Escherichia coli YtfE is a di-iron protein with an important function in assembly of iron–sulphur clusters

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Abstract

Our previous analysis of the transcriptome of *Escherichia coli* under nitrosative stress showed that the *ytfE* gene was one of the highest induced genes. Furthermore, the *E. coli* strain mutated on the *ytfE* gene was found to be more sensitive to nitric oxide than the wild-type strain. In the present work, we show that the mutation of the *ytfE* gene in *E. coli* yielded a strain that grows poorly under anaerobic respiratory conditions and that has an increased sensitivity to iron starvation. Furthermore, all examined iron–sulphur proteins have decreased activity levels in the strain lacking *ytfE*. Altogether, the results suggest a role for *ytfE* in iron–sulphur cluster biogenesis. YtfE was overexpressed in *E. coli* and it is shown to contain a di-iron centre of the histidine-carboxylate family.

Introduction

High concentrations of nitric oxide (NO) are used by the immune system, generated by the inducible nitric oxide synthase (iNOS), to kill invading prokaryotic pathogens and parasites. The mechanisms used by *Escherichia coli* to respond to this aggression have been extensively studied (Mukhopadhyay *et al*., 2004; Saraiva *et al*., 2004; Flatley *et al*., 2005). In particular, three proteins are proposed to metabolize NO: (i) flavorubredoxin (NorV), a flavodiiron NO reductase (Gardner *et al*., 2002; Gomes *et al*., 2002); (ii) flavohaemoglobin (HmpA) (Poole & Hughes, 2000; Gardner & Gardner, 2002); and (iii) pentaheme nitrite reductase (NrfA) (Poock *et al*., 2002). Analysis of the transcriptome of *E. coli* grown under nitrosative stress showed that the genes encoding for flavorubredoxin and flavohaemoglobin are indeed among the highest induced genes (Mukhopadhyay *et al*., 2004; Flatley *et al*., 2005; Justino *et al*., 2005). These results were found independent of the conditions tested, i.e. they were observed either in *E. coli* grown aerobically and treated with *S*-nitrosoglutathione (GSNO) (Mukhopadhyay *et al*., 2004) or in *E. coli* grown anaerobically and exposed to NO (Justino *et al*., 2005). Interestingly, in both studies, the *E. coli ytfE* gene was found to be very strongly induced. In fact, we found that an *E. coli* strain mutated on *ytfE* exhibited increased sensitivity to NO stress, and that the degree of growth impairment of the *E. coli ytfE*-mutated strain was higher than that observed for strains deleted in either flavorubredoxin or flavohaemoglobin (Justino *et al*., 2005). The *E. coli* K-12 *ytfE* gene is predicted to encode for a cytoplasmic protein, conserved in all enterobacteria, and that shares significant sequence identity with gene products of several other bacteria (Justino *et al*., 2005). To assess its role, *E. coli* YtfE was produced, biochemically and spectroscopically characterized and the phenotype of the *E. coli ytfE* mutant was analysed. The data showed a key role of YtfE in the anaerobic respiratory metabolism of *E. coli*.

Materials and methods

Bacterial strains and growth assays

The genotypes and sources of *Escherichia coli* K-12 strains are listed in Table 1. The cells were grown in Luria–Bertani (LB) or in minimal salts medium (MS) (Justino *et al*., 2005) that, when supplemented with L-arginine, L-threonine, L-histidine, L-leucine and L-proline at 40 μg mL^{-1} was denominated MSA. Glucose (40 mM), glycerol [0.4% (weight in volume, w/v)] and sodium acetate (40 mM) were used as carbon sources. Sodium nitrate, sodium fumarate, and dimethyl sulphoxide (DMSO) at 40 mM and sodium nitrite (5 mM) were used as electron acceptors while sodium pyruvate (2 mM) was used as electron donor. Cultures using glycerol as the carbon source contained 5% LB, except for glycerol/nitrite medium, which was prepared as in Tyson *et al*. (1997). Potassium gluconate [0.2% (w/v)] medium was

