Expression of the *Escherichia coli* *yfiD* gene responds to intracellular pH and reduces the accumulation of acidic metabolic end products

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The YfiD protein of *Escherichia coli* has been reported to be an acid-inducible protein. Here it is shown that expression of a *yfiD*::*lac* reporter fusion is enhanced up to 3.5-fold during acidic growth. The anaerobic transcription factor FNR was confirmed as the major regulator of *yfiD* expression, and ArcA was found to enhance anaerobic *yfiD* expression, probably by displacing a repressing FNR dimer in the –93.5 region of the promoter. Moreover, the pyruvate sensor PdhR was shown to act as a minor anaerobic repressor of *yfiD* expression. On the basis of its strong homology to the C-terminal region of pyruvate formate-lyase (PFL) it was predicted that YfiD would be a radical-containing enzyme. The YfiD radical was found to be introduced by the PFL-activase enzyme, but unlike PFL, AdhE did not deactivate radicalized YfiD. The extent of radical activation of YfiD was enhanced by low intracellular pH, and thus it was concluded that both *yfiD* expression and YfiD activity are affected by growth at low pH. The *yfiD* mutant strain JRG4033 excreted increased levels of organic acids compared to the parental strain when grown in chemostat culture under oxygen-starved conditions, consistent with the acid-inducibility of *yfiD* expression and the recently reported ability of YfiD to rescue the activity of oxygenolytically cleaved PFL.

Keywords: acid stress, radical protein, pH homeostasis, pyruvate formate-lyase, formate

INTRODUCTION

Much of the success of bacteria depends on the ability to survive and thrive in adverse conditions. *Escherichia coli* is an adaptable bacterium that has evolved mechanisms to allow it to cope with conditions as diverse as the gut and the sewer. These different environments present particular challenges such as wide variations in oxygen availability, pH and osmolarity. The ability to adapt to these ever changing environmental factors is ultimately rooted in altered patterns of gene expression. *E. coli* is a facultative anaerobe that uses combinations of transcription factors to control gene expression in response to environmental oxygen. Predominant amongst these

transcription factors is FNR (fumarate and nitrate reduction regulator), a direct oxygen sensor that functions mainly to activate the expression of genes required during anaerobic growth (Kiley & Beinert, 1999). FNR senses anaerobiosis through the acquisition of an oxygen-labile [4Fe–4S] cluster, which promotes dimerization of the protein and enhances site-specific DNA binding (Lazzazera *et al.*, 1996; Jordan *et al.*, 1997; Popescu *et al.*, 1998). Analysis of the cytosolic polypeptides produced during anaerobic growth of *E. coli* revealed that the *yfiD* gene product (YfiD) was a member of the FNR regulon (Green & Baldwin, 1997). Searches for other *E. coli* proteins with amino acid sequences related to YfiD revealed a strong homology (77% identity over a 64 aa overlap) to the C-terminal region of pyruvate formate-lyase (PFL), the product of another FNR-regulated gene, *pflB*. As well as *yfiD*, *E. coli* possesses three more genes whose products are predicted to be similar to PFL: *tdcE*, *pflD* and *f810*

Abbreviations: dO₂, dissolved oxygen tension; FNR, fumarate and nitrate reduction regulator; GST, glutathione S-transferase; PFL, pyruvate formate-lyase.

(Sawers & Watson, 1998). In *E. coli* the *yfiD* gene is located in the *srnB-ung* intergenic region and genes potentially encoding YfiD proteins have been identified in a wide range of Gram-negative and -positive bacteria including *Serratia* sp., *Serratia liquefaciens* and *Haemophilus influenzae*, as well as the bacteriophages T4 and T5. The *H. influenzae yfiD* gene is, like that of *E. coli*, close to the *ung* genes and for bacteriophage T4 and T5 it is close to the thymidine kinase gene and the tRNA gene cluster, respectively. In both *Serratia* species the *yfiD* gene is associated with the *phlA* gene, which encodes extracellular phospholipase A1. All the encoded proteins contain a highly conserved pentapeptide motif (RVXGY) around the C-terminal glycyl residue (G734 in PFL), which is the site of the glycyl radical produced during the activation of PFL (Sawers & Watson, 1998). The PFL-like proteins also contain the adjacent cysteinyl residues characteristic of the PFL active site. However, YfiD contains only one cysteine residue and an active site cannot be confidently predicted on the basis of its amino acid sequence. The *yfiD* promoter has an unusual architecture and has proved a useful tool yielding new insights into FNR-mediated repression (Green *et al.*, 1998; Green & Marshall, 1999; Marshall *et al.*, 2001). Expression of *yfiD* is activated by FNR acting at $-40\cdot5$, a conventional activation position, but is downregulated by a second FNR dimer at $-93\cdot5$, which leads to maximal *yfiD* expression during microaerobic growth (Green *et al.*, 1998; Marshall *et al.*, 2001).

The *yfiD* gene product has appeared in five proteomic screens. Firstly, as a polypeptide upregulated by expression of the heterologous oxygen-responsive FNR protein, HlyX, in anaerobically grown *E. coli* (Green & Baldwin, 1997). In the second analysis, YfiD was induced to high levels when the cytoplasm was acidified, i.e. during growth at pH 4.4, and upon the addition of propionate at pH 6 (Blankenhorn *et al.*, 1999). Interestingly, the effects of pH on gene expression are known to overlap with many other environmental factors, but particularly with oxygen availability (Slonczewski & Foster, 1996). It has been noted that the acid-induced expression of amino acid decarboxylases is enhanced under anaerobic conditions (Meng & Bennett, 1992; Neely *et al.*, 1994) and that expression of cytochrome *o* is repressed by acid stress (Cotter *et al.*, 1990). It was therefore suggested that there is a complex relationship between YfiD, pH and oxygen (Blankenhorn *et al.*, 1999). Thirdly, YfiD was decreased in gel-entrapped *E. coli*, and on the basis of its transient expression pattern it was suggested that YfiD is an early stationary phase protein (Perrot *et al.*, 2000). In the fourth analysis, enhanced *yfiD* expression was noted in strains harbouring heterologous polyhydroxyalkanoate biosynthesis genes (Han *et al.*, 2001). It was again suggested that YfiD was present under these particular conditions because the cytoplasm becomes more acidic. Finally, YfiD was detected as a phosphorylated protein in L-forms of *E. coli* (Freestone *et al.*, 1998). Thus, although the exact physiological role of YfiD has not yet been assigned, this protein has emerged from a range of studies related to

anaerobic and acidic growth. Therefore, because *yfiD* expression is regulated by FNR and the YfiD gene product is acid-inducible, we have investigated the effects of anaerobiosis, pH and growth phase on *yfiD* expression and activation of the YfiD protein. We find that the major regulator of *yfiD* expression is FNR, but that *yfiD* expression is induced at the level of transcription during growth under acidic conditions. Furthermore, the activation of YfiD by PFL activase is enhanced by growth at low pH in the presence of propionate or benzoate. In addition, accumulation of lactate, succinate, pyruvate and formate is greater for the *yfiD* mutant strain than for the parent, consistent with a role for YfiD in reducing exposure to acidic metabolic end products and in directing carbon flux.

METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* strains used were: the parental strain, MC1000 [$\Delta(lacIPZOYA)$ -X74 *galU galK str^r thi araD139 $\Delta(araABC-leu)$*]; an *fur* strain, JRG1728 [MC1000 $\Delta(tyrR-fur-rac-trg)17$ *zdd-230::Tn9*]; and a *pdhR*⁺ strain JRG2547 (Quail & Guest, 1995). The PFL activase (*pflA*) and deactivase (*adhE*) deficient strains were MC4100 [$\Delta ara \Delta(argF-lac) rpsL relA1 flbB deoC1 ptsF25$] derivatives, 234M11 (Heßlinger *et al.*, 1998) and RM235, respectively. The *arcA* strain was BW3218 $\Delta lac-169 \Delta(serB-trpR-arcA-thr)$, and this strain was complemented with pRB38, a multicopy plasmid encoding ArcA. *E. coli* strain W3110 was used as a prototroph to construct the *yfiD* null mutant (JRG4033) by homologous allelic exchange of chromosomal *yfiD* with *yfiD::kan^r*. This was achieved using a pMAK705 (Hamilton *et al.*, 1989) derivative, pGS1193, containing 1010 bp of 5' flanking DNA (beginning 44 bp downstream of the ATG start codon of *yfiD* and extending 966 bp upstream), a *kan^r* cassette (isolated from pUC4-KIXX, Amersham-Pharmacia) and 1262 bp of 3' flanking DNA (beginning 193 bp upstream of the *yfiD* TAA stop codon and extending 1069 bp downstream of the stop codon). Thus pGS1193 contained an insert in which 147 bp of *yfiD* coding sequence was replaced by a *kan^r* cassette. The direction of transcription of the *kan^r* cassette was shown to be colinear with *yfiD* by diagnostic restriction digests. Once integrated it is unlikely that the disrupted *yfiD* gene could cause any polar effects because it is not part of an operon, its transcriptional terminators remain intact and the flanking genes (*yfiK* and *ung*) are transcribed in the opposite direction to *yfiD*. *E. coli* W3110 was transformed with pGS1193 and grown at non-permissive (42 °C) and permissive (30 °C) temperatures in the presence or absence of antibiotic(s) as appropriate to effect plasmid integration and subsequent resolution of the mutant (Hamilton *et al.*, 1989). A representative strain, designated JRG4033, was identified after plasmid-curing as a *yfiD* null mutant on the basis of its stable *kan^r* phenotype, the size of DNA fragments produced from PCR amplification of the disrupted *yfiD* gene from genomic DNA (not shown), Southern hybridization (not shown) and Western blotting (see Fig. 1). *E. coli* BL21 [*F⁻ ompT hsdS (r_B m_B⁻) gal*] transformed with pGS1011, constructed by ligating the *yfiD*-containing *NcoI*-*Sall* fragment from pGS1036, a pUC118 derivative containing a PCR-generated *NcoI*-*Sall* flanked *yfiD* coding region, into pGEX-KG (Guan & Dixon, 1991), was used to overproduce the GST-YfiD fusion protein. The same *NcoI*-*Sall* fragment was transferred into pTac85 to create the *yfiD* expression plasmid pGS1207, which was used to assess the effects of

Fig. 1. Production of the YfiD protein is FNR-dependent. Western blot of stationary phase anaerobic cultures of the *yfiD* mutant, JRG4033, the parental strain, W3110 and an *fnr* mutant, JRG1728. YfiD was not detected in JRG4033 and was barely detectable in JRG1728. Two cross-reacting polypeptides were present in the extracts of the parental strain, equivalent to the full-length YfiD protein (YfiD) and a smaller product (YfiD'). The amount of YfiD' detected in these stationary phase cultures was low because activation of YfiD was found to be optimal in the exponential phase of growth (see Fig. 4).

mutations in the PFL activase and deactivase genes on YfiD. For monitoring expression of *yfiD* under a variety of conditions, the low copy number (~ 5 per chromosome) pRW50-based *yfiD::lac* reporter plasmid pGS1000 was used (Green *et al.*, 1998).

The media used to test the effects of pH on *yfiD* expression and YfiD activation was based on LBK broth (10 g tryptone l^{-1} , 5 g yeast extract l^{-1} , 6.4 g KCl l^{-1}) containing an appropriate buffer (100 mM: LBK/MOPS, pH 7.2; LBK/MES, pH 6.3; and LBK/HOMOPIPES, pH 4.5) (Blankenhorn *et al.*, 1999). These media were supplemented with propionic acid (50 mM) or benzoic acid (20 mM) as indicated to further depress the intracellular pH (Blankenhorn *et al.*, 1999). Cultures were grown in the indicated buffered media supplemented with appropriate antibiotics (200 μ g ampicillin ml^{-1} , 35 μ g tetracycline ml^{-1}) to maintain plasmids. Anaerobic growth was in sealed bottles at 37 °C for 16 h for most expression studies. When cultures were analysed at different points in the growth cycle, samples were taken in a Mk3 Anaerobic Workstation (Don Whitley). Aerobic cultures were grown in 250 ml conical flasks containing 10 ml medium with vigorous shaking at 37 °C for 16 h. β -Galactosidase activities were measured in duplicate for at least two independent cultures, as described by Miller (1972).

Protein purification. YfiD was initially purified as a glutathione *S*-transferase (GST)–YfiD fusion protein amplified in aerobically grown *E. coli* BL21(pGS1011). Cultures (500 ml) were grown at 37 °C with vigorous shaking in 2 $\times$ YT medium (Sambrook *et al.*, 1989) for 3 h, at which point the expression of the *gst-yfiD* fusion was initiated by addition of IPTG to a final concentration of 30 mg l^{-1} . Incubation was continued for a further 3 h before the cultures were harvested by centrifugation. Clarified French pressure cell extracts (up to 150 mg total protein, in 10 mM Tris/HCl, pH 8.0 containing 10 mM benzimidazole and 0.1 mM PMSF) were applied to a column (50 $\times$ 15 mm) of glutathione–agarose (Sigma) equilibrated with 50 mM Tris/HCl, pH 6.8 containing 0.5 mM DTT. The column was washed with 10 vols of the same buffer before eluting the GST–YfiD fusion with 50 mM Tris/HCl, pH 8.0 containing 10 mM reduced glutathione. Aliquots of the fusion protein were dialysed to remove glutathione and the pure YfiD protein was obtained by cleavage of the desalted GST–YfiD fusion with thrombin (14 U) for 2 h at 37 °C, followed by chromatography on glutathione–Sepharose to remove the released GST. The product contains an extra 15 aa, which are derived from the thrombin-sensitive linker, whose presence was confirmed by the total amino acid analysis and N-

terminal sequencing. Protein concentrations were estimated with the Bio-Rad protein reagent.

