl₂, 10% (w/v) glycerol]. The cells were disrupted by sonication using a Multi-Bead Shocker (Yasui). After centrifugation (20 000 g) at 4 °C for 15 min, the protein fraction in the supernatant was concentrated by 30% (w/v) ammonium sulfate precipitation, and dialysed against storage buffer [50 mM Tris/HCl (pH 7.5), 200 mM KCl, 5 mM MgCl₂, 500 µM MnCl₂, 50% (w/v) glycerol]. The Fur protein in the fraction was more than 90% pure based on SDS-PAGE analysis (data not shown).

The DNA fragments to be investigated were PCR-amplified and end-labelled with [γ -³²P]ATP by using T4 polynucleotide kinase, and a 100 pM aliquot of the end-labelled DNA fragments was solubilized in 10 µl binding buffer [10 mM Tris/HCl (pH 7.5), 40 mM KCl, 1 mM MgCl₂, 100 µM MnCl₂, 10% (w/v) glycerol, 0.1 mg BSA ml⁻¹]. To this solution, the *B. multivorans* Fur protein was added at a final concentration ranging from 0 to 1600 nM. After incubation at 30 °C for 30 min, the reaction mixture was loaded into a 5% (w/v) non-denaturing polyacrylamide gel in TBE buffer. After electrophoresis for 1 h at 100 V, the gel was dried and exposed to an imaging plate, and the gel image was analysed by a FLAS-T1500 system (Fujifilm).

Sequence analysis. The entire genomic sequence of *B. multivorans* ATCC 17616 has been determined (Y. Ohtsubo & others, unpublished). Detailed analysis of nucleotide sequences for this paper was done by using the GENETYX-MAC program version 13 (Genetyx). The BLAST programs (www.ncbi.nlm.nih.gov/BLAST/) were used for the analysis of sequence homology. A consensus sequence logo was generated using the WEBLOGO program (<http://weblogo.berkeley.edu/>).

RESULTS

Isolation and characterization of the *fur* gene of *B. multivorans* ATCC 17616

We have previously obtained approximately 5,100 derivatives of strain ATCC 17616 by random mutagenesis using a Tn5-based transposon, 'TnMod-RTp' (Komatsu *et al.*, 2003). The mutant library was screened for the constitutive production of siderophores on CAS agar plates. An orange halo around the colony of one mutant, BT163, was much larger than that of the wild-type strain (Fig. 1a). The addition of 30 µM FeCl₃ to the CAS agar plates resulted in a decrease in halo size for the wild-type strain, but no change for BT163. This suggested that strain ATCC 17616 represses the production of siderophores under iron-rich conditions and that BT163 constitutively produces an excess of siderophores. Cloning and subsequent analysis of the TnMod-RTp'-flanking genomic region of BT163 revealed that the transposon was inserted into an ORF whose product was identical to the Fur protein of *Burkholderia cenocepacia* 715j (Lowe *et al.*, 2001). We concluded that the mutated ORF in BT163 is the *fur* gene since (i) the wild-type phenotype on the CAS agar plate was restored by the introduction into BT163 of pCF1 which carries the wild-type allele of this ORF (Fig. 1a), (ii) the order of the genes flanking this ORF is conserved in other *Burkholderia* species (Lowe *et al.*, 2001), i.e. *omlA* (the gene for outer-membrane lipoprotein)-*fur*-*alla* (the gene for ureidoglycolate hydrolase), and (iii) no other genes homologous to the *fur* gene are found in the strain ATCC 17616 genome.

Fig. 1. Characterization of the *fur* mutation of *B. multivorans* ATCC 17616. (a) Siderophore production by strains ATCC 17616, BT163 (*fur*::TnMod-RTp'), and BT163 carrying pCF1 (pME6041::*fur*) on CAS agar plates with or without 30 µM FeCl₃. (b) and (c) Staining of SOD and catalase activities, respectively. 1, ATCC 17616; 2, DF1 (*fur* deletion mutant); 3, DF1 carrying pCF1. The supernatant containing a total of 75 µg protein was added into each well for electrophoresis. The value below each lane indicates the relative signal intensity of activity staining, taking that of strain ATCC 17616 as 1.

The transposon insert in the BT163 genome was between the 69th and 70th base positions in the 426-base *fur* gene. This insert led to the formation of a putative fusion ORF whose 3' portion encoded the C-terminal 119 amino acid residues of the Fur protein. It was theoretically possible that this presumptive fusion protein still had the ability to exert certain residual functions of Fur. Our attempt to completely remove the *fur* gene from strain ATCC 17616 genome led to successful generation of strain DF1, and its phenotype on the CAS agar plate was the same as that of strain BT163 (data not shown). Thereafter in this study, we used strain DF1 as a *fur* deletion mutant.