Table 1. Strains of *Escherichia coli* used in this study

Strain	Genotype	Source or reference
XL2-Blue	*E. coli recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacIqZΔM15* Tn*10* (Tetr)].	Stratagene
BL21-Gold(DE3)	*E. coli* B F⁻ *ompT hsdS*(rB⁻ mB⁻) *dcm*+Tetr *gal* λ(DE3) *endA* The	Stratagene
Wild type	K-12 ATCC 23716	ATCC
fur mutant	K-12 MG1655Δ*fur*::Tc^r	Simon Andrews
fnr mutant	*E. coli* RVΔ*nirB*Δ*lac*Δ*fnr*	Page (1990)
ytfE mutant	K-12 *ytfE*:: Cm^r	Justino *et al*. (2005)

made in MS with casamino acid [0.25% (w/v)]. All cultures were started with 2% inocula of overnight aerobic cultures in LB and grown, at 37 °C and 150 r.p.m., either aerobically in flasks filled with 1/5 of its volume, or anaerobically in rubber seal-capped flasks that, once filled with medium and closed, were extensively bubbled with nitrogen.

Enzymatic and iron starvation sensitivity assays

Cultures for enzymatic assays were grown to an $OD_{600\,nm}$ of 0.3–0.5 in the appropriate medium and assayed in duplicate.

The nitrate and nitrite reductase activities of cell extracts of *E. coli* wild-type and *ytfE* mutant, grown anaerobically in MSA/glucose with sodium nitrate or sodium nitrite, were assayed by the substrate-dependent oxidation of reduced methyl or benzyl viologen (Clegg *et al*., 2002).

For the aconitase activity, cultures of *E. coli* wild-type and *ytfE* mutant were grown aerobically in MSA/glucose, and subsequently treated and analysed as described in Gardner (2002).

The activity of isocitrate dehydrogenase was determined according to el-Mansi (1998) in cells grown aerobically in MSA supplemented with fumarate/glycerol or with pyruvate/acetate. The glutamate synthase activity was determined in cells grown aerobically in MS/glucose (Meister, 1985).

For the iron starvation assays, cultures of *E. coli* K-12 and LMS4209 were grown aerobically o/n in LB, diluted 1 : 100 in fresh medium, grown for 8 h and then used as inocula. The cultures were adjusted to a starting $OD_{600\,nm}$ of 0.05, and cell cultures were left untreated (control) or treated with the iron quelating agent 2,2′-dipyridyl to a final concentration of 100 and 300 μM. Cell turbidity was measured after 16 h of growth, at 37 °C and 150 r.p.m.

RNA extraction and RT-PCR analysis

Total RNA from cells grown to $OD_{600\,nm}$ of 0.3 was extracted with the Roche RNA kit, treated with DNaseI and analysed with the QIAGEN® OneStep Reverse Transcriptase (RT-PCR) Kit (Qiagen, Isaza, Portugal). NO treatment was performed as described in Justino *et al*. (2005). Primers that amplified products of 110 bp for *gapA*, 362 bp for *narG*, 798 bp for *narZ*, 676 bp for *napA*, 497 bp for *hmpA*, 504 bp for *norV* and 769 bp for *ytfE* were used in reactions performed with 200 ng of RNA, except for the analysis of the *hmpA* and *norV* transcription where 70 ng was used. *E. coli gapA* allowed evaluation of the RNA amounts.

Cloning, expression, purification, characterization of *Escherichia coli* recombinant *ytfE* and complementation studies