Characterization of YfiD. Total metal ion contents and electrospray mass spectrometry measurements were done with redissolved freeze-dried samples of isolated YfiD that had been dialysed against several changes of 1 mM ammonium acetate, pH 6.0. The native molecular mass of YfiD was also estimated by gel filtration on a Superdex 200 HR 10/30 column (Amersham-Pharmacia) equilibrated with 50 mM sodium phosphate buffer, pH 7.0 containing 0.15 M sodium chloride, and calibrated with a 6500–66000 molecular weight marker kit for gel filtration (Sigma). N-terminal amino acid sequencing was achieved by Edman degradation using an Applied Biosystems protein sequencer. Standard technologies were used for Western blotting. Polyclonal YfiD anti-sera were raised in rabbits (dwarf lop) and used at a final dilution of 1:20000. Detection of cross-reacting material was achieved using the Amersham-Pharmacia ECL detection system with horseradish peroxidase conjugated secondary antibodies, or by nitro blue tetrazolium/X-phosphate for alkaline phosphatase conjugated secondary antibodies, and was quantified using ImageMaster software (Amersham-Pharmacia).

Continuous culture. Chemostat cultures were grown in LH Fermentation chemostat vessels (either 1 l nominal capacity, 750 ml working volume or 2 l nominal capacity, 1130 ml working volume) maintained at 37 °C. The minimal medium had the following composition (g l^{-1}): (NH₄)₂SO₄, 2.0; K₂HPO₄, 1.0; NaH₂PO₄, 1.0; MgSO₄·7H₂O, 0.2; supplemented with trace element solution (Vishniac & Santer, 1957), 2 ml; and glucose, 30 mM. The pH was adjusted to 7.0 $\pm$ 0.2 by automatic titration with sterile 2 M KOH. Dissolved oxygen levels were monitored with galvanic oxygen electrodes. For aerobic cultures the culture agitation speed (400–500 r.p.m.) and air flow were adjusted to maintain a dissolved oxygen tension (dO₂) >50% air saturation or <5% for microaerobic cultures. For oxygen-starved cultures, the air supply was replaced by oxygen-free nitrogen. In some experiments air and nitrogen mixtures were used to gas the cultures. Inocula were grown as batch cultures overnight in the continuous culture medium.

Glucose in feed medium and culture filtrates was estimated enzymically with glucose oxidase (Bergmeyer & Bernt, 1968). Low molecular mass overmetabolites in culture filtrates were monitored by HPLC using a Shodex Ion pak KC-811 column (8 $\times$ 300 mm) at 50 °C. Samples were eluted with 0.1% (v/v) phosphoric acid at a flow rate of 2.0 ml min^{-1} through detectors for UV (210 nm) and refractive index (Waters 490E and Waters 410, respectively) coupled to a data module (Millennium 2010). Peak areas were proportional to concentrations, which were determined using standard solutions of each organic acid. Acetate, formate and ethanol were also estimated using enzyme-based Boehringer Mannheim kits, which also served to confirm these peak assignments in the HPLC elution profiles; the estimates obtained by this method were similar to those obtained by calibrated HPLC.

To investigate whether the *yfiD* null mutant is physiologically disadvantaged compared to the parent, a chemostat containing carbon-limited medium was inoculated with approximately equal numbers of W3110 and JRG4033 bacteria by combining equivalent overnight cultures of these strains. The mixed culture was allowed to grow as a batch culture in the fermentation vessel for ~ 8 h before switching to continuous growth with a dilution rate of 0.2 h⁻¹. Samples (~ 5 ml) were taken immediately after switching to continuous growth and subsequently every 5–10 generations. The proportion of each

strain in the culture vessel was determined by plating serial dilutions (in triplicate) of the culture on carbon-limited agar plates with and without kanamycin (30 mg l⁻¹). The plates were incubated for 40 h at 37 °C before counting the number of c.f.u. recovered. The numbers were averaged and the percentage of JRG4033 (kan^r bacteria) was calculated. The competition experiments were performed over 50 generations under aerobic (50 % dO₂) and microaerobic (5 % dO₂) conditions.

RESULTS

Expression of β -galactosidase from a *yfiD::lac* reporter is acid-inducible

The starting point for this work was the report that the *yfiD* gene product is induced by acid stress (Blankenhorn *et al.*, 1999). To test if this was caused by enhanced expression of *yfiD* during acidic growth we used our existing low copy number *yfiD::lac* reporter, pGS1000 (Green *et al.*, 1998), to monitor the expression of *yfiD* both aerobically and anaerobically at various pH values. The pH of batch cultures was set by buffering at the indicated pH, irrespective of the addition of 50 mM propionic or 20 mM benzoic acids, which act as membrane-permeant weak acids lowering the intracellular pH of *E. coli* (Blankenhorn *et al.*, 1999). The data obtained revealed that, at pH 7.2, *yfiD::lac* expression was induced >10-fold under anaerobic (3910 Miller U) compared to aerobic conditions (310 Miller U) (Table 1). Lowering the pH of the culture medium, or the intracellular pH by adding the weak acids propionate or benzoate, enhanced *yfiD* expression up to a further 3.5-fold anaerobically (3910 Miller U at pH 7.2, compared to 13590 Miller U at pH 6.3 in the presence of propionate) and 2.5-fold aerobically (310 Miller U at pH 7.2, compared to 790 Miller U at pH 6.3 in the presence of propionate). The β -galactosidase activities

of cultures with the lowest intracellular pH values (i.e. those grown at pH 6.3 plus benzoate, or at pH 4.5) were unexpectedly low, especially when grown anaerobically, indicating that β -galactosidase is unstable, *yfiD* expression is reduced, or that the reporter plasmid copy number has fallen to such an extent that β -galactosidase activity no longer faithfully reflects transcription of *yfiD* at these low pH values. To test which of these possibilities might be correct, the YfiD protein was purified (see below) and antibodies were raised to determine the amount of YfiD protein in the cultures directly by Western blotting. These experiments revealed that for anaerobic cultures the cellular content of YfiD protein followed the expected trend (YfiD content increased as *yfiD::lac* expression increased) except at the lowest pH values (pH 4.5 and at pH 6.3 in the presence of benzoate) where, contrary to expectations based on the *yfiD::lac* reporter data, the intracellular levels of YfiD were the greatest (Table 1). Thus it was concluded that at all but the lowest pH values, the β -galactosidase activities arising from the *yfiD::lac* reporter plasmid (pGS1000) reflected the production of YfiD protein. The possibility that the difference between the two measures of YfiD production at the lowest pH values may arise from a reduction in reporter plasmid copy number under these conditions cannot be excluded, but because it is known that β -galactosidase is sensitive to pH values below 6.0 (Degraeve *et al.*, 1996), the low reporter activities for cultures grown at the lowest pH values are most simply explained by reduced stability of β -galactosidase. An alternative explanation for the observations would require a reduction in *yfiD* expression combined with enhanced stability of YfiD protein in acidic cultures.