Sensitivity of the *fur* deletion mutant to ROS and nitrogen compounds

Disk assays showed that strain DF1 cells were more sensitive to H₂O₂ and paraquat than strain ATCC 17616 cells (data not shown). Crude cell extracts prepared from strains ATCC 17616 and DF1 were subjected to native PAGE and subsequent activity staining (Fig. 1b). A single band for SOD activity was detected from both strains, and the enzymic activity of this band from strain DF1 was 1.6-fold lower than that from strain ATCC 17616. Only one gene for cytoplasmic iron-containing SOD, *sodB*, was found in the ATCC 17616 genome. Northern analysis revealed that the amount of *sodB* mRNA was 1.6-fold lower in strain DF1 than in strain ATCC 17616 (data not shown). The catalase activity was also 1.7-fold lower in strain DF1 (Fig. 1c). Both activities were restored by the introduction of pCF1 into strain DF1 (Fig. 1b, c). These results indicated that the ATCC 17616 Fur protein is involved in the production of enzymes for removal of ROS.

To determine the involvement of Fur in the metabolism of inorganic nitrogen compounds, i.e. nitrate, nitrite and NO, the growth of ATCC 17616 and DF1 cells was investigated using EB broth or EB agar plates supplemented with one of the three inorganic nitrogen compounds. The sensitivity of strains ATCC 17616 and DF1 to nitrate was unchanged under aerobic and anaerobic conditions (data not shown). The sensitivity to nitrite under aerobic conditions was slightly higher for strain DF1 than for ATCC 17616 (Fig. 2a). However, strain DF1 was remarkably sensitive to nitrite under microanaerobic conditions (i.e. there was a 10⁴-fold decrease in the number of c.f.u. in comparison with ATCC 17616) (Fig. 2a). The sensitivity of strain DF1 was restored to that of ATCC 17616 by the introduction of pCF1. We investigated the aerobic growth of ATCC 17616 and DF1 cells in EB broth with and without the addition of acidified nitrite (Fig. 2b). Strain DF1 in EB broth grew slightly slower than ATCC 17616. The addition of acidified nitrite led to profound growth defects of both strains, but the defect in DF1 cells was much more severe than that in ATCC 17616 cells. These results indicate that Fur is necessary for the adaptation to nitrosative stress.

Fig. 2. Assay of sensitivity to nitrogen compounds. (a) Sensitivity of strain ATCC 17616 and DF1 cells to nitrite under aerobic and microanaerobic conditions was measured by the number of c.f.u. on EB agar plates with or without 5 mM sodium nitrite. Columns 1–6 indicate the various dilutions of late-exponential-phase cells from 5 × 10³ to 5 × 10⁸ c.f.u. ml⁻¹ in tenfold steps, respectively (see Methods). (b) Sensitivity to NO under aerobic conditions was analysed by measuring the OD₆₆₀ of cultures in EB at 30 °C with or without 2 mM acidified nitrite (see Methods). Error bars represent the SD from at least three independent experiments. ◆, ATCC 17616; ■, ATCC 17616 + acidified nitrite; ▲, DF1; ×, DF1 + acidified nitrite.

Carbon assimilation of strain ATCC 17616 and its *fur* deletion mutant

We also examined the effect of the *fur* mutation on the ability of cells to grow on M9 minimal agar containing any of 33 carbon sources that are available to the wild-type strain (14 sugar compounds, 12 organic acids and 7 aromatic compounds listed in Table 2). When ascorbate, gentisate and anthranilate were employed as carbon sources, the number of c.f.u. of DF1 cells was slightly lower (10- to 100-fold) than those of ATCC 17616 cells. Use of any of the remaining compounds resulted in a severe decrease in the number of c.f.u. of DF1 cells, but not ATCC 17616 cells, and these defects in strain DF1 were most remarkable with fructose, ribose, dulcitol, mannose and tartrate as carbon source. These growth defects in strain DF1 were complemented by the introduction of pCF1. In contrast to the growth-based assay, employment of Biolog GN2, a cellular respiration assay system, revealed

that the *fur* mutation caused reduced cellular respiration activities only when adonitol, arabinose, lactose, ketovaleate, leucine and carnitine were used as the substrates. However, cellular respiration of strain DF1 was still observed even when the substrate was any one of the 13 carbon compounds (fructose, galactose, glucose, inositol, mannitol, mannose, sorbitol, trehalose, acetate, citrate, malonate, succinate and ornithine) that produced a growth defect in strain DF1 (Table 2). These results suggest that although the *fur* mutation causes severe defects in the ability of the cell to use various carbon sources, these are not ascribable to the oxidative respiration of these carbon sources.