The coding region of *E. coli ytfE* (b4209) was amplified by PCR from *E. coli* K-12 ATCC 23716 genomic DNA using the primers 5′-AAGAATGAGGTATCACATATGGCTTATCGC-3′ and 5′-GGCTGTTTATTGGTAAGAATTCGGCTGCTG-3′, which incorporated *Nde*I and *Eco*RI restriction sites. The amplified gene was cloned into pET-24a (Novagen, Merck Biosciences VWR International, Portugal), and sequenced. Overexpression of the recombinant protein was achieved in *E. coli* Bl21Gold (DE3) cells grown aerobically (30 °C, 180 r.p.m.) in MS/glucose, $FeSO_4$ 10 μM and kanamycin 30 μg mL^{-1}, to an $OD_{600\,nm}$ of 0.3 and induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 8 h. Cells were ressuspended in buffer A (Tris-HCl 10 mM pH 7.6 and glycerol 9%) with NaCl 50 mM and disrupted. All purification steps were performed at a 4 °C. The soluble extract was loaded into a Q-Sepharose High-Performance column, and YtfE was eluted at ∼200 mM NaCl. Purification was achieved after loading into a Superdex S-75 gel filtration column (buffer A+150 mM NaCl), and into a Q-Sepharose HP column. Molecular mass determination of YtfE was performed in a Superdex 75 (10/300) GL (Tricorn GmbH, Portugal) column using standard proteins.

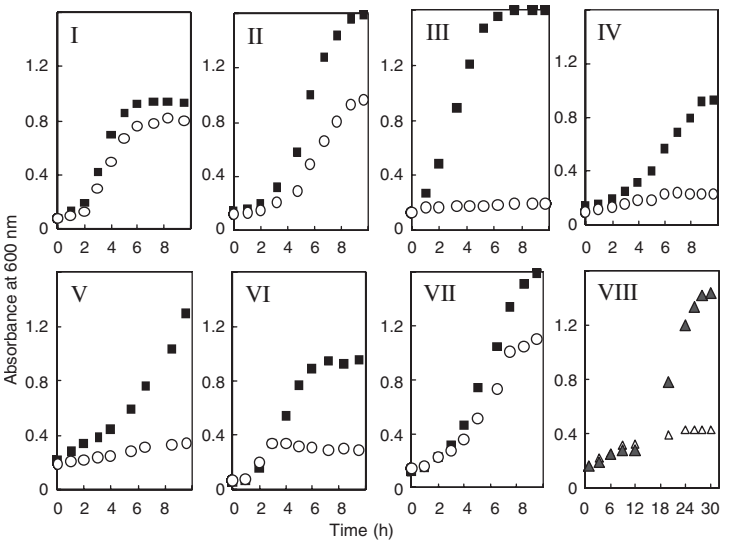
Protein concentration was assayed by the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) as the standard (Smith *et al*., 1985), and the iron content was determined by the TPTZ (2,4,6-tripyridyl-1,2,3-triazine) method (Fischer & Price, 1964). EPR spectra were obtained on a Bruker ESP 380 spectrometer, equipped with an Oxford instruments continuous flow helium cryostat. The redox titration followed by EPR (800 μM protein) was performed under anaerobic conditions, as described in Vicente & Teixeira (2005).

The *ytfE* gene excised from pET-YtfE was inserted into the expression plasmid pFLAG (Sigma). Cultures of *E. coli ytfE* mutant (LMS4209) transformed with pFLAG or pFLAG-YtfE were grown anaerobically in MSA/glucose/nitrate medium with IPTG (1 mM) and $FeSO_4$ (10 μM).

Results and discussion

The *ytfE* mutant strain grows very slowly under several anaerobic respiring conditions

Under fermentation conditions, the *Escherichia coli ytfE* mutant showed no major growth differences relative to the



Enzymatic activity of iron-sulphur proteins in the *ytfE* mutant

	NAR		NIR		ACO	GOGAT	ICDH	
Medium	Glu/NO_3^-	Glu/NO_2^-	Glu/NO_3^-	Glu/NO_2^-	MS/Glu	MS/Glu	Acet/Pyr	Gly/Fum
Strain (phenotype)								
E. coli K-12 (*wt*)	458	310	75	430	122	166	195	551
LMS4209 (*ytfE*$^-$)	9	13	44	190	30	105	199	550

Doubling Times (min^{-1})

