YeiL, the third member of the CRP–FNR family in *Escherichia coli*

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The *yeiL* open reading frame located at 48.5 min (2254 kb) in the *nfo–fruA* region of the *Escherichia coli* chromosome was predicted to encode a CRP and FNR paralogue capable of forming inter- or intra-molecular disulphide bonds and incorporating one iron–sulphur centre per 25 kDa subunit. Purified MBP–YeiL (a maltose-binding-protein–YeiL fusion protein) was a high-molecular-mass oligomer or aggregate which released unstable monomers (68 kDa) under reducing conditions. The MBP–YeiL protein contained substoichiometric amounts of iron and acid-labile sulphide, and an average of one disulphide bond per monomer. The iron and sulphide contents increased consistent with the acquisition of one [4Fe–4S] cluster per monomer after anaerobic NifS-catalysed reconstitution. By analogy with FNR and FLP (the FNR-like protein of *Lactobacillus casei*) it was suggested that the transcription-regulatory activity of YeiL might be modulated by a sensory iron–sulphur cluster and/or by reversible disulphide bond formation. A *yeiL–lacZ* transcriptional fusion showed that aerobic *yeiL* expression increases at least sixfold during stationary phase, requires RpoS, and is positively autoregulated by YeiL, positively activated by Lrp (and IHF in the absence of FNR) and negatively regulated by FNR. A regulatory link between the synthesis of YeiK (a potential nucleoside hydrolase) and YeiL was inferred by showing that the *yeiK* and *yeiL* genes are divergently transcribed from overlapping promoters. A 10–15% deficiency in aerobic growth yield and an enhanced loss of viability under nitrogen starvation conditions were detected with a *yeiL::kan^R* mutant, suggesting that YeiL might function as a post-exponential-phase nitrogen-starvation regulator.

Keywords: CRP–FNR protein superfamily, iron–sulphur proteins, transcriptional regulation, nitrogen starvation, stationary phase

INTRODUCTION

The CRP–FNR family of transcription regulators contains at least 70 members that respond to a variety of metabolic and environmental signals to control a diverse range of physiological functions in an equally diverse range of bacteria (Spiro, 1994; Guest *et al.*, 1996; Vollack *et al.*, 1999). The family can be divided into three broad phylogenetically distinct groups designated CRP, NtcA and FNR, although there is considerable variation within the groups and also some overlap between the groups with respect to functional specifi-

cities for signal recognition, DNA-binding, and RNA polymerase interaction (where known). The CRP group mainly contains transcription factors that regulate catabolite-sensitive genes in response to glucose starvation as signalled by cAMP (Busby & Kolb, 1996). The NtcA group is more diverse because it includes regulators of cyanobacterial nitrogen and sulphur metabolism as well as others concerned with CO metabolism and oxygen-dependent nitrate reduction in *Rhodospirillum* and *Bacillus* species, respectively. The FNR group is currently the largest group. It contains three phylogenetically distinct subgroups designated FnrN/AadR, FixK and DNR/FLP in addition to the FNR subgroup, which contains the archetypal FNR proteins that regulate oxygen-dependent gene expression via the anaerobic acquisition of oxygen-labile [4Fe–4S] clusters

Abbreviations: CRP, cAMP receptor protein; FNR, fumarate and nitrate reduction regulator; MALDI-TOF, matrix-associated laser desorption ionization-time of flight; MBP, maltose-binding protein.

Fig. 1. Map of the *nfo*–*fruA* region of the *E. coli* chromosome. The positions and polarities of the genes and unidentified reading frames from Blattner *et al.* (1997) are indicated together with the functions or predicted functions based on BLAST similarity searches.

(Kiley & Beinert, 1999). The FnrN/AadR subgroup represents a cluster of FNR proteins from nitrogen-fixing and photosynthetic bacteria whereas the FixK proteins regulate rhizobial nitrogen fixation by virtue of their own oxygen-dependent biosynthesis (Spiro, 1994). Finally, the DNR/FLP subgroup contains FNR-like proteins that regulate either denitrification in denitrifying bacteria (DNR, NNR) or oxidative stress in lactic acid bacteria (FLP, FlpA, FlpB). The FLP of *Lactobacillus casei* was the first Gram-positive member of the family and it was recently shown to respond to oxidative stress via the reversible formation of intra-subunit disulphide bonds (Gostick *et al.*, 1997, 1998). In each case the protein subunits are predicted to resemble CRP monomers by containing a C-terminal DNA-binding domain with a helix–turn–helix motif for site-specific DNA-recognition, a long α -helix that forms a dimer interface, and an N-terminal sensory domain based on a series of β -strands (Schultz *et al.*, 1991). The two structural domains also provide specific activating contacts with RNA polymerase (Busby & Ebright, 1997; Li *et al.*, 1998).