Analysis of Fur-binding regions of the ATCC 17616 genome

The *E. coli* strain H1780 is a *fur* deletion mutant, and carries a fusion of the *fiu* promoter and promoterless *lacZ* gene, the expression of which is repressed by Fur under iron-rich conditions (Hantke, 1987). Plasmid pEF1 carries the *fur* gene from *B. multivorans* ATCC 17616 (*fur*_{Bm}). Introduction of this plasmid into H1780 resulted in a drastic decrease in β -galactosidase activity under iron-rich but not iron-limiting conditions (data not shown), indicating that *fur*_{Bm} shares at least some functional exchangeability with the *E. coli fur* gene (*fur*_{Ec}). Based on this result, the FURTA system (Stojiljkovic *et al.*, 1994) was used to isolate the ATCC 17616 DNA sequences to which the Fur_{Ec} protein is able to bind with high affinity. In this assay, an *E. coli fur*⁺ strain (H1717) was used that carries a chromosomal copy of the Fur-repressed promoter (*fhuF* promoter) connected to a promoterless *lacZ* gene (Hantke, 1987). The introduction of a multicopy plasmid carrying a Fur-binding sequence (Fur box) into H1717 leads to the titration of Fur by the plasmid-specified Fur box, thus allowing the transcription of the fused *lacZ* gene.

The introduction of a pHSG398-based genomic library of ATCC 17616 into H1717 and screening of 9×10^4 clones on a MacConkey lactose agar plate containing 30 μ M FeCl₃ gave rise to 115 LacZ⁺ colonies. Subsequent sequence analysis of plasmids in these LacZ⁺ colonies revealed that 13 regions carried putative Fur-binding sequences (Table 3). All of these sequences were located within or very close to the putative promoter sequences, nine of which were followed by genes postulated to encode proteins for iron acquisition or cellular iron homeostasis (regions 2–7 and 10–12 in Table 3). Four such proteins encoded by regions 2, 6, 10 and 11 were putative TonB-dependent outer-membrane receptor proteins for siderophores of unknown origins, but their genes were not flanked by genes related to iron acquisition. In contrast, regions 3 and 12 apparently encode all the components necessary for the ABC-type ferric ion transporter system and TonB-dependent haemin uptake system, respectively (Fig. 3a and supplementary Table S2, available with the online version of this paper). Two genes (*fecI* and *orbS* in regions 4 and 5, respectively)

Table 2. Carbon assimilation of strains ATCC 17616 and DF1

The c.f.u. of strains ATCC 17616 and DF1 on EB and M9 agar plates containing one carbon source was measured as described in Methods. For simplicity, the following symbols are arbitrarily employed to indicate the relative c.f.u., taking that of strain ATCC 17616 on an EB plate as 1: + + +, between 1 and 10^{-1} ; + +, between 9×10^{-2} and 10^{-3} ; +, between 9×10^{-4} and 10^{-5} ; –, less than 9×10^{-6} .

Carbon source	Strain	
	ATCC 17616	DF1
Sugars		
Glucose	+ + +	+
Fructose	+ + +	+
Ribose	+ + +	+
Mannose	+ + +	+
Xylose	+ + +	+
Trehalose	+ + +	+
Galactose	+ + +	+
Arabinose	+ + +	+ +
Cellobiose	+ + +	+
Inositol	+ + +	+
Sorbitol	+ + +	+
Ribitol	+ + +	+
Mannitol	+ + +	+
Dulcitol	+ + +	+
Organic acids		
Pyruvate	+ + +	+ +
Succinate	+ + +	+
Isocitrate	+ + +	+ +
Citrate	+ + +	+
Fumarate	+ + +	+ +
Tartrate	+ + +	+
Acetate	+ + +	+
Malonate	+ + +	+
Ascorbate	+	–
Ornithine	+ + +	+
Shikimate	+ + +	+ +
Glycerophosphate	+ + +	+
Aromatic acids		
Salicylate	+	–
Gentisate	+	–
Hydroxyphenylpyruvate	+ + +	+ +
Hydroxybenzoate	+ + +	+ +
Benzoate	+	+
Anthranelate	+	+
Phthalate	+ + +	+
EB	+ + +	+ + +

were found that putatively encode extracytoplasmic function (ECF) sigma factors for iron acquisition (iron-starvation sigma factors). It is well known that (i) the transcription of genes for ferric-citrate transport in *E. coli* requires FecI, and (ii) FecR, a cytoplasmic membrane protein encoded by the second gene in the Fur-repressed *fecIR* operon which transmits an external iron signal to the cytoplasmic FecI protein (Braun *et al.*, 2003).