Strain (phenotype)	*E. coli* K-12 (*wt*)	LMS4209 (*ytfE*$^-$)
Anaerobic growth		
LB	78 ± 0.7	78 ± 1.1
MSA/Glu	110 ± 3.9	131 ± 3.5
LB/Nitrate	51 ± 2.4	103 ± 1.1
MSA/Glu/Nitrate	96 ± 12	SG
MSA/Glu/Nitrate (20 mM)	94 ± 6.3	SG
MSA/Gly/Nitrate	82 ± 1.2	SG
MSA/Glu/Nitrite	121 ± 1.2	175 ± 7.3
MSA/Gly/Nitrite	135 ± 8	192 ± 10
MSA/Gly/Fum	169 ± 3.0	SG
MSA/Gly/DMSO	183 ± 4.5	SG
MS/Gluconate	60 ± 2.7	SG
Aerobic growth		
LB	57 ± 3.5	63 ± 3.8
MSA/Glu	130 ± 1.5	324 ± 2.7
Acetate/Pyruvate	125 ± 0.5	164 ± 0.3

Fig. 1. Growth of the *Escherichia coli ytfE* mutant is inhibited under several growth conditions. Expression of YtfE restores the wild-type phenotype under anaerobic nitrate-growth conditions. The activities of iron–sulphur proteins are decreased in the *ytfE* mutant. *E. coli* wild-type strain (■), *E. coli ytfE* mutant (○), *E. coli ytfE*$^-$(pFLAG) (△) and *E. coli ytfE*$^-$(pFLAG-*ytfE*) (▲). (I) anaerobic growth (ANG) in Luria–Bertani (LB); (II) ANG in MSA/Glu/nitrite;(III) ANG in MSA/glycerol/nitrate; (IV) ANG in MSA/glycerol/fumarate; (V) ANG in MSA/glycerol/DMSO; (VI) ANG in MS/gluconate; (VII) aerobic growth in MSA/acetate/pyruvate; (VIII) ANG of *E. coli ytfE* mutant in the presence of nitrate by expression of plasmid-borne *E. coli ytfE*. The results are representative of at least three independent experiments. Tables: SG, slow growth; MS, minimal medium; Glu, glucose; Gly, glycerol; Fum, fumarate; DMSO, dimethylsulphoxide; NO_3^-, nitrate; NO_2^-, nitrite, Acet, acetate; Pyr, pyruvate; NAR, nitrate reductase; NIR, nitrite reductase; ACO, aconitase; GOGAT, glutamate synthase; and ICDH, isocitrate dehydrogenase. Activities in nmol min^{-1} mg^{-1} of protein.

parental strain (Fig. 1I). Although the *E. coli ytfE* mutant was able to sustain anaerobic growth in the presence of nitrite (Fig. 1II), it exhibited a 1.3-fold increase in the generation time when compared with the wild-type cells. In contrast, the *E. coli ytfE* mutant grew very slowly in the presence of nitrate (Fig. 1III), and the decrease in nitrate concentration did not stimulate the growth of the mutant. The reason why the *ytfE* mutant fails to grow under fermentation in glucose/nitrate medium remains unclear. The *E. coli ytfE* mutant also grew slowly anaerobically when fumarate or DMSO were used as electron acceptors (Figs 1IV and V), or when grown on gluconate, conditions that require the expression of the iron–sulphur containing gluconate dehydratase enzyme (Fig. 1VI). In contrast, the aerobic growth of *E. coli ytfE* mutant in a rich medium remained unchanged, while the aerobic growth in the minimal medium supplemented with acetate and pyruvate was similar to that found under fermentative conditions in minimal medium (Fig. 1VII).

Introduction of a plasmid containing the clone of the *ytfE* gene into the *E. coli ytfE* mutant strain restored the wild-type phenotype, as judged by the significant increase in the growth rate of the mutant strain observed under anaerobic nitrate-grown conditions (Fig. 1VIII). Figure 1VIII also shows that the growth behaviour of the *ytfE* mutant had not changed upon addition of iron to the medium.