That FNR is the major transcription regulator of *yfiD* expression was confirmed by Western blotting, which

Table 1. Expression of a *yfiD::lac* fusion and intracellular YfiD content during acid stress

β -Galactosidase activities of MC1000 (*fnr*⁺) bacteria containing the *yfiD* promoter cloned in pRW50 (pGS1000) are shown. Cultures were incubated at 37 °C until stationary phase. The media used were: LBK/MOPS, pH 7.2; LBK/MES, pH 6.3; and LBK/HOMOPIPES, pH 4.5 with the addition of propionate (50 mM) or benzoate (20 mM) as indicated. β -Galactosidase activities were measured in duplicate for stationary phase cultures and the values reported are the means of at least two cultures in which the values obtained varied by <15%. The final pH values of the culture media were also recorded. Samples of bacteria were obtained to allow the intracellular content of YfiD protein to be estimated by Western blotting combined with quantitative densitometry. The sample volumes were adjusted so that equivalent numbers of bacteria were loaded on to SDS-polyacrylamide gels and the amount of YfiD present after anaerobic growth at pH 7.2 was taken as 100%.

Growth conditions	Aerobic			Anaerobic		
	Final pH	Promoter activity	Intracellular YfiD content (%)	Final pH	Promoter activity	Intracellular YfiD content (%)
pH 7.2	7.53	310	< 10	7.07	3910	100
pH 7.2 + propionic acid	7.56	320	10–16	7.08	8490	170–173
pH 7.2 + benzoic acid	7.65	530	22	7.01	8890	147
pH 6.3	6.64	500	< 10	6.13	7890	136–146
pH 6.3 + propionic acid	6.39	790	23	6.36	13590	180–210
pH 6.3 + benzoic acid	6.19	500	20	6.13	1370	273–300
pH 4.5	–	–	–	4.70	660	238–247

Table 2. Effect of acidity on FNR, ArcA and PdhR activities

Expression of *yfiD* as monitored by the *yfiD::lac* reporter fusion pGS1000 in the presence or absence of the transcription factors FNR, ArcA and PdhR, and the effect of acidity on the activity of these regulators. As a control, the effects of the imposed genetic and environmental conditions on the expression of the FNR-regulated model promoter *FF-41.5* were also assessed. The parental strain for the *fnr* and *pdbR^c* mutants was MC1000 (Δlac). The *fnr* strain was JRG1728 ($\Delta fnr \Delta lac$) and the *pdbR* strain was JRG2547 (*pdbR^c Δlac*). The *arcA* strain was BW3218 ($\Delta arcA \Delta lac$) which was complemented with the multicopy *arcA* expression plasmid pRB38 to restore the *arcA*⁺ phenotype. The values given are the means of duplicate assays from at least two cultures that varied by less than 15 %.

Strain	Set pH	Aerobic		Anaerobic		
		Final pH	Promoter activity (<i>yfiD</i>)	Final pH	Promoter activity	
					<i>yfiD</i>	<i>FF-41.5</i>
Parent	7.2	7.53	310	7.06	2990	5970
<i>fnr</i>	7.2	7.51	70	7.01	200	210
<i>pdbR^c</i>	7.2	7.58	190	7.04	3740	–
Parent	6.3	6.65	500	6.12	4210	7340
<i>fnr</i>	6.3	6.75	90	5.95	230	170
<i>pdbR^c</i>	6.3	6.87	460	6.38	4950	–
<i>arcA</i> ⁺	7.2	7.56	110	7.05	6810	2760
<i>arcA</i>	7.2	7.55	150	7.04	2540	3140
<i>arcA</i> ⁺	6.3	6.66	173	6.25	9210	4060
<i>arcA</i>	6.3	6.89	89	6.27	4470	4440

showed that extracts of an *fnr* mutant strain (JRG1728) grown anaerobically at pH 7.2 contained very little YfiD protein compared to the parent strain, indicating that anaerobic expression of *yfiD* under these conditions is FNR dependent (Fig. 1). Immunoblots also revealed that the cellular content of YfiD was enhanced by growth at low pH under aerobic conditions (Table 1). In aerobic cultures the activity of the anaerobic transcription factor FNR is minimal, and consequently *yfiD::lac* expression is low, but still pH responsive (Table 1). This suggests either that acidic conditions can enhance FNR-mediated expression of *yfiD* under aerobic conditions or that an alternative acid-responsive regulator may be involved. Nevertheless, it can be concluded that acid stress does increase expression from the *yfiD* promoter (Table 1), and that FNR is the major regulator of *yfiD* expression (Fig. 1).

It has long been recognized that there are overlaps in bacterial responses to anaerobiosis and acid stress at the level of gene regulation (Cotter *et al.*, 1990; Meng & Bennett, 1992; Neely *et al.*, 1994; Slonczewski & Foster, 1996; Blankenhorn *et al.*, 1999). Therefore, we considered whether FNR and/or ArcAB, the major oxygen-sensing systems of *E. coli* contributed to the acid enhancement of *yfiD* expression. This could be achieved by enhancement of the activities of either, or both, regulatory systems at lower intracellular pH values. The data presented in Table 2 and Fig. 1 confirmed that FNR is responsible for the anaerobic induction of *yfiD* expression with dramatically reduced anaerobic

yfiD::lac expression reported for the *fnr* mutant strain (200 Miller U at pH 7.2) compared to the parent (2990 Miller U at pH 7.2). As expected from the data in Table 1, lowering the pH of the cultures increased anaerobic *yfiD::lac* expression for the parent (from 2990 Miller U at pH 7.2 to 4210 Miller U at pH 6.3) but there was only a small increase for anaerobic cultures of the *fnr* mutant (from 200 Miller U at pH 7.2 to 230 Miller U at pH 6.3). To test the possibility that FNR may be directly involved in the observed acid response, expression from the simple FNR-regulated synthetic promoter *FF-41.5* (Wing *et al.*, 1995) was investigated at pH 7.2 and 6.3. The experiments revealed that although FNR-dependent expression from this promoter was subject to a small but reproducible enhancement after growth at low pH, it is unlikely that FNR activity is modulated by intracellular pH (Table 2).

The suggestion that the acid-responsive *yfiD* expression is independent of FNR was supported by Western blot analyses of cell-free extracts of MC1000(pGS1000) (*fnr*⁺) and JRG1728(pGS1000) (*Δfnr*) prepared after aerobic and anaerobic growth at pH 7.2 in the presence or absence of benzoate. The data obtained indicated that the intracellular content of YfiD was increased in response to the presence of benzoate. This enhancement was independent of FNR and dissolved oxygen tension, because even in the *fnr* strain, JRG1728, grown aerobically in the presence of benzoate, the intracellular content of YfiD increased ~ fourfold compared to cultures grown in the absence of benzoate (not shown).

Furthermore, the FNR-independent aerobic increase in YfiD protein content at low pH correlated with enhanced expression of *yfiD::lac* in these cultures (110 Miller U for cultures without added benzoate, compared to 460 Miller U for those with benzoate), suggesting that although FNR may contribute to increasing *yfiD* expression during growth under acidic conditions it is not essential for acid induction of *yfiD* expression and that another factor is mediating this effect.