Table 3. Regions containing Fur-regulated genes of strain ATCC 17616

Region	First gene just downstream of Fur box						Putative Fur box				Ratio of transcription of the first gene (DFI/ ATCC 17616)
	Chr	From	To	Direction	Gene name	Putative function or product*	Fur-box element†	Match to <i>E. coli</i> Fur box	Palindrome length‡	Distance from translational start§	
1	I	185 075	185 809	+		Conserved hypothetical protein (<i>Burkholderia</i> sp. 383, 198/225, 88 %)	tgTAATGATAgcCATTcTC	14	4	88	50.1 ± 13.2
2	I	300 436	302 637	+		TonB-dependent outer-membrane siderophore receptor (<i>Burkholderia ambifaria</i> MC40-6, 605/733, 82 %)	tggAATGAgAAcCATTcTC	13	5	40	11.2 ± 5.08
3	I	1 225 998	1 227 041	—		Substrate-binding protein of ABC-type Fe ³⁺ transporter system (<i>Burkholderia ambifaria</i> MC40-6, 330/347, 95 %)	GATAATcATtccCATTtcg	12	3	119	92.2 ± 28.1
4	I	1 401 255	1 401 764	+	<i>fecI</i>	ECF subfamily RNA polymerase sigma factor (<i>Burkholderia</i> sp. 383, 156/169 (92 %)	GtatATaATAATCATTcTC	14	5	58	58.7 ± 13.9
5	I	1 748 437	1 749 078	+	<i>orbS</i>	ECF subfamily RNA polymerase sigma factor (<i>Burkholderia cenocepacia</i> , 190/220, 86 %)	GAgAATGATttgCgTTtaC	12	4	64	2.24 × 10 ³ ± 0.63 × 10 ³
6	I	2 334 542	2 336 755	+		TonB-dependent outer-membrane siderophore receptor (<i>Burkholderia</i> sp. 383, 625/737, 84 %)	tcaAATGAcAATgATTcTt	12	4	146	122 ± 15.6
7	I	2 346 617	2 346 853	+	<i>bfd</i>	Bacterioferritin-associated ferredoxin (<i>Burkholderia cenocepacia</i> AU 1054, 75/78, 96 %)	ttTAATGATAATaATTcgC	14	5	44	332 ± 88.8
8	I	2 352 051	2 352 296	+		Conserved hypothetical protein (<i>Burkholderia dolosa</i> AUO158, 74/81, 91 %)	GtaAATGggAATagTTATC	13	3	64	878 ± 167
9	I	3 349 867	3 351 105	—		Uncharacterized iron-regulated membrane protein (<i>Burkholderia dolosa</i> AUO158, 356/412, 86 %)	tAgAATGtcgCgCgTTgTC	10	4	185	9.30 ± 2.39
10	II	1 697 287	1 699 533	+		TonB-dependent outer-membrane siderophore receptor (<i>Burkholderia cepacia</i> AMMD, 692/748, 92 %)	agaAATcgTAATaATTccC	11	4	81	560 ± 202
11	II	1 958 624	1 960 828	—		TonB-dependent outer-membrane siderophore receptor (<i>Burkholderia ambifaria</i> MC40-6, 656/684, 95 %)	GATAccGATtcTCATTtgC	13	4	179	7.90 ± 5.93
12	II	2 250 111	2 252 378	+	<i>hmuR</i>	TonB-dependent outer-membrane haemin receptor (<i>Burkholderia cenocepacia</i> AU 1054, 666/755, 88 %)	GATAATaATtcTCATTtaC	14	5	140	4.88 × 10 ³ ± 1.88 × 10 ³

Table 3 cont.

Region	First gene just downstream of Fur box				Putative Fur box				Ratio of transcription of the first gene (DF1/ATCC 17616)		
	Chr	From	To	Direction	Gene name	Putative function or product*	Fur-box element†	Match to <i>E. coli</i> Fur box		Palindrome length‡	Distance from translational start§
13	II	2 406 835	2 408 940	+		Putative ligand-gated protein (<i>Burkholderia cepacia</i> AMMD, 675/701, 96 %)	GcaAATaActcTCATTtaC	11	6	117	7.91 ± 3.01

*Amino acid identities and their percentage to the most homologous proteins of other *Burkholderia* strains are indicated in parentheses.

†Putative Fur box sequences found in Fur-binding sequences. The nucleotides that match to those of the *E. coli* consensus Fur box (5'-GATAATGATAATCATTATC-3') are shown in upper-case letters.

‡The length of the palindrome of the putative Fur-box sequences. Note that the length of the palindrome of the *E. coli* consensus Fur-box is 9.

§The distance between the 5' end of the putative Fur box and the presumed translation start codon.

||The value indicates the relative transcriptional level of the first gene in strain DF1 under iron-rich conditions, taking the value in strain ATCC 17616 as 1. Each value with a SD was obtained by at least three independent qRT-PCR experiments.