Ralstonia eutropha NorA (Pohlmann *et al.*, 2000) and *Pseudomonas stutzeri* DnrN (Vollack & Zumft, 2001) are two homologues of *E. coli* YftE, whose genes are induced by NO. The *R. eutropha* NorA-null mutant showed no phenotypic alteration when cultivated under denitrifying conditions, and the disruption of *dnrN* in *P. stutzeri* caused alteration of the kinetics of the transcriptional response of the *nisSTB* operon towards nitrite (Vollack & Zumft, 2001). Although these proteins also seem to have a role in nitrogen oxide metabolism, the different phenotypes may be due to the fact that denitrifying bacteria are not closely related to *E. coli*.

However, the functions of NorA and DnrN remain to be clarified (Kim *et al.*, 2003).

Analysis of the *Salmonella enterica* response to NO allowed the identification of a locus containing the *nipC* gene (Kim *et al.*, 2003), another homologue of *E. coli ytfE*. The *S. enterica nipC* mutant did not show growth defects under the growth conditions tested (different media, pHs, electron acceptors such as oxygen, nitrite and nitrate and carbon sources) (Kim *et al.*, 2003). We also did not observe significant differences between the wild-type strain and the *E. coli yftE* mutant in LB or minimal medium, in the presence or absence of oxygen (Fig. 1). Furthermore, when compared with growth in minimal medium supplemented with nitrate, the differences of anaerobic growth in LB/nitrate between the *ytfE* mutant and the wild-type strain were much smaller. Hence, appropriate growth conditions are crucial for a difference in phenotype between the mutant and the wild-type strains to be observed.

The activities of iron–sulphur proteins are decreased in the *ytfE* mutant

The mutation of *ytfE* resulted in a decrease in the total nitrate reductase-specific activity, to values that were very low when compared with the wild-type strain (Fig. 1). Moreover, the recombinant YtfE did not itself exhibit a significant nitrate reductase activity ($< 0.04\ s^{-1}$). The nitrite reductase activity of cells of the *ytfE* mutant grown in nitrite was ~40% of the wild-type strain, a result in agreement with the ~30% increase on the generation time of the *ytfE* mutant strain under nitrite-grown conditions. Two *E. coli* enzymes account for the utilization of nitrite as a substrate, namely the [4Fe-4S]/siroheme containing nitrite reductase NirB (Cole, 1996), and the decaheme periplasmic nitrite reductase (NrfA) complex (Wang & Gunsalus, 2000), whose genes are expressed in the presence of nitrate and nitrite (Simon, 2002). The fact that nitrite reductase activity of the *ytfE* mutant extract is approximately half that of the parental strain suggests that in the absence of YtfE, at least one of the two nitrite reductase enzymes has reduced activity. To further clarify this issue, UV-visible spectra of the soluble fraction from mutant and wild-type cells grown in nitrite were recorded, showing that in both strains equal amounts of *c*-type haemes are expressed (data not shown). Therefore, the lower nitrite reductase activity of the *ytfE* mutant is not because of a decreased expression of the catalytic subunit of NrfA.

Furthermore, in the *ytfE*-deficient strain, the glutamate synthase activity was ~37% lower when compared with the wild-type strain, while the aconitase activity decreased by ~75% (Fig. 1). In contrast, the activity of isocitrate dehydrogenase, an enzyme that does not contain iron–sulphur centres, was unaffected by the mutation (Fig. 1). Hence, all examined iron–sulphur proteins had decreased levels of specific activities in the *E. coli* mutant *ytfE* strain and it can be inferred from the growth behaviour of the *ytfE* mutant that other iron–sulphur containing enzymes such as siroheme nitrite reductase, DMSO reductase, fumarate reductase and gluconate dehydratase enzymes were are also affected by the *ytfE* mutation (Fig. 1).

