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Effect of iron limitation and *fur* gene inactivation on the transcriptional profile of the strict anaerobe *Clostridium acetobutylicum*

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Iron is a nutrient of critical importance for the strict anaerobe *Clostridium acetobutylicum*, as it is involved in numerous basic cellular functions and metabolic pathways. A gene encoding a putative ferric uptake regulator (Fur) has been identified in the genome of *C. acetobutylicum*. In this work, we inactivated the *fur* gene by using insertional mutagenesis. The resultant mutant showed a slow-growing phenotype and enhanced sensitivity to oxidative stress, but essentially no dramatic change in its fermentation pattern. A unique feature of its physiology was the overflowing production of riboflavin. To gain further insights into the role of the Fur protein and the mechanisms for establishment of iron balance in *C. acetobutylicum*, we characterized and compared the gene-expression profile of the *fur* mutant and the iron-limitation stimulon of the parental strain. Not surprisingly, a repertoire of iron-transport systems was upregulated in both microarray datasets, suggesting that they are regulated by Fur according to the availability of iron. In addition, iron limitation and inactivation of *fur* affected the expression of several genes involved in energy metabolism. Among them, two genes, encoding a lactate dehydrogenase and a flavodoxin, were highly induced. In order to support the function of the latter, the *ribDBAH* operon responsible for riboflavin biosynthesis was also upregulated significantly. Furthermore, the iron-starvation response of *C. acetobutylicum* involved transcriptional modifications that were not detected in the *fur* mutant, suggesting that there exist additional mechanisms for adaptation to low-iron environments. Collectively, these results demonstrate that the strict anaerobe *C. acetobutylicum* senses and responds to availability of iron on multiple levels using a sophisticated system, and that Fur plays an important role in this process.

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INTRODUCTION

Because of the nature of its redox potential and co-ordination capacity, iron is a microelement of immense importance for almost all micro-organisms, playing a key role in a large number of biological processes (Andrews *et al.*, 2003). Although abundant in most natural habitats, aerobic and pathogenic bacteria struggle with its inaccessibility caused either by oxidation of Fe^{2+} to Fe^{3+}, which is highly insoluble, or by sequestration as a result of the mechanisms of host defence (Braun & Killmann, 1999; Ratledge & Dover, 2000). To cope with this limitation, bacteria have evolved a collection of powerful tools, such as synthesis and export of chelators, termed siderophores, that capture ferric iron with high affinity, or reduction by ferric reductases (Schröder *et al.*, 2003; Wandersman & Delepelaire, 2004). On the other hand, overload with iron may promote the formation via the Fenton reaction of reactive oxygen species (ROS) that can cause damage to key cellular components (Touati, 2000). Therefore, micro-organisms must adequately regulate expression of the genes involved in iron uptake and metabolism in order to achieve optimal levels of intracellular unincorporated iron that comply with the cellular demands.

In most bacterial species, the Fur (ferric uptake regulator) protein represents the major system for maintenance of iron homeostasis. Fur senses excess intracellular Fe^{2+} and binds to the promoter regions of the genes participating in

Abbreviations: DP, 2,2′-dipyridyl; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; RF, riboflavin; ROS, reactive oxygen species.

Four supplementary figures and three supplementary tables are available with the online version of this paper.

iron acquisition, thereby obstructing their transcription. Conversely, when iron availability is restricted, derepression of the corresponding genes is observed. In addition to iron transport, Fur controls other processes such as redox-stress resistance, energy metabolism, flagellar chemotaxis and metabolic pathways (Escolar *et al.*, 1999; McHugh *et al.*, 2003).

The role of Fur in the bacterial iron response has been an area of active investigation in the last decade. However, the molecular mechanisms for maintenance of iron homeostasis in strictly anaerobic bacteria have remained largely uncharacterized. The Gram-positive endospore-forming bacterium *Clostridium acetobutylicum* is a representative of this group. A unique feature of its fermentative metabolism is the ability to switch from synthesis of the organic acids acetate and butyrate during exponential growth to production of the solvents butanol, acetone and ethanol upon transition to stationary phase (Jones & Woods, 1986). The need for iron in this micro-organism is unquestionable, given the presence of a high number of iron-containing proteins comprising the main pathways of energy metabolism. Although *C. acetobutylicum* emerged and thrives in anaerobic niches where a bioavailable form of iron is expected to be sufficiently accessible, previous physiological and biochemical studies implied a complex iron-dependent response (Bahl *et al.*, 1986; Junelles et al., 1988; Peguin & Soucaille, 1995). Deciphering the entire genome of this bacterium provides us with the opportunity to interpret such results in light of the coding sequence (Nölling *et al.*, 2001). Differential expression of genes putatively involved in iron transport and metabolism in *C. acetobutylicum* has been reported for the first time as a response to oxidative stress (Hillmann *et al.*, 2009). Moreover, it has been demonstrated that this micro-organism is far from defenceless and can survive limited aerobic exposure (Kawasaki *et al.*, 2004; O'Brien & Morris, 1971). To what extent *C. acetobutylicum* and other strictly anaerobic bacteria are able to adapt to aeration in their natural habitats is still not known (Imlay, 2008). Thus, it could be speculated that transition to an oxygenic atmosphere about 2.0 billion years ago might have triggered the development of a more sophisticated system for maintenance of iron homeostasis. Such a system would sense and respond to the special requirements of a strictly fermentative anaerobe.

The genome of *C. acetobutylicum* revealed three genes encoding Fur-like proteins. CAC1682 showed the highest percentage of similarity in amino acid sequence to the Fur orthologue of the closely related *Bacillus subtilis* (Hillmann *et al.*, 2008). In the present work, we started to investigate the mechanisms for maintenance of iron homeostasis in *C. acetobutylicum* by characterization of the role of Fur, as well as the response of this micro-organism to conditions of iron limitation via a combined physiological, transcriptomic and proteomic approach. Our results demonstrate that Fur plays a major role in the maintenance of adequate iron status in this micro-organism.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Cultures of *Escherichia coli* were grown aerobically in Luria–Bertani medium at 37 °C. All strains of *C. acetobutylicum* were stored as spore suspensions at −20 °C and incubated under anaerobic conditions at 37 °C either in complex CGM or chemically defined minimal medium MS-MES. Preparation of both media is described elsewhere (Hillmann *et al.*, 2008). In order to achieve iron-limiting conditions, increasing concentrations, ranging from 100 to 200 μM, of the iron chelator 2,2′-dipyridyl (DP; Sigma-Aldrich) dissolved in 96 % pure ethanol were added to complex medium, whereas minimal medium was supplemented with 6 μM $FeSO_4$. For isolation of total RNA and protein, mid-exponential cells (OD_{600} 0.8–0.9), grown in iron-replete (40 μM $FeSO_4$) or -deplete minimal MS-MES medium, were used. Where necessary, growth medium contained antibiotics [chloramphenicol (25 μg ml^{-1}), tetracycline (50 μg ml^{-1}), thiamphenicol (15 μg ml^{-1}), erythromycin (30 μg ml^{-1})] or X-Gal.