In *B. multivorans* ATCC 17616, the *fecI* gene is followed by *fecR* (Fig. 3a and supplementary Table S2). We constructed a knockout mutant of the strain ATCC 17616 *fecI* gene, but its subsequent analysis using iron-limiting and the CAS plates revealed no apparent effects of the mutation on iron acquisition (data not shown). The *orbS* gene in strain ATCC 17616 is followed by the genes encoding the biosynthesis of an ornibactin-like siderophore of unknown structure (Fig. 3a and supplementary Table S2). The involvement of these genes in the biosynthesis was confirmed by our observation in which the TnMod-RTp' insertion mutant in *orbI* did not support its growth on iron-limiting agar, nor form the orange halo zone on the CAS plate (data not shown). The same phenotypes were also observed for the ATCC 17616 *orbS* mutant (data not shown), supporting the idea that this gene product is required for the biosynthesis of a siderophore. The putative Fur-binding sequence in region 7 is followed by *bfd* that putatively encodes bacterioferritin (Bfr)-associated ferredoxin (Fig. 3a and supplementary Table S2), and is considered to act as a Bfr reductase, mediating the release of iron from this iron storage protein (Andrews *et al.*, 2003). Downstream of the *bfd* gene is the *tonB-exbB-exbD* cluster which encodes, in other bacteria, a system to transduce cellular energy to outer-membrane receptors for siderophores and haemin (Schalk *et al.*, 2004).

To confirm that the Fur_{Bm} protein was indeed capable of binding to the putative Fur box-like sequences obtained above, this protein was purified as described in Methods. EMSA analysis confirmed that the purified Fur_{Bm} protein binds to all 13 putative Fur box-like sequences (Fig. 3 and data not shown). The predicted Fur-binding sequences obtained by FURTA were used to generate a consensus logo sequence (Fig. 4). This logo sequence indicates that the AAT (residues 4–6) and ATT (residues 14–16) motifs are well conserved and form palindromic sequences. These sequences are also conserved in the Fur box of *E. coli*, strongly suggesting that Fur_{Bm} and Fur_{Ec} recognize these common sequences. qRT-PCR was carried out to investigate transcription of the genes located just downstream of the Fur boxes in strains ATCC 17616 and DF1 (Table 3) under iron-rich conditions. All of these genes in strain DF1 were transcribed at levels 7.9- to 4900-fold higher than those in ATCC 17616. These results clearly indicate that the Fur_{Bm} protein acts as a repressor for expression of these genes in strain ATCC 17616.

DISCUSSION

In this study we isolated an insertion mutant of the ATCC 17616 *fur* gene, and demonstrated that its deletion mutant is viable under conventional cultivation conditions. This dispensability of Fur in ATCC 17616 contrasts with the essentiality of the *fur* gene in *B. pseudomallei* (Loprasert *et al.*, 2000), and this is the first report of a successful construction of a *fur* deletion mutation in a *Burkholderia* species. Null mutations of *fur* can be isolated in *E. coli* and

Fig. 3. Fur-regulated gene clusters of *B. multivorans* ATCC 17616. I, II, III, and IV correspond to regions 4, 5, 7, and 12 described in Table 3. (a) Genetic maps of the Fur box-containing regions. The genes located just downstream of the Fur boxes are shown in grey. A detailed description of the genes is given in supplementary Table S2. (b) Relative position of Fur box (open box) to the -10 and -35 sequences of the putative promoter and the putative start codon (bold type). The arrow indicates the direction of transcription. (c) EMSA analysis for the binding of Fur_{Bm} to the Fur box-containing sequence. The concentration of Fur_{Bm} (nM) used for the analysis is listed at the bottom of each lane.

Neisseria species, but not, for example, in *P. aeruginosa* (Vasil, 2007). It is still unknown why Fur is essential for some bacterial strains but not for other related strains. A detailed comparison of iron- and Fur-regulated genes in the two *Burkholderia* genomes might clarify the reason(s) for this difference.

Our results strongly suggest that Fur is necessary to adapt to nitrosative stress in strain ATCC 17616. The involve-

Fig. 4. Sequence logo of the Fur box of *B. multivorans* ATCC 17616. The Fur boxes listed in Table 3 were used to determine the consensus nucleotide frequencies by using the WEBLOGO program.

ment of Fur in nitrogen metabolism has been reported in other bacteria. The Fur protein in *Neisseria meningitidis* activates the *panI* and *norB* genes, which encode nitrite and NO reductases, respectively (Delany *et al.*, 2004), and the *E. coli fur* mutant exhibits a severe growth delay under aerobic conditions in the presence of nitrosylated glutathione or acidified nitrite (Mukhopadhyay *et al.*, 2004). In contrast, we showed in this study that the *fur* mutation in strain ATCC 17616 causes hypersensitivity to nitrite under microaerobic conditions and also acidified nitrite (an NO producer) under aerobic conditions. Nitrite and NO are reactive nitrogen species that induce bacterial damage through interaction with DNA and proteins containing iron/zinc-sulfur clusters, haem, thiols, tyrosyl radicals, tyrosine residues or amines (Lundberg *et al.*, 2004). Cells of strain DF1 might fail to remove nitrite and/or NO, thereby resulting in high sensitivities to these compounds.