The complete genome of *Escherichia coli* (Blattner *et al.*, 1997) was recently shown to encode a third member of the CRP–FNR family (YeiL) when searched with a sequence profile derived from a multiple alignment of five members of the family: CRP and FNR from *E. coli*; FLP from *L. casei*; and FlpA and FlpB from *Lactococcus lactis* (Gostick *et al.*, 1999). The YeiL protein (SWISS-PROT accession no. P33023) is the 219-residue product of the 657 bp *yeiL* open reading frame (GenBank accession no. U00007). A BLAST analysis showed that YeiL is more closely related to members of the CRP–FNR family (16–22% identical, 42–45% similar) than to other known proteins. A phylogenetic tree derived from 38 members of the CRP–FNR family indicated that YeiL is a distant relative of the CRP group (Gostick *et al.*, 1999).

The *yeiL* gene is located in the *nfo*–*fruA* region of the *E. coli* linkage map at 48.57 min (co-ordinates 2253.38 to 2254.03 kb; Blattner *et al.*, 1997), where it is flanked by a cluster of unidentified reading frames, URFs (Fig. 1). Putative functions have here been assigned to the products of these URFs by similarity searches. They include two potential guanosine kinases, YeiC (P30235) and YeiI (P33020), which are 38% identical (61% similar), and two potential pyrimidine nucleoside transport proteins, YeiJ (P33021) and YeiM (P33024), which are 88% identical (95% similar). There is also a potential nucleoside hydrolase, YeiK (P33022), which is 40% identical (66% similar) both to YbeK (P41409; an unlinked *E. coli* URF) and to the inosine-uridine nucleoside hydrolase of *Crithidia fasciculata* (Degano *et al.*, 1996). At least 26 of the 27 residues surrounding the catalytic site of this trypanosomal purine salvage pathway enzyme are conserved in YeiK and YbeK. Thus it would appear that several genes involved in nucleoside metabolism are located in the *nfo*–*fruA* region of the *E. coli* chromosome together with a novel paralogue of CRP and FNR.

The aim of the present work was to characterize the YeiL protein and to define its function by investigating the physiological consequences of *yeiL* gene inactivation and the regulation of *yeiL* gene expression.

METHODS

Bacterial strains, plasmids and phages. These are listed in Table 1. An isogenic *yeiL*::*kan*^R derivative of W3110, JRG3827, was constructed as follows, using standard molecular biological procedures (Sambrook *et al.*, 1989). The *yeiL* gene with 1028 bp and 722 bp flanking segments was PCR-amplified from W3110 chromosomal DNA as a 2.41 kb fragment using two primers incorporating useful restriction sites (shown in bold): S449 (*Pst*I *Sal*I, 5'-TTTAACTGCAGTCGACTTGCCAAGAACGCCAG-3') and S450 (*Sma*I *Bam*HI, 5'-TTTAAACCCGGGATCCTACCTGTTAGCGGCATC-3'). The entire fragment was cloned into pGEM-T Easy (Promega) to generate pGS1109 and after propagating in GM242 (*dam*) a 40 bp *Clal* fragment in the *yeiL* coding region was replaced by inserting a *kan*^R cassette (1.2 kb *Sma*I fragment) from pUC4-KIXX (Pharmacia) between end-filled *Clal* sites to generate pGS1110. The location of the *kan*^R