Analytical procedures. Measurements of OD_{600} and quantification of the fermentation products (ethanol, acetone and butanol) in culture supernatants were performed as described previously (Fischer *et al.*, 2006). Flavin concentration in culture supernatants was quantified by measuring both absorption (A_{444}) and fluorescence with an exitation beam of 450 nm (Spectramax ME2; Molecular Devices) with use of a standard curve.

Generation of *fur* knockout mutant in *C. acetobutylicum*. The *fur* insertional mutant was obtained through the ClosTron system (Heap *et al.*, 2007, 2010). In order to retarget the group II intron for insertion into the *fur* coding sequence, a splicing by overlap extension (SOEing) PCR was performed using a set of primers (fur-IBS-271a, fur-EBSd1-271a and fur-EBS2-271a) designed via the Targetron software tool (Sigma-Aldrich), the EBS universal primer and the plasmid pMTL007 as an intron template DNA. Sequences of all oligonucleotides used for mutagenesis are listed in Table S1 (available with the online version of this paper). Next, the amplified 350 bp fragment was digested with *Hin*dIII and *Bsr*GI and cloned into the pMTL007CE-2 vector. The resultant plasmid was introduced into *E. coli* DH5α for amplification and positive clones were identified by test restriction. From these, two clones were selected and the corresponding plasmids were subjected to sequencing using the fur-IBS-271a and fur-EBS1d-271a primers, in order to exclude the presence of any point mutations in the retargeting region. The purified plasmid carrying the retargeted intron was electroporated into *E. coli* ER2275 (pAN-2) for *in vivo* methylation, followed by electrotransformation into *C. acetobutylicum*. To select for transformants, cells were plated on RCA agar containing thiamphenicol (15 μg ml^{-1}). Single colonies were used to induce insertion of the retargeted intron by growing them in liquid CGM medium supplemented with 7.5 μg thiamphenicol ml^{-1} at 37 °C. After 4 h, aliquots were plated on RCA agar plates containing 30 μg erythromycin ml^{-1}. PCR employing gene-specific primers (fur_verif_fw and fur_verif_rev; see Table S1), which hybridize outside (upstream and downstream) of the insertion site, was used for screening of the resistant colonies. For further verification of the mutation, DNA from the positive clones and the wild-type (WT) was isolated as described previously (Fischer *et al.*, 2006) and Southern hybridization was performed using the amplified *fur* gene carrying the inserted intron as a probe (see Fig. S1).

For complementation, a fragment including the *fur* gene and its promoter region, spanning from positions −500 to +456 relative to the translational start site, was PCR-amplified from *C. acetobutylicum* genomic DNA using primers fur_prom_XmaI_fw and fur_prom_NcoI_rev (Table S1), introducing *Xma*I and *Nco*I sites at the 5′ and 3′ ends, respectively. The PCR product was subsequently cloned into

Table 1. Bacterial strains and plasmid vectors used in this study

Abbreviations: WT, wild-type; *cat*$^+$, chloramphenicol resistance; *erm*$^+$, erythromycin resistance; *tet*$^+$, tetracycline resistance; *tm*$^+$, thiamphenicol resistance; *recA*$^-$, homologous recombination abolished; *mcr*BC, lacking methylcytosine-specific restriction system.

Strain or plasmid	Relevant characteristics	Reference or source
C. acetobutylicum		
ATCC 824	WT	ATCC
fur::int	Derivative of strain ATCC 824; *fur*::intron; *erm*$^+$	This study
fur::int compl	Derivative of strain *fur*::int; pMTL85141Fur_{Cac}; *erm*$^+$; *tm*$^+$	This study
E. coli		
DH5α	F$^-$; ϕ80dlacZ M15; *end*A1; *rec*A1; *hsdR*17; *supE*44; thi-l, λ^-; *gyrA*96; *relA*1; Δ(*lacZYA-argF*); U169	Hanahan (1983)
ER2275	*rec*$^-$, *mcr*BC$^-$	New England Biolabs
DH5α pMTL007C-E2 *fur*::271a	DH5α; plasmid pMTL007C-E2 *fur*::271a; *cat*$^+$	This study
ER2275 pMTL007C-E2 *fur*::271a	ER2275; plasmid pMTL007C-E2 *fur*::271a; pAN-2 *cat*$^+$; *tet*$^+$	This study
Plasmids		
pAN-2	Φ*3tI*; p15A, oriR; *tet*$^+$	Heap *et al.* (2007)
pMTL007	Group II intron; ErmBtdRAM2 and *ltrA* ORF from pMTL20lacZTTErmBtdRAM2; *cat*$^+$	Heap *et al.* (2007)
pMTL007CE-2	*cat*$^+$; *tm*$^+$; *erm*$^+$	Heap *et al.* (2010)
pMTL007CE-2 *fur*::271a	Derivative of pMTL007CE-2; *fur*::271a	This study
pMTL85141	pIM131; *cat*$^+$; ColE11; MCS	Heap *et al.* (2009)
pMTL85141 Fur_{Cac}	Derivative of pMTL85141; *fur*; *fur* promoter	This study

pMTL85141 (Heap *et al.*, 2009) and the resulting construct, after verification by sequencing, was introduced into the *fur* mutant strain via the same procedure as described above, giving a complementation strain – *fur*::int compl.

Oxidative-stress experiments. Assessment of the sensitivity of *C. acetobutylicum fur*::int to oxidative stress in comparison to the WT was performed as described previously (Hillmann *et al.*, 2008).

Visible and fluorescence spectra, qualitative TLC. Cell-free culture supernatant from *C. acetobutylicum fur*::int strain was tested spectrophotometrically and fluorometrically. An aqueous solution of riboflavin (RF) (⩾99 %; Sigma-Aldrich) was used as a flavin standard. Visible absorption spectra were obtained on a Spectramax ME2 (Molecular Devices) between 310 and 550 nm. Fluorescence emisssion spectra were measured using the same device with an excitation beam at 450 nm and scanning was performed between 480 and 650 nm.

For the purposes of analytical TLC, the WT and the *fur* mutant were grown for 80 h in MS-MES medium. Cells were removed by centrifugation (9000 ***g***, 15 min) and the culture supernatants were aliquotted, lyophilized and stored in the dark at room temperature. Standards of flavin mononucleotide (FMN) (⩾70 %), flavin adenine dinucleotide (FAD) (⩾95 %) and RF (⩾99 %) (all from Sigma-Aldrich), as well as the lyophilized supernatants, were dissolved in 96 % pure ethanol. Five micrograms was loaded on silica gel plates (TLC Silica gel 60 F_{254}; Merck) and chromatographed in the dark with *n*-butanol : acetic acid : water at ratio of 4 : 1 : 5 as a solvent system. The spots were visualized using 302 nm UV light.