As has been observed in other bacterial strains, the *fur* mutant of ATCC 17616 was more sensitive to ROS than the wild-type strain, and the enzymic activities of SodB and

catalase for the removal of ROS were reduced. Our observation that the amount of *sodB* mRNA in DF1 cells was slightly lower than that in ATCC 17616 cells suggests that Fur directly or indirectly activates the transcription of *sodB*. Fur binds directly to the *sodB* promoter to activate *sodB* transcription in *N. meningitidis* (Sebastian *et al.*, 2002). In contrast, small regulatory RNA molecules (sRNAs) are involved in inhibiting translation of the *sodB* gene in *E. coli* and *P. aeruginosa* (Masse & Gottesman, 2002; Wilderman *et al.*, 2004). In these bacteria, the active form of Fur under iron-rich conditions binds Fur boxes that are located upstream of sRNAs thus repressing their transcription and allowing the translation of *sodB* mRNA. Since no Fur-box like sequences were found at the region upstream of the strain ATCC 17616 *sodB* gene, it is most likely that the Fur protein indirectly activates the expression of *sodB*.

The *fur* mutant of ATCC 17616 showed, when compared to the wild-type strain, a reduced ability to assimilate various carbon sources (Table 2). The *fur* mutation in *E. coli* causes a loss of the ability to use non-fermentable carbon sources such as succinate, acetate and fumarate (Andrews *et al.*, 2003; Hantke, 1987). However, strain ATCC 17616 was unique in that its *fur* mutant could not efficiently assimilate either fermentable or non-fermentable carbon sources. Another unique property revealed by the carbon assimilation tests was that many compounds that could not be readily employed as carbon sources in the *fur* mutant were still available as substrates for cellular respiration. This implies that the growth defect of the mutant was not primarily due to defect(s) in its respiration. Our preliminary results indicated that the growth defect of DF1 cells on an M9 glucose agar plate was in part suppressed under iron-limiting and microaerobic conditions (data not shown). Since large amounts of ROS (e.g. H₂O₂ and hydroxy radical) are produced by the Fenton reaction in cells grown under iron-rich and aerobic conditions (Andrews *et al.*, 2003), the growth defect of DF1 on M9 glucose agar might be due to the accumulation of ROS. The Fur proteins in *E. coli* and *P. aeruginosa* apparently activate the genes for succinate dehydrogenase under iron-rich conditions through the transcriptional repression of antisense sRNAs against these dehydrogenase genes (Masse & Gottesman, 2002; Mellin *et al.*, 2007; Vasil, 2007; Wilderman *et al.*, 2004). It would be of interest to know whether a similar mechanism is involved in the expression of such dehydrogenase genes in *B. multivorans* ATCC 17616.

Many Gram-negative bacterial species have the ability to use various kinds of exogenous siderophores that they do not produce themselves, and these abilities depend on the expression of genes encoding outer-membrane receptors specific to the exogenous siderophores (Andrews *et al.*, 2003). *B. multivorans* ATCC 17616 may also have such abilities because four Fur-repressive genes encoding putative siderophore receptors were identified in regions 2, 6, 10 and 11 (Table 3). However, it is uncertain whether

these genes have the expected functions, since they are not similar to other genes clearly involved in iron acquisition. Another putative siderophore receptor gene, *orbA*, in region 5 is expected to be functional because it is located within the biosynthetic gene cluster for an ornibactin-like siderophore, and *orbI* in this cluster is required for the production of this siderophore (Table 3, Fig. 3a and supplementary Table S2). However, no Fur boxes are found at the positions upstream of *orbA* and other genes, with the exception of *orbS*. Therefore, it is most plausible that (i) the transcription of siderophore biosynthetic genes and *orbA* requires the *orbS* product as an ECF sigma factor, and (ii) *orbS* transcription is repressed under iron-rich conditions by the active form of Fur. Such a two-step regulation process has been demonstrated in the ornibactin biosynthetic genes in *B. cenocepacia* 715j (Agnoli *et al.*, 2006). *orbS* is an orthologue of *pvdS* (an ECF sigma factor gene for pyoverdine synthesis) from *P. aeruginosa*, and PvdS is essential for the production of various virulence factors (Beare *et al.*, 2003; Lamont *et al.*, 2002; Visca *et al.*, 2002). It would be of interest to examine whether OrbS in *B. multivorans* ATCC 17616 also plays pleiotropic roles in the production of various proteins.

Tuanyok *et al.* (2005) have carried out transcriptome analyses of *Burkholderia mallei* and *B. pseudomallei* under iron-rich and iron-limiting conditions, and the transcriptional levels of more than 200 genes in each species were reported to be drastically changed, depending on the iron concentration. Many homologues of Fur-regulated ATCC 17616 genes identified in our current study are listed in the data of Tuanyok *et al.* (2005) and one of these genes is *bfd*. However, there has been no further detailed analysis of this gene. In contrast, our study clearly demonstrates that the transcription of the *bfd* gene in ATCC 17616 is directly repressed by Fur, and such definitive experimental data have not been obtained even in *E. coli* (Andrews *et al.*, 2003).