Table 1. Bacterial strains, plasmids and phages

Strain, plasmid or phage	Relevant genotype	Source or reference
W3110	<i>trpR icl^c</i> (prototroph)	Lab. collection
JC7623	<i>recB21 recC22 sbcB15 sbcC201</i>	Kushner <i>et al.</i> (1971)
JRG3826	JC7623 <i>yeiL::kan^R</i>	This work
JRG3827	W3110 <i>yeiL::kan^R</i>	This work
RK4353	$\Delta(\arg F-lac)U169$	V. Stewart*
RK5279	$\Delta(\arg F-lac)U169 fnr-250$	V. Stewart*
JRG3885	RK4353(λ G271)	This work
JRG3886	RK5279(λ G271) <i>fnr-250</i>	This work
JRG3887	RK4353(λ G271) <i>yeiL::kan^R</i>	This work
JRG3888	RK5279(λ G271) <i>fnr-250 yeiL::kan^R</i>	This work
JRG3889	RK4353(λ G271) <i>rpoS359::Tn10</i>	This work
JRG3890	RK5279(λ G271) <i>fnr-250 rpoS359::Tn10</i>	This work
JRG3891	RK4353(λ G271) <i>ihfAΔ82::Tn10</i>	This work
JRG3892	RK5279(λ G271) <i>fnr-250 ihfAΔ82::Tn10</i>	This work
JRG3893	RK4353(λ G271) <i>lrp::Tn10</i>	This work
JRG3894	RK5279(λ G271) <i>lrp::Tn10</i>	This work
CAG626	<i>lon lacZ_{am} trp_{am} pho_{am} supC_{ts} mal_{am} rpsL</i>	NEB
GM242	<i>dam-3</i>	Marinus & Morris (1973)
pGS1109	pGEM-T Easy with 2.41 kb <i>yeiL</i> containing PCR fragment; Ap ^R	This work
pGS1110	pGS1109, with 40 bp <i>Clal</i> deletion and 1.2 kb <i>kan^R</i> insertion in the <i>yeiL</i> gene; Ap ^R Km ^R	This work
pGS1145	pGEM-T Easy with 1046 bp PCR-generated <i>yeiL</i> promoter region; Ap ^R	This work
pGS1224	1,042 bp <i>EcoRI-BamHI yeiL</i> promoter fragment of pGS1145 cloned in pRS415; Ap ^R	This work
pGS1225	682 bp PCR product containing the <i>yeiL</i> coding region cloned in pGEM-T Easy; Ap ^R	This work
pGS1226	<i>yeiL</i> coding region in 673 bp <i>BamHI-SalI</i> fragment of pGS1225 cloned in pMal-c2; Ap ^R	This work
pGS1376	pSU19 with 2.39 kb <i>yeiL</i> containing <i>PstI-BamHI</i> fragment; Cm ^R	This work
λ G271	λ RZ5 derivative carrying the <i>yeiL-lacZ</i> fusion	This work

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cassette was confirmed and its polarity shown to be the same as *yeiL* by sequence analysis before using pGS1110 to construct a *yeiL::kan^R* mutant of *E. coli* JC7623 (*recBC sbcBC*) according to Oden *et al.* (1990). The presence of the mutation was verified first by colony PCR with Km^R Ap^R transformants using S449 and S450 as primers, which showed that the amplified product was 1.2 kb larger than with wild-type DNA, and second by Southern blot analysis of *EcoRI*- and *PstI*-digested DNA from a representative mutant (JRG3826) and the parental strain at high stringency (0.1 % SDS; 72 °C) with gel-purified and ³²P-labelled 2.39 kb *Sall-BamHI* fragment of pGS1109 as a *yeiL* hybridization probe (Ready-To-Go DNA labelling kit and [α -³²P]dCTP; Amersham-Pharmacia). P1vir-mediated transduction was used to transfer the *yeiL::kan^R* mutation to W3110 to generate JRG3827, and from the latter

to RK4353 and RK5297 (Table 1). The *rpoS*, *ihfA* and *lrp* mutations of laboratory stocks were likewise transferred by P1 transduction in the construction of JRG3889–JRG3894 (Table 1). The 2.39 kb *PstI-BamHI* fragment of pGS1109 containing the *yeiL* promoter and coding region was transferred to pSU19 (Martinez *et al.*, 1988) to provide a low-copy (10 to 12) plasmid for complementation tests.