Mutation of *ytfE* increases the sensitivity of *Escherichia coli* to iron starvation

To further confirm the involvement of *ytfE* in iron metabolism, the ability of the *ytfE* mutant to grow under iron-starvation conditions was analysed (Fig. 2). The two strains showed similar growth in the absence of the iron chelator, independent of the oxygen supply. Upon the addition of 100 μM of dipyridyl, the *ytfE* mutant strain underwent a decrease in growth higher than the wild-type strain (Fig. 2). Furthermore, the effect of iron starvation on the *ytfE* mutant strain is highly accentuated under anaerobic conditions when compared with the wild-type strain (Fig. 2). The sensitivity to iron starvation under aerobic conditions of the *ytfE* mutant at the two dipyridyl concentrations tested is greater than the one observed for the *iscS* mutant and lower than that of the *sufABCDSE* mutant, the two *E. coli* systems implicated in the assembly of iron–sulphur centres (Outten *et al.*, 2004). Interestingly, and as observed for *ytfE*, the transcription of *sufA* and *iscA* is also elevated under NO stress conditions (Mukhopadhyay *et al.*, 2004; Flatley *et al.*, 2005; Justino *et al.*, 2005).

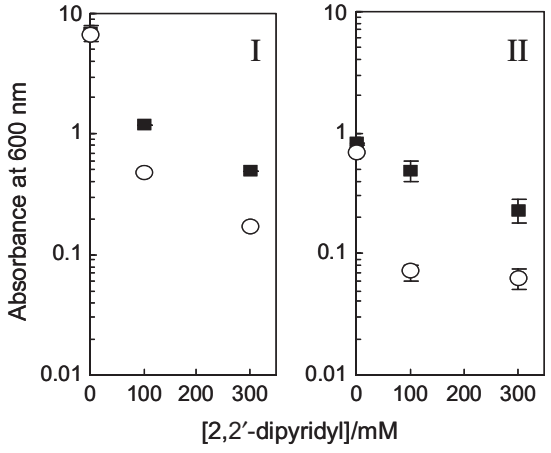


Fig. 2. The *Escherichia coli ytfE* mutant has increased sensitivity to iron starvation. Cultures of the *E. coli* wild-type strain (■) and of the mutant *ytfE* strain (○), grown in Luria–Bertani aerobically (I) or anaerobically (II). Error bars are ± 1 standard deviation for experiments carried out at least three times.

Fnr and Fur regulate *ytfE* transcription

In the *fnr* null *E. coli* strain, the *ytfE* mRNA level increased (Fig. 3I), indicating that the transcription of *ytfE* is negatively regulated by Fnr. As YtfE is an iron-containing protein and because of its putative involvement in iron metabolism, the possible control exerted by the global regulator of iron metabolism Fur was also analysed. The results show that Fur acts as a repressor in the transcription of *ytfE* (Fig. 3I).

As the analysis of the promoter regions upstream of *ytfE* revealed no obvious Fnr or Fur binding site motifs, it is possible that the effect of Fnr and Fur on *ytfE* transcription is due to an indirect effect on the regulation of iron uptake or iron metabolism.

YtfE does not regulate the *Escherichia coli* genes involved in NO detoxification

The *E. coli ytfE* and the genes that share a high sequence similarity with *ytfE* are annotated in the databases as regulators of cell morphology and NO sensing. In our previous work, we reported that *E. coli* cells mutated in *ytfE* did not present different morphological characteristics when compared with the wild-type *E. coli* K-12 (Justino *et al.*, 2005). Here, we show that YtfE does not act as a regulator of any of the *E. coli* genes so far known to be involved in NO detoxification, namely *hmpA* and *norV*, as the induction caused by NO in the transcription of *norV* and *hmpA* was still observed in the *E. coli ytfE* mutant strain (Fig. 3II). Moreover, the di-iron centre in YtfE does not seem to act as a redox-active site for NO chemistry because the recombinant protein did not exhibit a significant NO reductase activity (data not shown).

In spite of the important role of YtfE in *E. coli* nitrate metabolism, the nitrate reductases genes *narG*, *narZ* and *napA* are not regulated by YtfE, as judged by the equal levels of induction caused by nitrate on the transcription of these genes in the wild type and in the *ytfE* mutant strains (Fig. 3III).