Since the genome sequence of *B. multivorans* ATCC 17616 has been determined (Y. Ohtsubo & others, unpublished), use of this strain and its *fur* and *orbS* mutants for transcriptome and subsequent qRT-PCR analyses will clarify (i) the direct and indirect target genes of these two regulator products and (ii) how the extracellular iron signal is transmitted to each member of the Fur regulon.

ACKNOWLEDGEMENTS

This work was supported by Grant-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Agriculture, Forestry and Fisheries (HC-06-2323), Japan.

REFERENCES

- Abdul-Tehrani, H., Hudson, A. J., Chang, Y. S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R. & Andrews, S. C. (1999). Ferritin mutants of *Escherichia coli* are iron deficient and

- growth impaired, and *fur* mutants are iron deficient. *J Bacteriol* **181**, 1415–1428.
- Agnoli, K., Lowe, C. A., Farmer, K. L., Husnain, S. I. & Thomas, M. S. (2006). The ornibactin biosynthesis and transport genes of *Burkholderia cenocepacia* are regulated by an extracytoplasmic function sigma factor which is a part of the Fur regulon. *J Bacteriol* **188**, 3631–3644.
- Amann, E. & Brosius, J. (1985). ATG vectors for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene* **40**, 183–190.
- Andrews, S. C., Robinson, A. K. & Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol Rev* **27**, 215–237.
- Ausubel, F. M., Brent, R., Kingston, E. R., Moore, D. D., Seidman, G. J., Smith, A. J. & Struhk, K. (1991). *Current Protocols in Molecular Biology*. New York: John Wiley & Sons.
- Banin, E., Vasil, M. L. & Greenberg, E. P. (2005). Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A* **102**, 11076–11081.
- Beare, P. A., For, R. J., Martin, L. W. & Lamont, I. L. (2003). Siderophore-mediated cell signalling in *Pseudomonas aeruginosa*: divergent pathways regulate virulence factor production and siderophore receptor synthesis. *Mol Microbiol* **47**, 195–207.
- Braun, V., Mahren, S. & Ogierman, M. (2003). Regulation of the FecI-type ECF sigma factor by transmembrane signalling. *Curr Opin Microbiol* **6**, 173–180.
- Chang, A. C. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134**, 1141–1156.
- Cheng, H. P. & Lessie, T. G. (1994). Multiple replicons constituting the genome of *Pseudomonas cepacia* 17616. *J Bacteriol* **176**, 4034–4042.
- Chiarini, L., Bevivino, A., Dalmastri, C., Tabacchioni, S. & Visca, P. (2006). *Burkholderia cepacia* complex species: health hazards and biotechnological potential. *Trends Microbiol* **14**, 277–286.
- Delany, I., Rappuoli, R. & Scarlato, V. (2004). Fur functions as an activator and as a repressor of putative virulence genes in *Neisseria meningitidis*. *Mol Microbiol* **52**, 1081–1090.
- Dennis, J. J. & Zylstra, G. J. (1998). Plasmids: modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. *Appl Environ Microbiol* **64**, 2710–2715.
- Escolar, L., Perez-Martin, J. & de Lorenzo, V. (1999). Opening the iron box: transcriptional metalloregulation by the Fur protein. *J Bacteriol* **181**, 6223–6229.
- Hall, H. K. & Foster, J. W. (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.
- 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.
- Heeb, S., Itoh, Y., Nishijyo, T., Schnider, U., Keel, C., Wade, J., Walsh, U., O'Gara, F. & Haas, D. (2000). Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. *Mol Plant Microbe Interact* **13**, 232–237.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host-range FLP-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86.
- Komatsu, H., Imura, Y., Ohori, A., Nagata, Y. & Tsuda, M. (2003). Distribution and organization of auxotrophic genes on the multichromosomal genome of *Burkholderia multivorans* ATCC 17616. *J Bacteriol* **185**, 3333–3343.
- Lamont, I. L., Beare, P. A., Ochsner, U., Vasil, A. I. & Vasil, M. L. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **99**, 7072–7077.
- Lefebvre, M. & Valvano, M. (2001). *In vitro* resistance of *Burkholderia cepacia* complex isolates to reactive oxygen species in relation to catalase and superoxide dismutase production. *Microbiology* **147**, 97–109.
- Lessie, T. G., Hendrickson, W., Manning, B. D. & Devereux, R. (1996). Genomic complexity and plasticity of *Burkholderia cepacia*. *FEMS Microbiol Lett* **144**, 117–128.
- Litwin, C. M. & Calderwood, S. B. (1993). Role of iron in regulation of virulence genes. *Clin Microbiol Rev* **6**, 137–149.
- Loprasert, S., Sallabhan, R., Whangsuk, W. & Mongkolsuk, S. (2000). Characterization and mutagenesis of *fur* gene from *Burkholderia pseudomallei*. *Gene* **254**, 129–137.
- Lowe, C. A., Asghar, A. H., Shalom, G., Shaw, J. G. & Thomas, M. S. (2001). The *Burkholderia cepacia fur* gene: co-localization with *omla* and absence of regulation by iron. *Microbiology* **147**, 1303–1314.
- Lundberg, J. O., Weitzberg, E., Cole, J. A. & Benjamin, N. (2004). Nitrate, bacteria and human health. *Nat Rev Microbiol* **2**, 593–602.
- Mahenthalingam, E., Urban, T. A. & Goldberg, J. B. (2005). The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat Rev Microbiol* **3**, 144–156.
- Masse, E. & Gottesman, S. (2002). A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A* **99**, 4620–4625.
- Mellin, J. R., Goswami, S., Grogan, S., Tjaden, B. & Genco, C. A. (2007). A novel *fur*- and iron-regulated small RNA, NrrF, is required for indirect *fur*-mediated regulation of the *sdhA* and *sdhC* genes in *Neisseria meningitidis*. *J Bacteriol* **189**, 3686–3694.
- Mey, A. R., Wyckoff, E. E., Kanukurthy, V., Fisher, C. R. & Payne, S. M. (2005). Iron and *fur* regulation in *Vibrio cholerae* and the role of *fur* in virulence. *Infect Immun* **73**, 8167–8178.
- Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A. & Storz, G. (2004). Prominent roles of the NorR and Fur regulators in the *Escherichia coli* transcriptional response to reactive nitrogen species. *Proc Natl Acad Sci U S A* **101**, 745–750.
- Nagata, Y., Matsuda, M., Komatsu, H., Imura, Y., Sawada, H., Ohtsubo, Y. & Tsuda, M. (2005). Organization and localization of the *dnaA* and *dnaK* gene regions on the multichromosomal genome of *Burkholderia multivorans* ATCC 17616. *J Biosci Bioeng* **99**, 603–610.
- Ohtsubo, Y., Goto, H., Nagata, Y., Kudo, T. & Tsuda, M. (2006). Identification of a response regulator gene for catabolite control from a PCB-degrading beta-proteobacteria, *Acidovorax* sp. KKS102. *Mol Microbiol* **60**, 1563–1575.
- Parke, J. L. & Gurian-Sherman, D. (2001). Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. *Annu Rev Phytopathol* **39**, 225–258.
- Qing, G., Ma, L. C., Khorchid, A., Swapna, G. V., Mal, T. K., Takayama, M. M., Xia, B., Phadtare, S., Ke, H. & other authors (2004). Cold-shock induced high-yield protein production in *Escherichia coli*. *Nat Biotechnol* **22**, 877–882.
- Sambrook, J. & Russell, W. D. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schalk, I. J., Yue, W. W. & Buchanan, S. K. (2004). Recognition of iron-free siderophores by TonB-dependent iron transporters. *Mol Microbiol* **54**, 14–22.
- Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.