RNA isolation, microarray experiments, semiquantitative RT-PCR and Northern hybridization. Total RNA was isolated from two independent cultures of the WT, which were grown under either iron-replete or -deplete conditions, and two independent cultures of the *fur* mutant, grown under iron-replete conditions. After verification of its quality and quantification of its concentration, RNA was reverse-transcribed into cDNA, labelled with Cy3 and Cy5 and purified as described previously (Hillmann *et al.*, 2009). Microarray experiments were performed in duplicate (n=2) on array slides that contain 3840 oligonucleotides, representing 99.8 % of all annotated protein-coding genes in *C. acetobutylicum*, as reported by Grimmler *et al.* (2011). In order to exclude dye-specific effects, each RNA sample was labelled both with Cy3 and Cy5 and corresponding dye-swap experiments were performed. Thus, eight transcriptional scores were attained for each gene. A gene was considered to be expressed differentially when the average expression ratio (ratio of medians) from all eight datasets was ⩾3 or ⩽0.33 and all eight transcriptional scores were >2.00 or <0.50, respectively. The microarray design and data have been deposited in the ArrayExpress database under accession number E-MEXP-3401. The reliability of the microarray data was confirmed by semiquantitative RT-PCR (see Fig. S2). Furthermore, expression levels of the *ribDBAH* operon in *C. acetobutylicum* WT, grown under iron-replete and -deplete conditions, the *fur* mutant and the complementation strain (*fur*::int-compl) were analysed by Northern hybridization. All oligonucleotides used for generation of probes for Northern hybridization and conduct of semiquantitative RT-PCR are listed in Table S1.

Promoter analysis and motif identification. The promoter regions (within 250 bp of the translation start codon) and the coding sequences of the genes expressed differentially in the *fur* mutant were searched for the presence of a Fur-binding motif by using the Virtual footprint software tool connected to the PRODORIC database (Münch *et al.*, 2003; http://prodoric.tu-bs.de/vfp/). The scanning was performed employing a predetermined positional weight matrix from *Pseudomonas aeruginosa* (cut-off score >7.5). A sequence logo was generated via multiple alignment of all the putative Fur-binding sites identified in this study, using the web-based software tool WebLogo (Crooks *et al.*, 2004; http://weblogo.berkeley.edu/logo.cgi).

2D gel electrophoresis. Isolation of intracellular protein content, preparation for analysis, 2D gel electrophoresis, detection and analysis of the spots were accomplished as described previously (Janssen *et al.*, 2010).

RESULTS

C. acetobutylicum fur mutant strain exhibits reduced growth and enhanced sensitivity to oxidative stress

In order to evaluate the role of Fur in the lifestyle of the strict anaerobe *C. acetobutylicum*, we generated a *fur* (CAC1682)-knockout strain (*fur*::int) through insertional mutagenesis employing the ClosTron system (Heap *et al.*, 2007, 2010); the mutation was confirmed by Southern hybridization (see Fig. S1). In a first set of experiments, we monitored the growth pattern of the *fur*::int strain under various conditions relative to the WT. When grown anaerobically for 48 h at 37 °C on complex agar medium, the mutant formed smaller colonies than the parental strain, suggesting that loss of Fur leads to a major physiological effect (Fig. 1a). Similarly, upon cultivation in liquid complex (CGM) and minimal (MS-MES) media, the mutant exhibited a slower growth rate and a lower final yield (Fig. 1b, c). To investigate the behaviour of the *fur*::int strain further, we added increasing concentrations of the iron chelator DP to complex medium in order to mimic iron depletion. Application of 100 μM DP did not lead to a significant change in the growth rate of the WT strain, whereas 150 and 200 μM DP affected it in a gradual manner (Fig. S3). Interestingly, under all three tested concentrations of DP, growth inhibition of the *fur* mutant was more pronounced (Fig. S3), showing clearly that it does not tolerate iron-limitation conditions better than the WT, in contrast to what has been demonstrated for other species (Yang *et al.*, 2008).

Another basic aspect of the physiological characterization of *C. acetobutylicum* is quantification of the accumulated fermentation products in culture supernatant. Previous studies have reported that iron deficiency leads to a change in the metabolic profile of *C. acetobutylicum* (Bahl *et al.*, 1986; Junelles et al., 1988; Peguin & Soucaille, 1995). However, a gross difference in the product spectrum of the *fur*::int strain relative to the WT was not detected (data not shown).

Considering the limited aerobic resistance of *C. acetobutylicum*, this micro-organism might experience varying degrees of aeration in its natural habitats. Therefore, a coordinated control of iron acquisition that would diminish formation of deleterious ROS via the Fenton reaction is required. The crucial role of Fur in this regulation was demonstrated by the reduction in the viability of the *fur* mutant upon exposure to H_2O_2 and O_2 relative to the WT (Fig. 2).

fur::int strain overproduces RF

Upon cultivation of the *fur* mutant in liquid medium, the supernatant appeared bright yellow in colour. Flavin accumulation has been reported to be induced upon iron deficiency in plants (Susín *et al.*, 1993, Vorwieger *et al.*, 2007), yeasts (Fedorovich *et al.*, 1999, Tanner *et al.*, 1945) and bacteria (Crossley *et al.*, 2007, Demain, 1972, Worst *et al.*, 1998). Therefore, we analysed cell-free culture supernatant from the *fur*::int strain and it displayed visible and fluorescense emission spectra characteristic for flavins (Fig. 3a, b). Testing the composition through TLC revealed the presence of RF as a single constituent (Fig. 3c). It has been reported recently that overexpression of the *ribDBAH* operon in *C. acetobutylicum* leads to accumulation of about 70 mg RF l^{-1} in culture (Cai & Bennett, 2011). Considering the industrial potential of this bacterium, it was tempting to quantify the amounts generated by the *fur*::int strain. After