Further analysis of the *yfiD* promoter revealed the absence of consensus sites for regulators such as PhoP and OmpR that are known to be involved in the response of *E. coli* to acid. However, two overlapping putative ArcA sites (7/10 bases matching the consensus centred at -97, AGTTgATgtA, and -94, TGTaAAacAA; non-consensus bases in lower case) were detected within the far upstream (-93.5) FNR site that is responsible for the anaerobic downregulation of *yfiD* expression (Green *et al.*, 1998), suggesting that ArcA might prevent FNR binding at this location and thereby increase *yfiD* expression. Therefore, the effects of an *arcA* mutation on *yfiD::lac* expression were tested (Table 2). Anaerobic expression of *yfiD* was 2–2.7-fold lower in an *arcA* mutant strain (2540 Miller U compared to 6810 Miller U for the same strain expressing *arcA* from the plasmid pRB38 at pH 7.2, and 4470 Miller U compared to 9210 Miller U at pH 6.3), indicating that ArcA has a positive effect on *yfiD* expression. The simplest explanation for this data, based on our knowledge of FNR-mediated regulation of *yfiD* expression (Green *et al.*, 1998; Green & Marshall, 1999; Marshall *et al.*, 2001), is that ArcA relieves the repression mediated by the far upstream FNR dimer by competing for occupation of the -93 region of the *yfiD* promoter. Expression of the solely FNR-regulated model promoter *FF-41.5* (Wing *et al.*, 1995) was tested in the *arcA* background to determine whether there was any effect of removing ArcA on a simple FNR-driven promoter. Deletion of *arcA* had no significant effect on FNR-mediated expression from *FF-41.5* at both pH 7.2 and pH 6.3, and thus the ArcA-mediated enhancement of *yfiD* expression (Table 2) is probably mediated by ArcA competing with FNR for occupancy of the -93 region of the *yfiD* promoter, where FNR can act negatively and ArcA positively, and that the positive effects of ArcA are magnified by the presence of *arcA* in multiple copies. However, this mechanism is not directly responsible for the anaerobic acid induction of *yfiD* expression because the *yfiD* promoter remains pH-responsive in the *arcA* background (2540 Miller U at pH 7.2, compared to 4470 Miller U at pH 6.3) (Table 2).

Analysis of the *yfiD* promoter region revealed the presence of a near consensus (cAaTGGTTTACCAATT, 12/14, non-consensus bases in lower case) PdhR binding site (Quail & Guest, 1995) centred at +17 relative to the transcript start. The PdhR protein is responsible for repressing the expression of the pyruvate dehydrogenase complex, and thus plays a key role in the regulation of the central metabolic pathways in *E. coli*. PdhR senses the status of the intracellular pyruvate pool

by binding the co-effector, pyruvate, and because the PdhR–pyruvate complex cannot bind target DNA, expression of the *pdhR–aceEF–lpd* operon, encoding pyruvate dehydrogenase, is derepressed when pyruvate is available (Quail & Guest, 1995). The expression of the *yfiD::lac* reporter was tested in JRG2547, a strain that carries the *pdhR^c* mutation that prevents PdhR binding to target DNA (Quail & Guest, 1995). The data show that, as might be predicted from the position of the PdhR box, PdhR acts as a weak anaerobic repressor of *yfiD* expression (2990 Miller U at pH 7.2 for the parent compared to 3740 Miller U for the *pdhR^c* mutant). Furthermore, because acidic growth conditions still enhance *yfiD* expression in a *pdhR^c* background (3740 Miller U at pH 7.2 compared to 4950 Miller U at pH 6.3), it would appear that PdhR does not have a major role in acid-induced *yfiD* expression (Table 2).

A *yfiD* null mutant accumulates increased levels of acidic metabolic end products

To attempt to define a role for the YfiD protein, a *yfiD* null mutant strain (JRG4033) was created by internal deletion and insertion of a kanamycin resistance cassette into the *yfiD* gene of *E. coli* W3110. After Western blotting to confirm that JRG4033 was unable to produce YfiD protein (Fig. 1), a range of phenotypic tests were applied to the mutant, including: monitoring growth and viability of cultures grown aerobically and anaerobically on a variety of carbon sources; sensitivity to UV irradiation, ethanol and freeze–thaw cycling; and morphological differences under light and electron microscopy. However no obvious phenotype was indicated by these tests. To investigate whether YfiD plays a role in resisting acid stress, the responses of the parent (W3110) and the *yfiD* mutant (JRG4033) to acid shock were monitored. Under aerobic and anaerobic conditions the parent and mutant responded similarly in viability tests to acid shock at pH 2.5 (inorganic acid) or at pH 4.5 (weak organic acid), after growth at pH 7.2 or pH 5.7 (not shown), indicating no fundamental role for YfiD in resisting, or adapting to, acid stress. Therefore, it was concluded that the role of YfiD must be more subtle than could be revealed by the simple tests employed.

Because the tests described above failed to reveal a phenotype, and because *yfiD* expression is maximal under microaerobic conditions (Marshall *et al.*, 2001), the properties of the *yfiD* mutant (JRG4033) and parent (W3110) strains were assessed in aerobic (50% dO₂) and microaerobic (5% dO₂) continuous culture competition experiments. When grown aerobically, the proportions of parent and mutant in the culture were essentially constant over 50 generations. However, when cultured microaerobically (<5% dO₂) the proportion of the *yfiD* null mutant, as estimated by the relative number of kanamycin-resistant (*yfiD* null mutant) c.f.u., was reduced from 49% to 29%, and from 45% to 23%, in two independent experiments, within 35 generations. These data indicate that the *yfiD* mutant strain has a microaerobic growth defect. To investigate this further,

Table 3. Physiological parameters of oxygen-starved chemostat cultures

The *E. coli* strain W3110 and its *yfiD* null mutant derivative, JRG4033, were grown under oxygen starvation on glucose-minimal medium in continuous culture. Once steady state had been achieved, the culture medium was analysed for overmetabolites. The values provided are the means of duplicate assays. Acetate, ethanol and formate were determined enzymically and by HPLC (the estimates from the enzymic assays are given) and pyruvate, lactate and succinate were estimated by HPLC measurements alone.

Strain	Metabolites produced (mM)					
	Formate	Acetate	Pyruvate	Lactate	Succinate	Ethanol
Parent	1.4	14.7	0.05	< 0.05	< 0.05	11.5
$\Delta yfiD$	10.8	17.3	0.80	7.0	0.75	10.6
Ratio (mutant:parent)	7.7	1.2	16.00	> 140	> 15.00	0.9

the properties of the mutant (JRG4033) and parent (W3110) were compared in chemostat cultures under both aerobic and oxygen-starved conditions. The measured growth parameters ($\mu_{\max}$ and $Y_{\max}$, max growth rate and max growth yield respectively) indicated that the disruption of the *yfiD* gene did not have a profound effect on the growth of *E. coli* under oxygen-starved conditions, confirming the observations made in batch culture. However, analysis of the culture medium of the *yfiD* mutant strain showed that it contained elevated levels of lactate, succinate, pyruvate and formate compared to the parental strain (Table 3). The concentrations of other metabolic end products (carbon dioxide, acetate and ethanol) were similar for both strains. During growth in the presence of oxygen there was no significant accumulation of any of the overmetabolites by either strain (not shown). These observations indicate that, directly or indirectly, YfiD acts to prevent the accumulation of lactate, succinate, pyruvate and formate during oxygen-starved growth, either by routing carbon flux away from these products or by removing them once they have formed. Such a role is in accord with the anaerobic- and acid-inducibility of *yfiD* expression, and also with the roles of other acid-inducible genes whose products appear to remove, or to prevent the accumulation of, acidic compounds.