- Sebastian, S., Agarwal, S., Murphy, J. R. & Genco, C. A. (2002).** The gonococcal *fur* regulon: identification of additional genes involved in major catabolic, recombination, and secretory pathways. *J Bacteriol* **184**, 3965–3974.
- Stojiljkovic, I., Bäumlér, A. J. & Hantke, K. (1994).** Fur regulon in Gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a *fur* titration assay. *J Mol Biol* **236**, 531–545.
- Studier, F. W. & Moffatt, B. A. (1986).** Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113–130.
- Takeshita, S., Sato, M., Toba, M., Masahashi, W. & Hashimoto-Gotoh, T. (1987).** High-copy-number and low-copy-number plasmid vectors for *lacZ* alpha-complementation and chloramphenicol- or kanamycin-resistance selection. *Gene* **61**, 63–74.
- Thompson, D. K., Beliaev, A. S., Giometti, C. S., Tollaksen, S. L., Khare, T., Lies, D. P., Nealson, K. H., Lim, H., Yates, J., III & other authors (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.
- Touati, D., Jacques, M., Tardat, B., Bouchard, L. & Despied, S. (1995).** Lethal oxidative damage and mutagenesis are generated by iron in delta *fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. *J Bacteriol* **177**, 2305–2314.
- Tuanyok, A., Kim, H. S., Nierman, W. C., Yu, Y., Dunbar, J., Moore, R. A., Baker, P., Tom, M., Ling, J. M. & Woods, D. E. (2005).** Genome-wide expression analysis of iron regulation in *Burkholderia pseudomallei* and *Burkholderia mallei* using DNA microarrays. *FEMS Microbiol Lett* **252**, 327–335.
- Vasil, M. L. (2007).** How we learnt about iron acquisition in *Pseudomonas aeruginosa*: a series of very fortunate events. *Biomaterials* **20**, 587–601.
- Visca, P., Leoni, L., Wilson, M. J. & Lamont, I. L. (2002).** Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. *Mol Microbiol* **45**, 1177–1190.
- Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P. C., Gottesman, S., Ochsner, U. A. & Vasil, M. L. (2004).** Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc Natl Acad Sci U S A* **101**, 9792–9797.

Edited by: J. W. B. Moir