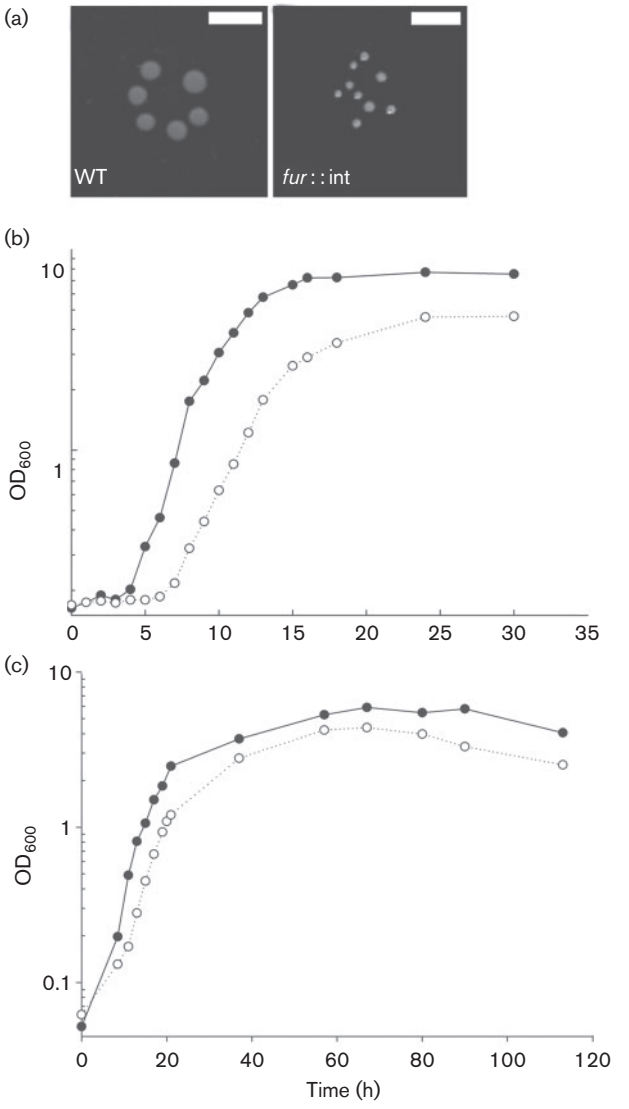

Fig. 1. Comparative growth profiling of *C. acetobutylicum fur* mutant with the WT. Colonies of both strains grown anaerobically for 48 h on solid complex medium (a) and representative growth curves (n=3) obtained in complex CGM medium (b) and minimal MS-MES medium (c) are shown (●, WT; ○, *fur* mutant). Bars [in (a) only], 5 mm.

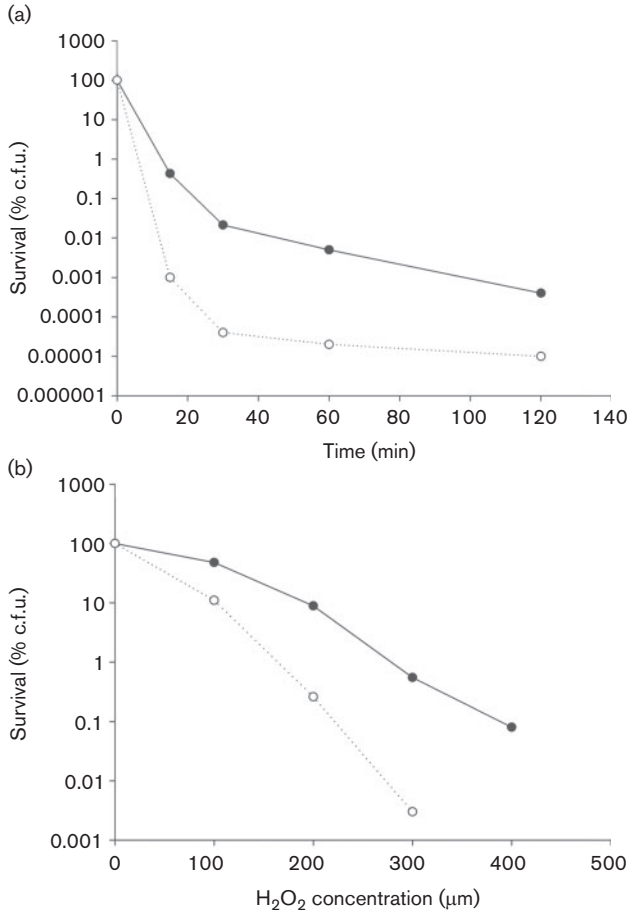

Fig. 2. Tolerance of *C. acetobutylicum fur* mutant strain (○) and WT (●) to oxidative stress. Cultures grown anaerobically at 37 °C in complex medium up to mid-exponential phase were incubated either (a) aerobically at 37 °C and 180 r.p.m. on a rotary shaker or (b) with increasing concentrations of H_2O_2 for 30 min.

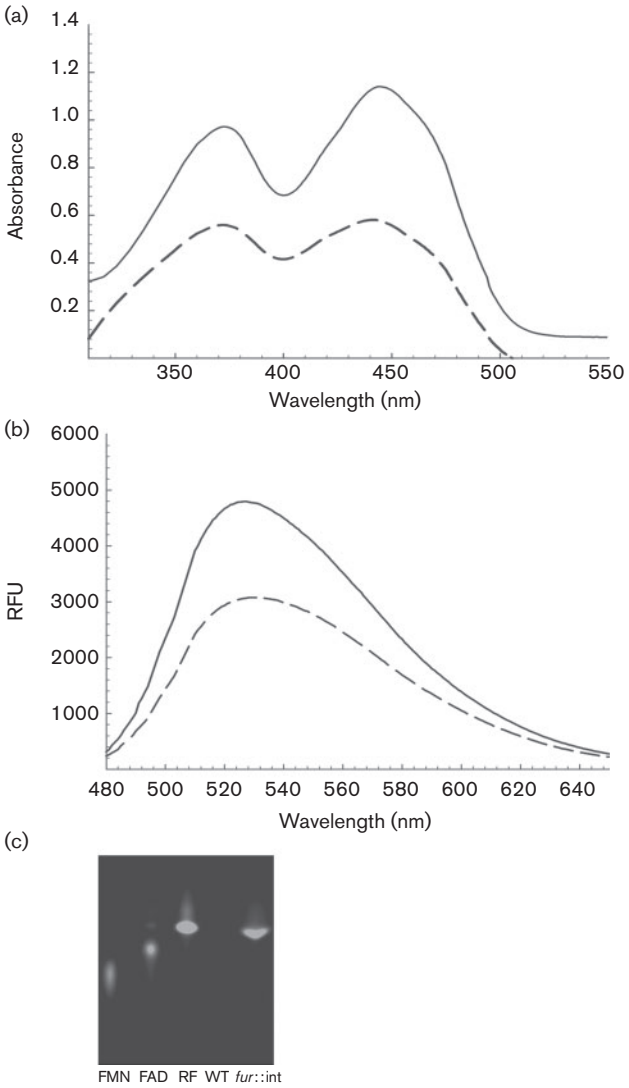

Fig. 3. Identification of RF in culture supernatants of *C. acetobutylicum fur*::int. (a) Absorption and (b) fluorescence emission spectra (RFU, relative fluorescence units) of an aqueous RF standard solution (solid line) and cell-free culture supernatant from *C. acetobutylicum fur*::int (dashed line). (c) TLC of flavin standards (FMN, FAD and RF) and lyophilized culture supernatant from the WT and *fur*::int strain were dissolved in 96 % pure ethanol and spotted on a TLC plate, developed with a mixure of butanol : acetic acid : water (4 : 1 : 5) and subsequently visualized with a UV illuminator.

120 h cultivation in minimal MS-MES medium, the mutant produced a mean of 78 mg RF l^{-1}. Moreover, Cai & Bennett (2011) showed that overproduction of RF did not influence the solvent production significantly, which is in agreement with our results.