Aerobic overproduction of the YfiD protein

The *lac* reporter work described above shows that *yfiD* expression is dramatically increased during anaerobiosis in an FNR-dependent manner and that the imposition of acidic growth conditions further enhances anaerobic *yfiD* expression. However, in a previous proteomic analysis of acid-inducible *E. coli* proteins it was reported that although YfiD was acid induced (in accord with the transcription data reported here) it was present at similar levels in both aerobically and anaerobically grown cultures (Blankenhorn *et al.*, 1999). One possible explanation for this original observation is based on the prediction that under anaerobic conditions, YfiD is a radical-containing protein, and that exposure to oxygen

Fig. 2. SDS-PAGE analysis of overproduced YfiD protein. Left, molecular mass markers; right, purified YfiD protein (10 μ g). Polypeptides were visualized by Coomassie blue staining.

would cause the activated protein to be cleaved in a manner analogous to that observed for PFL. Alternatively, intracellular pH may be influencing the extent of radical incorporation and hence activation of YfiD. Either of these possibilities could cause the YfiD spot on the 2D gels to appear similar under aerobic and anaerobic conditions. To test these possibilities it was necessary to isolate the YfiD protein to allow the predicted radical-activation mechanism to be investigated and to raise antibodies to determine the extent of YfiD activation under acidic growth conditions. Therefore, the YfiD protein was overproduced as a GST fusion protein from pGS1011. After induction with IPTG, the GST–YfiD fusion formed up to 16% of the soluble cell protein and the yield of pure YfiD was 19 mg (l culture)^{−1}. Isolated overproduced YfiD had a subunit M_r of 15 000 and was estimated to be $\geq 95\%$ pure after densitometric analysis of Coomassie brilliant blue stained SDS-polyacrylamide gels (Fig. 2). Because of the position of the thrombin cleavage site in the GST–YfiD fusion protein, and the presence of an engineered *Nco*I site overlapping the initiating ATG codon, the released YfiD protein has an additional 15 N-terminal amino acids (which derive from the vector-encoded linker) and an I2V substitution. The N-terminal amino acid sequence (GSPGISGGGGGILDSMVTGIQ) of the YfiD

Fig. 3. Temporal expression of *yfiD::lac* during aerobic and anaerobic growth of *E. coli*. Cultures of MC1000 (Δlac) carrying the *yfiD::lac* reporter plasmid, pGS1000, were grown aerobically and anaerobically in medium buffered at pH 7.2 for 2 days at 37 °C. At intervals the cultures were sampled, the extent of growth estimated by measuring the OD₆₀₀ and *yfiD* promoter activity was determined by measuring β -galactosidase activity (Miller, 1972). Aerobic cultures (solid lines): growth ($\blacktriangle$) and β -galactosidase activity ($\bullet$). Anaerobic cultures (broken lines): growth ($\blacksquare$) and β -galactosidase activity ($\blacklozenge$).

protein confirmed the presence of the additional amino acids and that the purified protein was YfiD.

Properties of aerobically isolated YfiD

The native molecular mass of YfiD estimated by gel filtration after purification from aerobic cultures indicated that it was a monomeric protein of $M_r \sim 15000$. Electrospray mass spectrometry of YfiD revealed a molecular mass of 15486 Da, which closely matches the 15482 Da predicted from the nucleotide sequence of *yfiD* plus the linker sequence, and indicates the absence of any post-translational modification. The UV-visible and EPR (electron paramagnetic resonance) spectra of these preparations were featureless, except for a maximum at 276 nm ($\epsilon_{276} 15.7 \text{ mM}^{-1} \text{ cm}^{-1}$) in the absorption spectra. Furthermore, total metal ion analysis revealed that YfiD was metal-ion free except for a trace of Zn (0.1 mol Zn per mol of YfiD).

Expression of *yfiD* is enhanced in stationary phase

Previous studies indicated that *yfiD* is expressed throughout the anaerobic growth cycle but that expression increased a further 1.7-fold in stationary phase cultures compared to exponential phase (Green *et al.*, 1998). A more thorough analysis of the growth phase-dependency of *yfiD::lac* expression was undertaken following the suggestion that YfiD is an early stationary phase protein (Perrot *et al.*, 2000). The data revealed that in cultures buffered at pH 7.2, aerobic expression of *yfiD::lac* was low throughout the growth cycle, but increased as growth proceeded (Fig. 3). During the anaerobic growth cycle of similarly buffered cultures,

Fig. 4. The effect of intracellular pH and growth phase on cytoplasmic YfiD levels and radical activation. Cultures of MC1000 were grown anaerobically in LBK/MOPS, pH 7.2 in the absence or presence of propionate (50 mM). The cultures were sampled in exponential (7 h) and stationary phase (24, 28, 31 h) of the growth cycle, lysed in SDS-PAGE loading buffer and applied to SDS-polyacrylamide gels (20%) such that the same number of bacterial cells were present in each track. YfiD protein was detected by Western blotting with polyclonal anti-YfiD serum.

yfiD::lac expression was much higher than observed aerobically, but followed the same pattern, with maximum β -galactosidase activity being observed in stationary phase. Accordingly, under these conditions, YfiD protein was at the limits of detection by Western blotting of whole cell samples obtained at intervals during aerobic growth (< 1000 molecules per cell), but accumulated in anaerobically grown cultures reaching a concentration of approximately 100000 molecules per cell (or 3.4% of total protein) after 7 h growth (not shown). This level of YfiD protein was maintained even after 48 h, indicating that YfiD is not an early stationary phase protein under these growth conditions, but that it accumulates throughout the growth cycle and is present for at least 48 h after growth has ceased.