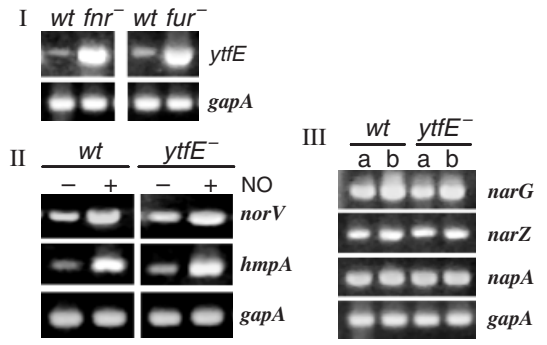


Fig. 3. *ytfE* is negatively regulated by Fnr and Fur. (I) Transcriptional level of *ytfE* assayed in RNAs extracted from cells of *Escherichia coli* wild (*wt*), *E. coli fnr* mutant (*fnr*⁻) and *E. coli fur* mutant (*fur*⁻), grown anaerobically either in MSA/glucose for the analysis of the *fnr* mutation or in Luria–Bertani (LB) for the analysis of the *fur* mutation. For each experiment, the transcriptional level of the internal gene control *E. coli gapA* in the RNAs used is also shown. (II) Level of transcription of the *E. coli* flavorubredoxin (*norV*) and flavohaemoglobin (*hmpA*) in the wild-type (*wt*) and *ytfE* mutant (*ytfE*⁻) strains, cultured anaerobically in MSA/glucose in the absence (−) or in the presence of 50 μM nitric oxide, NO (+). (III) Levels of mRNA in *E. coli* wild-type strain (*wt*) and *ytfE* mutant (*ytfE*⁻) of the nitrate reductases genes *narG*, *narZ* and *napA*. Cells were grown anaerobically in minimal medium/glucose (a) or in glucose/nitrate medium (b).

YtfE is a binuclear non-haeme iron protein

The recombinant *E. coli* YtfE was isolated as a mixture of ~24 kDa monomeric and ~50 kDa dimeric forms containing ~1.5 atoms of iron per monomer. The UV/visible spectrum of YtfE is characteristic of a nonhaeme iron protein with a broad band at *c.* 360 nm (Fig. 4a). These data suggested the presence of a di-iron centre in YtfE that was confirmed by EPR spectroscopy, which showed a spectrum with all of the expected principal *g*-values below 2.0, $g = 1.96$, 1.92 and 1.88 (Fig. 4b), optimally detected at *c.* 15 K. No other resonances were observed, in either perpendicular or parallel modes, namely those generally attributed to high-spin ferric iron (data not shown). The EPR *g*-values are typical of non iron–sulphur di-iron centres in the mixed valence Fe(III)–Fe(II) state (Kurtz, 1997). The intensity at the three EPR lines was measured as a function of the solution redox potential (Fig. 4c), yielding a bell-shaped curve, with $E = +260$ and $+110$ mV. This behaviour is also characteristic of di-iron centres (Solomon *et al.*, 2000). In either the fully reduced (Fe(II)–Fe(II)) or the fully oxidized (Fe(III)–Fe(III)) states, no further resonances were observed, again in parallel or perpendicular mode. Together, the electronic and the EPR spectra show clearly that the iron ions form a di-iron centre, corroborating our earlier proposal that the conserved histidines and carboxylates are ligands to the iron atoms (Justino *et al.*, 2005).

Conclusion

In summary, we observed that the expression of *Escherichia coli ytfE*, a novel type of di-iron protein, is stimulated by nitrosative stress and iron starvation, that *E. coli* strains deficient in *ytfE* grow slowly under several respiratory conditions and are sensitive to iron starvation. Furthermore, all examined iron–sulphur proteins had decreased activity levels in the strain lacking *ytfE*. Hence, *E. coli* YtfE may represent a novel system involved in the biosynthesis of iron–sulphur clusters that seems to be particularly important in cells submitted to stress conditions and clearly has a key role in anaerobic respiratory metabolism.