Conditions of iron limitation and knockout of *fur* in *C. acetobutylicum* result in extensive transcriptional reshaping

Motivated by the marked phenotype of the *fur*::int strain and in order to gain insight into the mechanisms for maintenance of iron homeostasis in *C. acetobutylicum*, we determined and compared the transcriptional profiles in response to iron limitation and inactivation of *fur*. Total RNA was isolated from mid-exponential cultures of the WT propagated in low-iron minimal medium (6 μM $FeSO_4$) and the *fur*::int strain. The amount of iron was chosen in concert with previous studies that demonstrated iron concentrations of up to 10 μM to be limiting for growth of *C. acetobutylicum* and *Clostridium pasteurianum* (Bahl *et al.*, 1986; Dabrock *et al.*, 1992). For comparison of the transcript levels, a culture of the WT grown under iron-replete conditions (40 μM $FeSO_4$) was used. Conditions of iron limitation affected the transcription of 156 genes significantly, with 79 being upregulated and 77 downregulated. Inactivation of *fur*, on the other hand, resulted in a pronounced effect on the

level of transcription of 157 genes. Among these, 73 were induced and 84 were downregulated.

Of special interest were genes that were regulated both upon iron limitation and in the *fur* mutant. Comparison of both microarray datasets allowed us to identify an overlapping collection of 32 upregulated and seven downregulated genes (see Table 2). In order to identify potential direct targets of Fur, the promoter regions (within 250 bp of their start codon) and the coding sequences of these genes were searched for presence of Fur-binding sequences using the 'Virtual footprint' software and a positional weight matrix (PWM) generated for *P. aeruginosa* (Münch *et al.*, 2003). Multiple alignment of all identified candidate binding sites was used to generate a sequence logo (Fig. S4), representing the most conserved bases within the Fur box. In addition to the genes regulated in parallel in both experiments, analysis of the transcriptomic data revealed a number of genes that were affected by either iron deficiecy (Table S2) or mutation of *fur* (Table S3).

Genes regulated in response to iron limitation as well as *fur* mutation

Consistent with the predicted role of Fur as a regulator of the intracellular iron status, among the genes upregulated most highly in response to iron starvation and inactivation of *fur* in *C. acetobutylicum* were several genes associated with transport of iron. A Feo-type system, which is putatively responsible for uptake of Fe^{2+} (CAC1029–CAC1032), and a ferrichrome system, *fhu*BDC (CAC0788–CAC0791), which is predicted to be involved in transport of Fe(III)–siderophore complexes, appeared to be induced strongly in both microarray datasets. This finding is corroborated by the fact that both operons are associated with candidate Fur-binding sequences. In bacteria, cyanobacteria and algae, iron deficiency leads to dramatically reduced levels of ferredoxins, which is compensated by induction of flavodoxins (Knight & Hardy, 1966; Mayhew & Massey, 1969; Sandmann & Malkin, 1983). Therefore, it was suggested that flavodoxin acts as an electron-carrier counterpart of ferredoxin in the context of iron deficit (Knight *et al.*, 1966). This assumption is supported further by our results, as a gene encoding a flavodoxin (CAC0587) was among the most notably induced (above 200-fold) under iron-limiting conditions and in the *fur* mutant (see Table 2). Presumably the flavodoxin-encoding gene forms a bicistronic operon with another highly upregulated gene (CAC0588), encoding a hypothetical protein, due to the absence of promoter sequence in the intergenic region. In agreement with these results, the flavodoxin protein appeared as a new spot on a 2D protein gel from the *fur* mutant, unidentified so far in *C. acetobutylicum* (Fig. 4). Furthermore, a putative high-score Fur-binding sequence was identified in the promoter region of this operon. The switch from an iron-containing protein like ferredoxin to an FMN-cofactored flavodoxin as an electron transporter upon iron limitation necessitates increased production of RF. Accordingly, in both microarray datasets the transcript levels of the genes belonging to the *ribGBAH* operon, which are involved in synthesis of riboflavin, were among the most strongly upregulated. This transcriptional pattern was confirmed using Northern hybridization. Both iron deficiency and the absence of Fur resulted in a strong hybridization signal that correlated well with the appearance of RF in culture supernatant, whereas introduction of a functional copy of the *fur* gene resulted in partial complementation (Fig. 5). Analytical 2D PAGE analysis of the *fur* mutant, using IPG-Strips pI 4–7 (Bio-Rad), revealed significant upregulation of 6,7-dimethyl-8-ribityllumazine synthase (RibH) in comparison to the WT (Fig. 4). The other enzymes of the pathway could not be identified under these conditions, because their pI is out of range. A putative Fur-binding sequence was identified within the first ORF of the *rib* operon. However, expression of the *rib* genes in Gram-positive bacteria has been shown to be controlled by a conserved FMN-sensing RFN element (riboswitch) (Mironov *et al.*, 2002). Therefore, an indirect mechanism of regulation is likely.

Previous studies demonstrated that lactate is the predominant metabolic product during the acidogenic phase upon iron limitation in *C. acetobutylicum* (Bahl *et al.*, 1986). In agreement with these findings, we found a Fur box-associated gene encoding L-lactate dehydrogenase (CAC0267) to be induced under both tested conditions. Other candidate direct targets of Fur–Fe^{2+} repression included an operon (CAC1602–CAC1603) encoding a diverged CheY domain-containing protein and a hypothetical protein, a putative methyltransferase-encoding gene, a B_{12} biosynthesis operon and, unexpectedly, a gene encoding a 30S ribosomal protein. In addition, a low-score Fur box (score 7.5) was identified in the promoter region of an operon encoding barnase and barnase inhibitor, which was induced moderately in both microarray datasets.

To add to the picture of iron-dependent response in *C. acetobutylicum*, a gene (CAC3650) encoding a protein that possesses an HD-GYP domain, predicted to exhibit cyclic di-GMP hydrolysis activity, was induced in both microarray datasets. This finding indicates that a change in concentration of second messengers could mediate some aspects of the response to iron limitation. Analysis of the promoter sequence of this gene failed to identify a putative Fur-binding sequence, suggesting an indirect regulation. Further upregulated genes without an apparent Fur box were two genes predicted to be involved in purine biosynthesis (CAC0954–CAC0955), a gene encoding a putative nitrogenase and an operon (CAC3622–CAC3627) encoding proteins with predicted functions in amino acid metabolism and cofactor synthesis.