YfiD is a radical protein that shares the PFL activase

In initial experiments, using samples from anaerobic cultures grown at pH 7.2, two cross-reacting species were observed in Western blots; YfiD itself ($M_r \sim 14000$), and a second, higher mobility, YfiD' species ($M_r \sim 11000$), (Figs 1 and 4). This species accounted for 27–34% of cross-reacting material in the 2–16 h growth period but then fell to 9–12% in the 24–31 h section of the anaerobic growth cycle. By analogy with PFL and the size of YfiD', it was reasoned that YfiD' is one of the products arising from oxidative cleavage of active, radical-containing YfiD protein. This interpretation was supported by the recent demonstration that YfiD is a glycyl radical protein (Wagner *et al.*, 2001) and by the failure to detect YfiD' in a PFL activase deficient strain of *E. coli* (Fig. 5), indicating that insertion of the radical is required to generate YfiD'. Moreover, this latter observation indicates that YfiD does not have a unique activase but shares that of PFL. It would also appear that in anaerobic cultures at pH 7.2 during exponential growth and early stationary phase, only one third of the YfiD protein present in the cell is in the active state and that this falls to about 10% in late stationary phase. Thus, although the level of YfiD protein is maintained upon cessation of growth it is very likely that its activity changes. Furthermore, the extent of YfiD activation was

Fig. 5. The effect of the PFL activase (PflA) and deactivase (AdhE) on YfiD. Cultures of 234M11 (*pflA*, lacking PFL activase) and RM235 (*adhE*, lacking PFL deactivase) transformed with pGS1207 were grown anaerobically in LBK/MES, pH 6.3, containing 40 mM nitrate as a terminal electron acceptor, 50 mM propionate, 200 µg ampicillin ml⁻¹ and 100 µg IPTG ml⁻¹, to ensure maximal YfiD expression and activation. Samples were removed from the cultures in exponential (24 and 28 h for 234M11; 8 and 24 h for RM235) and stationary (31 and 48 h for 234M11; 28 and 31 h for RM235) phases of the growth cycle, lysed in SDS-PAGE loading buffer and applied to SDS-polyacrylamide gels (20%) such that the same numbers of bacterial cells were present in each track. A sample taken from a stationary phase culture of MC1000 (labelled WT; expressing both PFL activase and AdhE) is shown for comparison. YfiD protein was detected by Western blotting with polyclonal anti-YfiD serum.

affected by lowering the intracellular pH of the cultures by the addition of propionate (Fig. 4). After 7 h of growth in the presence of propionate, YfiD' now represented 57% of the total YfiD protein detected in Western blots compared to only 19% in the absence of propionate. Even in stationary phase when the proportion of YfiD' falls, it remained twice as high in the propionate-exposed cultures (Fig. 4). Thus it is concluded that lower intracellular pH not only enhances *yfiD* expression, but also enhances YfiD activity.

Having established that YfiD is a radical-containing protein that shares the PFL activase it was of interest to investigate whether YfiD, like PFL, could be deactivated by the multifunctional alcohol and acetaldehyde dehydrogenase, AdhE (Kessler *et al.*, 1992). Therefore, the extent of YfiD activation was estimated in an *adhE* mutant strain of *E. coli* after incubation in the presence of nitrate to allow anaerobic respiratory growth. The data obtained showed that the extent of oxygenolytic cleavage of YfiD to YfiD' was similar in the presence or absence of AdhE suggesting that, unlike PFL, YfiD is not deactivated by AdhE (Fig. 5).

DISCUSSION

The investigations reported here were prompted by a proteomic analysis of acid-inducible proteins in *E. coli* (Blankenhorn *et al.*, 1999). This indicated that the *yfiD* gene product was upregulated both aerobically and anaerobically after exposure to acid stress (Blankenhorn *et al.*, 1999). Here it has been shown that the anaerobic transcription factor FNR is the major activator of *yfiD* expression, but that the extent of FNR-mediated activation can be modulated by the indirect oxygen sensor ArcA and the pyruvate sensor PdhR. Moreover, in accord with the previous proteomic analysis, expression

Fig. 6. Regulation of the *yfiD* gene of *E. coli* showing the effects of global regulators on the activity of the *yfiD* promoter. The pyruvate responsive regulator PdhR acts as a minor repressor of *yfiD* expression when the intracellular pyruvate pool is low. The anaerobic regulators FNR and ArcA combine to allow maximum YfiD expression during anaerobic growth. Aerobically, FNR is largely inactive, neither FNR site is occupied and *yfiD* expression is low. Microaerobically, active FNR begins to appear in the bacterial cytoplasm and the high affinity activating FNR site (FNR I) becomes occupied, allowing *yfiD* expression. Under these conditions there is insufficient active FNR to occupy the upstream repressing FNR site (FNR II) and furthermore, ArcA binding to overlapping sites (solid bars) within FNR II ensures that this is so. Anaerobically, the intracellular concentration of active FNR is now sufficient to bind at the FNR II site by displacing ArcA and thereby inhibit the transcription activation mediated by the FNR dimer bound at FNR I. At low pH, an as yet uncharacterized acid-responsive regulator (indicated by ? and broken arrows) enhances *yfiD* expression and intracellular YfiD content in acid cultures in the presence and absence of FNR. + indicates a positive effect; - a negative effect on *yfiD* expression.

from a *yfiD::lac* reporter fusion and the intracellular content of YfiD was found to be greatest during growth at low pH for both aerobic and anaerobic cultures. Therefore, from the data presented here, the model describing the regulation of *yfiD* expression in *E. coli* can be adapted to include three new features (Fig. 6). The first is competition between FNR (acting negatively) and ArcA (acting positively) for occupation of the -93.5 region of the promoter (FNR II; Fig. 6). This competition is consistent with the observation that *yfiD* expression is maximal under microaerobic conditions (Marshall *et al.*, 2001). Thus, aerobically, FNR is largely inactive, neither FNR site (FNR I or FNR II; Fig. 6) within the *yfiD* promoter is occupied and *yfiD* expression is low. Microaerobically, a low level of active FNR appears in the bacteria, and the high affinity activating FNR site (FNR I) becomes occupied, allowing *yfiD* expression. Under these conditions there is insufficient active FNR to occupy the low affinity upstream repressing FNR site (FNR II), and the data presented here suggests that ArcA binding to this region ensures that this is so, thereby facilitating maximal *yfiD* expression. Because the level of *yfiD* expression observed when ArcA is present in multiple copies is similar to that reported for expression from a modified *yfiD* promoter that lacks the FNR II site (Green *et al.*, 1998), it is most

likely that ArcA acts as an anti-repressor, minimizing FNR occupation of the FNR II site during microaerobic growth. Anaerobically, the intracellular concentration of active FNR becomes sufficient to allow occupation of the FNR II site by displacing ArcA, and thereby inhibit the transcription activation mediated by the FNR dimer bound at FNR I. The next phase of investigation should consider the relative binding affinities of FNR and ArcA for the -93 region of the *yfiD* promoter. Such studies will have to be done *in vitro* because mutagenesis of either type of upstream binding site would inevitably affect the properties of the other because of the large sequence overlap. The second feature is the PdhR-mediated repression of *yfiD* indicating a link to pyruvate/central metabolism. Previous reports have indicated that intracellular YfiD levels are enhanced by growth in the presence of pyruvate (Blankenhorn *et al.*, 1999), which may now be explained (at least in part) by the relief of PdhR-mediated repression. The third feature is that *yfiD* expression is enhanced during growth at acid pH. It is well known that the effects of pH on gene expression overlap with other environmental factors and particularly with oxygen availability (Cotter *et al.*, 1990; Meng & Bennett, 1992; Neely *et al.*, 1994; Slonczewski & Foster, 1996; Blankenhorn *et al.*, 1999) and it now appears that *yfiD* expression responds to both oxygen starvation and acidic pH via FNR, ArcA and an as yet unknown acid-responsive component.