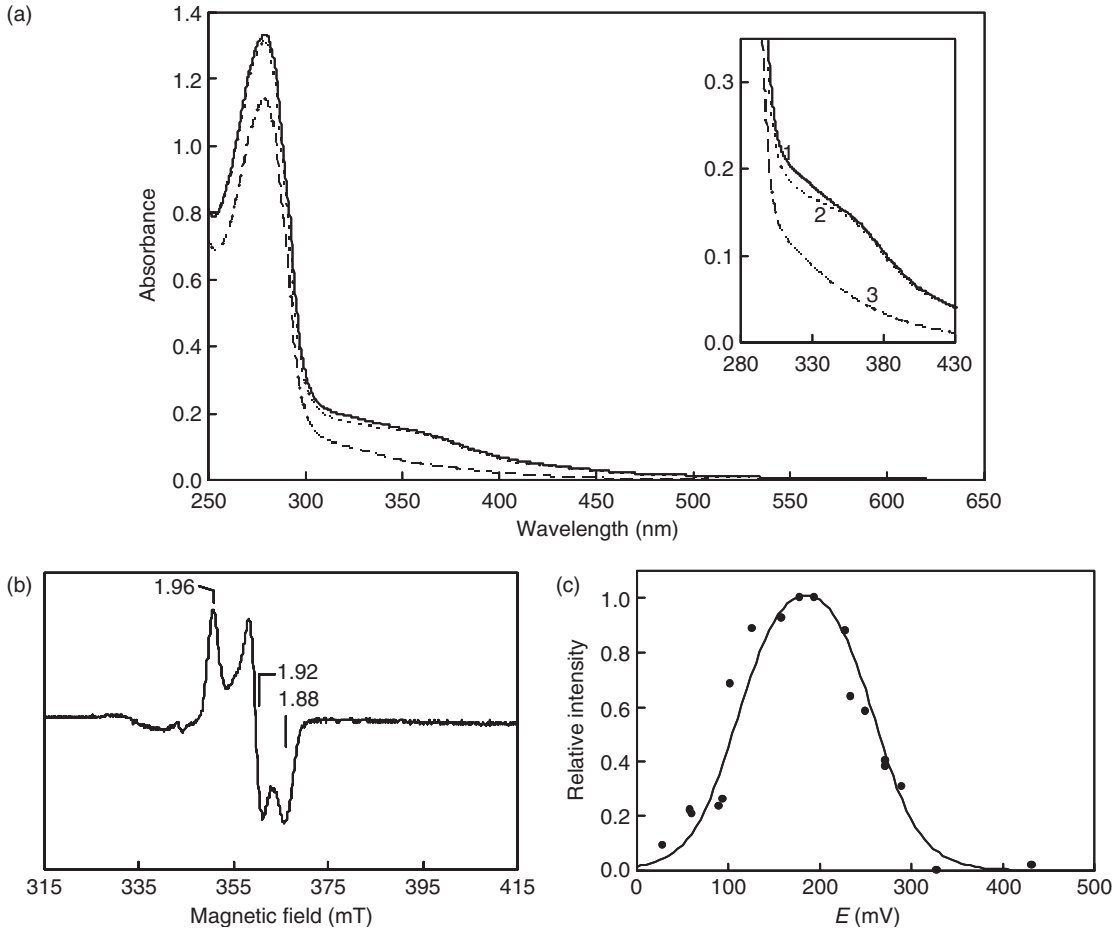


Fig. 4. *Escherichia coli* YtfE protein is a di-iron protein. (A) UV/Visible spectra of YtfE after purification (1, —), oxidized with potassium persulphate (2, · · ·) and reduced with sodium dithionite (3, – – –). The inset shows the spectral differences in detail. (B) EPR spectrum of as-prepared YtfE, at 14 K. Microwave frequency: 9.644 GHz; microwave power: 2.4 mW; modulation amplitude:1 mT. (C) Redox titration of YtfE di-iron centre. Full line was calculated with the Nernst equation for two monoelectronic consecutive processes, with reduction potentials of +260 and +110 mV.

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