In addition to its well-established role as a repressor, Fur has been shown to act as a transcriptional activator via direct binding to the promoter regions of the target genes (Delany *et al.*, 2004; Yu & Genco, 2012). Our search did not detect Fur-binding sites in any of the genes downregulated

Table 2. Subset of genes affected by both iron limitation (−Fe) and *fur* gene mutation in *C. acetobutylicum*

ORF no.*	Gene name	Annotated function	Expression ratio*		COG†
			−Fe/+Fe	*fur*::int/WT	
CAP0141		Periplasmic hydrogenase small subunit	**0.6**	0.1	C
CAP0142		Periplasmic hydrogenase large subunit	**0.5**	0.1	C
CAP0143		Hydrogenase maturation protease δ subunit, HyaD-like	0.3	0.1	C
CAP0144		Steroid-binding protein	0.3	0.1	R
CAP0145		Hypothetical protein	0.3	0.1	X
CAP0146		Hypothetical protein	0.2	0.1	O
CAC0267	*ldh*	L-Lactate dehydrogenase	18.5	11.2	C
CAC0546		Uncharacterized membrane protein	0.3	0.2	X
CAC0567		Putative methyltransferase	5.7	7.2	H
CAC0570		PTS enzyme II, ABC system	0.3	0.2	G
CAC0582		Cobalamin biosynthesis protein	3.0	4.7	H
CAC0583		CbiK protein (chain A, anaerobic cobalt chelatase)	**2.8**	5.5	H
CAC0584		Precorrin-6B methylase 1 CobL1/CbiE	**2.5**	4.2	H
CAC0587		Flavodoxin	275.7	312.4	C
CAC0588		Hypothetical protein	365.9	188.0	X
CAC0590	*ribD*	Pyrimidine deaminase and pyrimidine reductase	65.4	78.9	H
CAC0591	*ribB*	Riboflavin synthase subunit α	55.3	57.2	H
CAC0592	*ribA*	GTP cyclohydrolase/3,4-dihydroxy-2-butanone 4-phosphate synthase	44.2	61.9	H
CAC0593	*ribH*	6,7-dimethyl-8-ribityllumazine synthase	41.0	49.0	H
CAC0594		Pyridoxal biosynthesis lyase PdxS	4.8	9.1	H
CAC0595		Glutamine amidotransferase subunit PdxT	7.0	9.6	H
CAC0787		Uncharacterized conserved protein	53.9	20.4	S
CAC0788	*fhuB*	Ferrichrome transport permease	207.1	33.6	P
CAC0789	*fhuD*	Permease	199.9	37.4	P
CAC0790	*fhuC*	Ferrichrome-binding periplasmic protein l	86.7	54.4	P
CAC0791		Ferrichrome ABC transporter ATP-binding protein	122.2	67.0	P
CAC0843		RNase precursor (barnase), secreted	4.2	3.3	F
CAC0844		Barstar-like protein RNase (barnase) inhibitor	3.8	3.3	K
CAC1029		FeoA-like protein, involved in iron transport	100.5	140.5	P
CAC1030		FeoA-like protein, involved in iron transport	113.8	154.7	P
CAC1031	*feoB*	FeoB-like GTPase, responsible for iron uptake	246.7	235.9	P
CAC1032		Transcriptional regulator	218.1	159.9	X
CAC1033		Hypothetical protein, CF-31 family	3.3	5.5	X
CAC1478		30S ribosomal protein S4	9.6	7.2	J
CAC1602		Diverged CheY domain-containing protein	29.9	22.4	S
CAC1603		Hypothetical protein	32.0	28.3	J
CAC2905		Uncharacterized protein	0.3	0.1	X
CAC3314		Nitroreductase family protein	3.6	3.8	R
CAC3622		Benzoyl-CoA reductase/2-hydroxyglutaryl-CoA dehydratase	11.0	28.5	E
CAC3623		2-Hydroxyglutaryl-CoA dehydratase activator	ND	16.7	E
CAC3624		6-Pyruvoyl tetrahydrobiopterin synthase	ND	ND	H
CAC3625		MoaA family Fe–S oxidoreductase	13.8	24.1	O
CAC3626		GTP cyclohydrolase I	6.9	10.1	H
CAC3627		PP-loop superfamily ATPase	4.8	7.3	R
CAC3650		HD-GYP domain-containing protein	6.7	6.9	T

*Bold type indicates genes whose expression ratio is below the threshold for significance, but which belong to the same operon as differentially expressed genes. ND, No data available.

†Cluster of orthologous groups (Tatusov *et al.*, 2003).

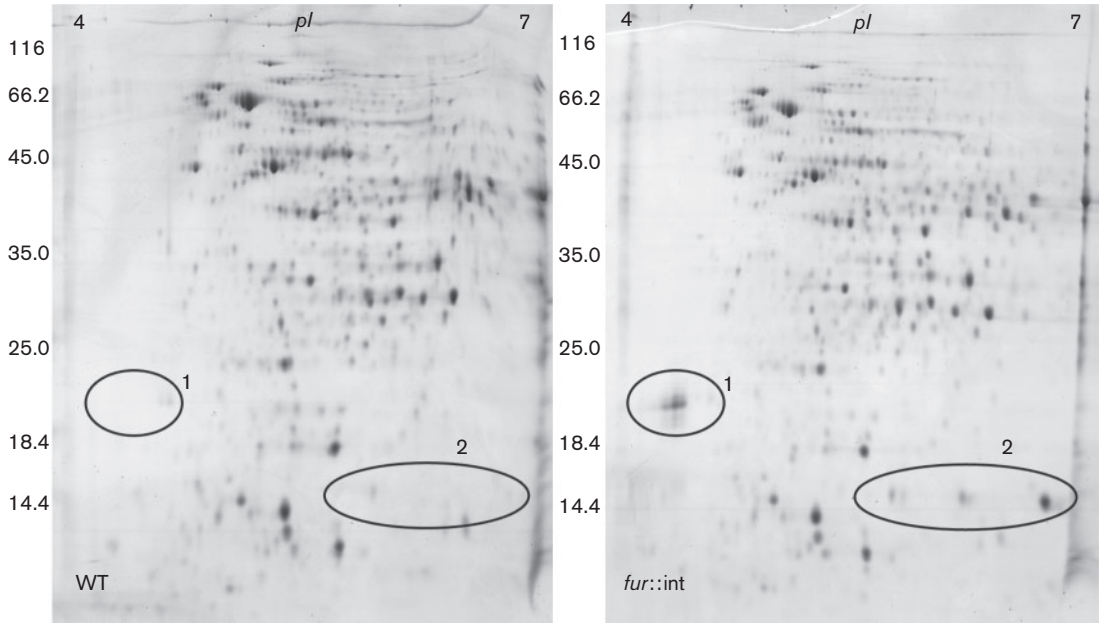

Fig. 4. Protein-expression profile using 2D gel electrophoresis with colloidal Coomassie staining of the *fur*::int mutant strain in comparison to the WT. 1, Flavodoxin; 2, 6,7-dimethyl-8-ribityllumazine synthase (RibH).