The pattern of regulation observed for *yfiD* has some features in common with those of the *pflB* gene, which also encodes a radical-containing protein, PFL. The *pflB* gene is transcribed from multiple promoters, and promoters 6 and 7 are, like the *yfiD* promoter, positively regulated in response to anaerobiosis by both FNR and ArcA (Sawers, 1993). The *pflB* gene is part of an operon consisting of *focA-pflB* and it has been reported that expression of *pflB* is positively regulated by intracellular pyruvate (Sawers & Bock, 1988). However, analysis of the *pflB* locus revealed no good matches to the PdhR binding site close to promoter 6 or promoter 7, and so they are unlikely to be directly influenced by intracellular pyruvate pools via PdhR. A potential PdhR site was found within the *pflB* coding region, some 1787 bp upstream of the PFL activase gene (*pflA*) transcript start, and it is thus unlikely that this potential PdhR site is transcriptionally relevant. However, from the PdhR-mediated repression of *yfiD* described here, the report that growth in the presence of pyruvate increases the intracellular content of YfiD (Blankenhorn *et al.*, 1999), and the pyruvate-mediated induction of *pflB* (Sawers & Bock, 1988), it appears that *yfiD* and *pflB* could be coordinately regulated in response to both pyruvate and anaerobiosis. It will be of interest to determine whether *pflB* expression is also acid responsive. An indication that this may be so was provided by the presence of a cross-reacting species of $M_r \sim 85\,000$ (consistent with the size of PFL) in our Western blots, the intensity of which increased as intracellular pH decreased (not shown).

The overlapping pattern of regulation of *yfiD* and *pflB*

has acquired new significance because during the reviewing of this work it has been shown that YfiD can act as a 'spare part' by providing an active glycyl radical domain to restore activity to oxygenolytically cleaved PFL (Wagner *et al.*, 2001). PFL cleaves pyruvate to form acetyl-CoA (which is subsequently converted to acetate) and formate (which is converted to carbon dioxide and hydrogen) during anaerobic growth. Thus, it is suggested that YfiD may act to maintain/restore PFL activity to bacteria that have been exposed to oxygen (Wagner *et al.*, 2001). Such a role is consistent with FNR-mediated regulation tuned for maximum *yfiD* expression in microaerobic conditions (Marshall *et al.*, 2001), and with PdhR-mediated repression of *yfiD* expression in response to low intracellular pyruvate. Moreover, the chemostat studies with the *yfiD* mutant provide further support for the 'spare part' hypothesis, which predicts that in the absence of YfiD under microaerobic conditions, carbon flux through PFL would be reduced due to an inability to repair oxygenolytically cleaved PFL. Reduced flux through PFL could lead to increased excretion of pyruvate, succinate and lactate, but not acetate, ethanol or formate. The data presented here indicate that a lesion in *yfiD* leads to excretion of increased levels of pyruvate (16-fold greater than for the parent strain), succinate (> 15 -fold higher) and lactate (> 140 -fold higher) whereas the levels of ethanol and acetate were similar for both the parent and *yfiD* mutant strains (Table 3). All these features are consistent with YfiD acting to maintain high levels of PFL activity during microaerobic growth. The sevenfold increase in formate excretion for the *yfiD* mutant cultures does not at first sight appear to be consistent with this role. However, because the growth medium contains limited amounts of oxygen and no other exogenous electron acceptors, it could be that the reduced flux through PFL, caused by the inability to rescue the activity of oxygen-damaged PFL because of the absence of YfiD, has not allowed sufficient formate to accumulate to activate the FhlA-regulated formate hydrogen-lyase system in the mutant cultures. Previous studies have shown that maximal anaerobic expression of *hycB* (a component of formate hydrogen-lyase) at pH 7.0 *in vivo* requires the presence of 30 mM formate in the culture medium (Rossmann *et al.*, 1991). Moreover, 10 mM formate has been shown to be necessary for maximum activity of FhlA *in vitro* (Hopper *et al.*, 1994). Although these studies are not directly comparable to those described here, they do indicate that substantial formate accumulation is required for the optimal induction of formate hydrogen-lyase. Consequently, if formate hydrogen-lyase is not induced in the *yfiD* mutant strain, due to subinductive intracellular formate concentrations resulting from reduced flux through PFL, then formate would not be disproportionated to carbon dioxide and water. This could lead to the detection of increased levels of formate in the *yfiD* mutant cultures compared to the parental cultures, where, because the carbon flux through PFL is uninhibited, sufficient formate is produced to induce a functional formate hydrogen-lyase. Other possibilities

that cannot be discounted are that YfiD may contribute directly or indirectly to the metabolism of formate, perhaps by reversing the PFL reaction, or that the formate accumulating in the *yfiD* mutant cultures arises from a source other than PFL. Further work will be needed to resolve these questions.

Consistent with the proposal that YfiD acts as a radical domain for damaged PFL and possibly other radical proteins in *E. coli*, the YfiD radical shares many features with that of PFL. The location of the YfiD radical was presumed to be G102 by analogy with PFL (Wagner *et al.*, 1992); and this has recently been shown to be the case (Wagner *et al.*, 2001). Here it is shown that the YfiD radical is inserted by the PFL activase, PflA. Therefore, *yfiD* and *pflB* not only share similar regulation at the transcriptional level, but the activation, and thus the activities, of the two proteins are also linked. In PFL the glycyl radical acts as a 'radical store' when the protein is in the resting state (Becker *et al.*, 1999) and it would now seem that a similar function is fulfilled by G102 in YfiD (Wagner *et al.*, 2001). The observation that the radical content of YfiD is increased at low pH suggests that activated PFL is more easily oxygenolytically cleaved under such conditions and consequently more YfiD is activated to restore activity to the damaged PFL protein. Whether the YfiD protein can, under certain conditions, have activity *per se* or whether its function is only to repair oxidatively damaged PFL is as yet unknown. The reaction mechanism of PFL requires an amino acid triad consisting of G734, C418 and C419. In the resting state the radical resides at G734 but during initiation of the PFL reaction it is transferred to C419 and then to C418 (Becker *et al.*, 1999). This sequence can be reversed to return the protein to the resting state, allowing multiple turnovers without regeneration of the glycyl radical. In contrast to PFL, the YfiD protein has only one cysteine residue (C31) and thus if it has independent activity it must use a different catalytic mechanism to that of PFL. Other glycyl radical enzymes are known that lack the adjacent cysteine motif of PFL (Sawers & Watson, 1998; Selmer & Andrei, 2001). The failure to observe increased oxygenolytic cleavage of YfiD from cultures unable to express the PFL deactivase AdhE (a multifunctional alcohol dehydrogenase), which acts to quench the PFL radical and thereby prevent oxidative cleavage occurring is consistent with the proposed role of YfiD, because YfiD would need to be activated under conditions that could damage PFL. Thus, the work reported here describes strong links between the regulation of expression and activity of YfiD and PFL, in accord with the proposed role of YfiD as a radical-containing module that can be plugged into oxidatively damaged PFL to restore activity to this key enzyme.

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