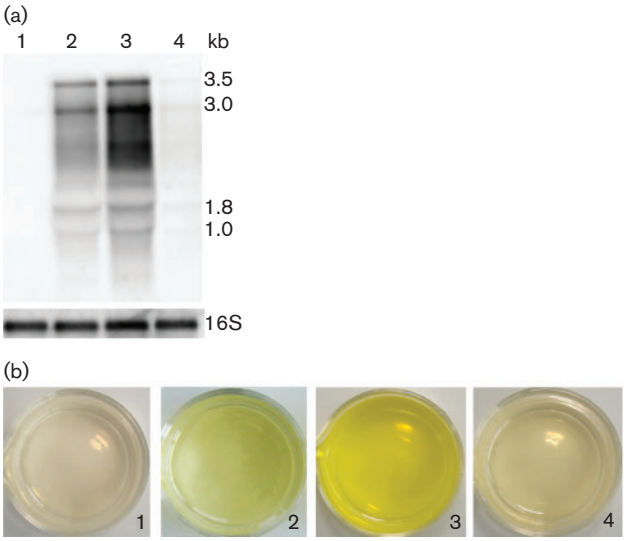

Fig. 5. Lack of Fur and iron limitation lead to induction of the *ribDBAH* operon, responsible for RF biosynthesis, in *C. acetobutylicum*. (a) Northern hybridization analysis was performed with RNA harvested from mid-exponential cultures of *C. acetobutylicum* WT grown under iron-replete (lane 1) and iron-deplete (lane 2) conditions; *fur*::int strain, lacking a functional Fur protein (lane 3); and a complementation strain (*fur*::int compl) (lane 4). Hybridization with a probe specific for 16S rRNA was used as a control for equal loading. Transcript sizes are indicated. (b) Cell-free supernatants from the four tested strains (1, WT; 2, iron-deficient WT; 3, *fur*::int; 4, *fur*::int compl), showing RF pathway upregulation at the metabolomic level, in concert with transcriptional induction of the *ribDBAH* operon.

in both microarray datasets, making it unlikely that their transcription is regulated directly by Fur.

Additional transcriptional changes in response to either iron limitation or *fur* gene mutation

Altogether, 117 genes (45 upregulated and 72 downregulated) involved in a variety of functions displayed either an iron-induced or an iron-repressed mode of transcriptional regulation that was not affected significantly in the *fur* mutant (see Table S2). A large proportion of these genes encode hypothetical or uncharacterized proteins. Among the genes that could be assigned a function, the most striking group was represented by those involved in amino acid metabolism and transport. The category of iron-repressed genes whose transcription was increased in the iron-deficient WT included a cluster of genes responsible for biosynthesis of arginine. Interestingly, a second predicted Feo-type iron-uptake system (CAC0447–CAC0448) was upregulated slightly (about 3-fold). In the group of downregulated (iron-induced) genes were those composing a large operon (CAC0253–CAC0261) encoding the components of a putative nitrogenase. Curiously, also the transcription of an operon predicted to be involved in transport of ferric iron (CAC1988–CAC1990) was down- instead of upregulated under these conditions.

Finally, 116 genes (38 upregulated and 78 downregulated) were expressed differentially in the *fur* mutant, but were not affected during iron deficiency in the WT strain (see Table S3). One explanation that might account for this transcriptional pattern is that the iron limitation used in

this study has not been sufficient to alter the expression of these genes above the threshold defined by us for significance. Therefore, we analysed the promoter regions of both upregulated and downregulated genes for the presence of Fur boxes in an attempt to identify putative direct targets of Fur–Fe^{2+}-mediated repression and activation, respectively. Fur-binding sequences were detected in none of the upregulated genes. However, putative binding sites were identified upstream of several downregulated genes (see Fig. S4).

DISCUSSION

The strictly anaerobic bacterium *C. acetobutylicum* was earlier believed to be fully intolerant to oxygen, inhabiting entirely anaerobic niches. In such environments, a bioavailable form of iron is abundant and the lack of oxygen avoids the formation of deleterious ROS. Therefore, the need for sophisticated mechanisms of an iron-dependent response was questionable. Undoubtedly, anaerobic environments where the redox potential is favourable would provide optimal growth. However, previous studies have demonstrated that *C. acetobutylicum* is able to cope with limited aerobic exposure (Kawasaki *et al.*, 2004; O'Brien & Morris, 1971). Such observations imply that this microorganism might experience varying degrees of aeration in its natural habitats. Therefore, a fine-tuned network for maintenance of iron homeostasis that satisfies the high demands for iron and is linked with oxidative-stress defence is necessary. We investigated this network in *C. acetobutylicum*. The role of Fur was studied by insertional inactivation of the *fur* gene. Absence of the ferric-uptake regulator protein in other strictly anaerobic bacteria, such as *Dichelobacter nodosus* and *Desulfovibrio vulgaris*, was reported not to cause any significant changes in their growth behaviour (Bender *et al.*, 2007; Parker *et al.*, 2005). Moreover, a Fur-like protein has been shown to be non-functional in the strictly anaerobic archaeon *Thermococcus kodakarensis* (Louvel *et al.*, 2009). In contrast, lack of this regulator in *C. acetobutylicum* caused a growth deficiency. Furthermore, susceptibility of the *C. acetobutylicum fur* mutant to oxidative stress demonstrated the importance of Fur for maintaining the intracellular iron balance.

In this study, we determined the transcriptional changes upon iron limitation and knockout of *fur* in *C. acetobutylicum* by using DNA microarrays. Comparison of the transcriptomic data from both experiments allowed us to outline the Fur-dependent response to iron in this microorganism. As expected, de-repressed in both microarray datasets were two operons with predicted functions in uptake of ferrous iron and Fe(III)–siderophore complexes. Siderophore synthesis has not been observed so far in *C. acetobutylicum* and, except for a single operon (CAC2004–CAC2006), no other obvious genes encoding systems for siderophore production have been annotated in its genome. Changes in the expression of the latter were not detected either under iron starvation or in the *fur* mutant. Thus, utilization of xenosiderophores synthesized by other species is more likely. Interestingly, a second putative ferrous iron-uptake system (CAC0447–CAC0448) was slightly upregulated in the iron-deficient WT. For another anaerobe, *Porphyromonas gingivalis*, it has been demonstrated that a second Feo-type system mediates manganese transport (Dashper *et al.*, 2005). A phylogenetic tree of FeoB proteins implies that FeoB2 in clostridia might also function as a manganese transporter (Cartron *et al.*, 2006). The slight upregulation under iron starvation could be explained by the fact that manganese can substitute for iron in a few cases (Martin & Imlay, 2011). The genome of *C. acetobutylicum* revealed the presence of only three additional operons encoding orthologues of known iron-transport systems (CAC1988–CAC1990, CAC2441–CAC2444 and CAC2877–CAC2878). However, while CAC1988–CAC1990 was downregulated in the iron-deprived WT, the other two operons did not exhibit a differential transcriptional response to either iron deficiency or *fur* gene inactivation. Therefore, it is possible that, despite their homology to iron-uptake systems, these three transporters might play a different role. Altogether, these results indicate that Fur is the primary regulator that mediates control of iron transport in *C. acetobutylicum*.

Major parts of the metabolism of *C. acetobutylicum* are iron-dependent, due to the participation of iron-rich proteins. Although regulation of enzymic activities at some key metabolic steps has been determined, data for the mechanisms of control at transcriptional level are scarce. The iron–sulfur-containing pyruvate : ferredoxin oxidoreductase (PFOR) and its physiological electron acceptor ferredoxin are central for the metabolism of *C. acetobutylicum*. We have shown here that regulation of two genes encoding a lactate dehydrogenase (CAC0267) and a flavodoxin (CAC0587), probably aimed to provide non-iron alternatives to these proteins under conditions of iron deficiency, is mediated by Fur. In order to support the function of flavodoxin, RF biosynthesis genes were also highly upregulated. The transcriptional profiling of another strict anaerobe, *D. vulgaris*, and the closely related *B. subtilis* showed significant upregulation of genes encoding flavodoxins as a response to iron limitation and deletion of *fur*. However, induction of the *rib* genes involved in synthesis of RF was not detected in these micro-organisms (Baichoo *et al.*, 2002; Bender *et al.*, 2007).

In *C. acetobutylicum*, reduced ferredoxin is an electron donor for hydrogenase. Two Fe-only hydrogenases (HydA1 and HydA2) and one putative [NiFe] hydrogenase are encoded in its genome (Demuez *et al.*, 2007; Gorwa *et al.*, 1996). It has been shown previously that iron limitation leads to a significant reduction in hydrogenase activity (Junelles *et al.*, 1988). However, upon analysis of our transcriptomic data, we did not observe differential expression of *hydA1* and *hydA2* under iron limitation or in the *fur* mutant. The transcription of an operon (CAP0141–CAC0146) encoding the components of the putative [NiFe] hydrogenase, though, was downregulated in both microarray

datasets. No Fur-binding sequence was detected in the promoter region of this operon, suggesting that indirect mechanisms of Fur regulation might be present in *C. acetobutylicum*. Repression of iron-containing proteins and iron-utilization pathways upon iron deprivation has been reported to be mediated by a Fur-regulated small RNA in numerous bacteria (Massé & Arguin, 2005; Gaballa *et al.*, 2008; Mellin *et al.*, 2007).

Considering the large number of Fe–S-containing proteins and their central role in the metabolism of *C. acetobutylicum*, inevitably a question arises as to how Fe–S clusters are repaired upon iron limitation. *E. coli* responds to iron starvation and oxidative stress by recruiting Suf (sulfur formation) machinery for Fe–S cluster assembly (Outten *et al.*, 2004). The *suf* operon was shown to be regulated directly by Fur and OxyR, respectively (McHugh *et al.*, 2003; Zheng *et al.*, 2001). Similarly, an operon encoding the components of a putative Suf system (CAC3288–CAC3290) has been reported to be induced under oxidative stress in *C. acetobutylicum* (Hillmann *et al.*, 2009). However, in our study, significant change in the level of transcription of this operon was not observed either under iron limitation or in the *fur* mutant. Therefore, the mechanisms for repair of Fe–S clusters under iron limitation in *C. acetobutylicum* remain unclear.

Our microarray analysis showed that the iron-starvation response of *C. acetobutylicum* involved a number of transcriptional changes that were not affected by inactivation of *fur*. The most dramatically upregulated was a cluster of genes involved in arginine biosynthesis. In *B. subtilis*, the AhrC protein senses a high intracellular concentration of arginine and represses genes involved in its biosynthesis (Czaplewski *et al.*, 1992). The genome of *C. acetobutylicum* showed a gene (CAC2074) encoding an orthologue of AhrC, which possibly exhibits control on arginine synthesis in a similar fashion. The reason for the de-repression of arginine-biosynthesis genes under iron limitation is currently unclear. Among the most dramatically downregulated under iron limitation, perhaps as an element of an iron-sparing response, was an operon putatively involved in synthesis and maturation of iron-containing nitrogenase (CAC0253–CAC0261). Explanation of all regulatory aspects is beyond the scope of this work. However, a hypothesis that might account for some of the transcriptional changes in the iron-deficient WT can be advanced. For example, other iron-sensing regulators might operate in *C. acetobutylicum*. Although Fur is the classical regulator that responds to intracellular iron levels, DtxR (diphtheria toxin repressor) has been identified in some bacteria as a major iron-dependent repressor (Boyd *et al.*, 1990; Dussurget *et al.*, 1996; Hill *et al.*, 1998). The genome of *C. acetobutylicum* revealed two ORFs (CAC1469 and CAC2616) encoding DtxR-like proteins. A DtxR-related protein (MntR) in the closely related *B. subtilis* was characterized as the main regulator of intracellular manganese homeostasis (Que & Helmann, 2000). Therefore, further investigation is needed to clarify the metal specificity of these putative regulators in *C. acetobutylicum*. Fur has also been shown to function as an iron-free repressor (apo-Fur) in *Helicobacter pylori* (Delany *et al.*, 2001; Ernst *et al.*, 2005), leading to downregulation of the target genes only under iron limitation. This could explain the transcriptional repression of some of the genes in the iron-deprived WT. Further experimentation is necessary to determine whether the *C. acetobutylicum* Fur protein also exhibits repression in its iron-free form.

Finally, the expression of a number of genes was changed exclusively in the *fur* mutant, but was not affected in the iron-limited culture. Putative Fur-binding sites were predicted upstream of three genes encoding a Cu/Zn superoxide dismutase (CAC1363), an ATP-dependent serine protease (CAC2135) and a spore cortex-lytic enzyme (CAC3081), as well as one operon (CAC2352–CAC2354), all of which were downregulated in the *fur* mutant. However, except for this finding, there is no further evidence from our study to support a role of Fur as a direct transcriptional activator in *C. acetobutylicum*. Secondary effects, such as reduced growth, deregulated iron transport and excess production of RF, are likely to account for a proportion of the observed transcriptional changes in the *fur* mutant. For example, withdrawal of GTP from the cellular pools for RF overproduction, as well as GTP-dependent Feo-mediated iron transport, might be a signal that inactivates the repression activity of the global regulator CodY (Ratnayake-Lecamwasam *et al.*, 2001). Therefore, it is possible that some of the transcriptional effects in the *fur* mutant strain might be due to de-repression of members of the CodY regulon. Also upregulated in the *fur* mutant were several genes involved in the heat-shock response, including *grpE*, *dnaK* and *hsp90*. Similarly, knockout of *fur* in *Neisseria meningitidis* resulted in induction of heat-shock response genes in an iron-independent manner (Delany *et al.*, 2006).

In summary, impact of *fur* mutation on the physiology of the strictly anaerobic *C. acetobutylicum* demonstrated that maintenance of iron homeostasis is an important aspect of the lifestyle of this micro-organism. Transcriptomic analysis of the iron-deprived WT and the *fur* mutant, as well as a search for putative direct targets of Fur, indicated that this regulator exhibits control not only on iron uptake, but also on basic features of energy metabolism in *C. acetobutylicum*. Our data also implied that *C. acetobutylicum* Fur may mediate indirect mechanisms of control. The diverse transcriptional response to iron starvation, lack of Fur, or both, highlights the need for further studies to better understand the iron-responsive networks in *C. acetobutylicum* and strict anaerobes in general.

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