Media, growth conditions and viable counts. The rich media were Luria–Bertani (LB) and peptone broth (Green *et al.*, 1997) supplemented with glucose (0.2 % for aerobic growth and 1.0 % anaerobic, unless stated otherwise) and antibiotics where required: kanamycin (50 μ g ml⁻¹); ampicillin (150 μ g ml⁻¹); tetracycline (15 μ g ml⁻¹); and chloramphenicol (normally 20 μ g ml⁻¹ but reduced to 10 and 5 μ g ml⁻¹ with low-copy plasmid transformants in rich and minimal media,

respectively). The citrate-containing minimal medium E (Vogel & Bonner, 1956) was used routinely with sugars (0.2%) and organic acids (40 mM) as carbon sources, thiamin (15 µM), and appropriate amino acid supplements. Cultures were grown from 1% stationary-phase inocula at 37 °C, either aerobically in conical flasks (with 0.1 vol. medium shaking at 200 r.p.m.) or anaerobically in sealed tubes (10 ml without shaking).

For carbon, phosphorus and nitrogen starvation, M9 medium was used with 0.02% glucose (normally 0.4%), 0.25 mM phosphate with 0.1 M Tris/HCl buffer (pH 7.6) and 0.2 mM NH₄Cl, respectively (Davis *et al.*, 1986), and the cultures (600 ml) were maintained at pH 7.4, 90% air saturation (stirring speed, 200 r.p.m.) and 37 °C in chemostat vessels for 7 d. The inocula (1%) were derived from single colonies from freshly streaked minimal plates, grown to stationary phase in M9 medium with 0.4% glucose. Serial dilutions of daily samples, plated in triplicate on pre-warmed LB agar and scored after 18 h at 37 °C, were used to determine viable counts (c.f.u. ml⁻¹), which were then expressed as percentages relative to the 24 h values. All of the findings were reproducible in two or three independent experiments.

Protein purification. The *yeiL* coding region was PCR-amplified from W3110 chromosomal DNA using forward primer S556 (5'-TTAAGGATCCCATATGAGTGAATCCGCGTTTAAGG-3') and reverse primer S559 (5'-TATGT-CGACTTACGTCATCATCCCGGAGAATTTAT-3'), each incorporating flanking restriction sites. The 682 bp product was cloned initially in pGEM-T Easy (Promega) and then between the *Bam*HI-*Sal*I sites of pMal-c2 (NEB) to place it downstream and 'in frame' with the maltose-binding protein (MBP) coding region and Factor Xa sensitive linker, to generate pGS1226. After confirmatory resequencing, pGS1226 transformants of *E. coli* CAG626 were used for overproduction and purification of the MBP-YeiL fusion protein. Soluble MBP-YeiL was purified from aerobic 2-litre cultures grown initially at 30 °C in LB plus 0.2% glucose and ampicillin to OD₆₀₀ 0.2, and then at 37 °C for 1 h after adding IPTG (0.3 mM). The bacteria harvested from one culture were washed and resuspended in 10 ml buffer containing 20 mM Tris/HCl (pH 7.4), 200 mM NaCl and 1 mM DTT, for ultrasonic disruption. The clarified cell-free extract was applied to an amylose column (10 ml; NEB), washed with 20 vols of the same buffer, and eluted with buffer containing 10 mM maltose.

Reconstitution of iron-sulphur clusters, and biochemical assays. The fusion protein was reconstituted using NifS from *Azotobacter vinelandii* according to Green *et al.* (1996). Typically, 1 ml of the purified protein at a concentration of 1 mg ml⁻¹ in a quartz cuvette was equilibrated in an anaerobic glove-box for 3–4 h on ice before adding DTT (2.5 mM), L-cysteine (1 mM), Fe²⁺ [40 mol (NH₄)₂Fe(SO₄)₂ per mol MBP-YeiL monomer] and NifS (0.7 µM) to the stated final concentrations, and then incubated in the stoppered cuvette for up to 16 h at 4 °C. Spectra were obtained with a Unicam UV-2 spectrophotometer (Pye-Unicam). The reconstituted protein was desalted anaerobically, using a Sephadex G25 column to remove excess reagent. Iron and sulphur contents were determined by the methods of Woodland & Dalton (1984) and Beinert (1983), respectively. Reactive thiol groups and the disulphide contents of the native protein were assayed according to Thelander (1973) and Thannhauser *et al.* (1987), respectively.

Protein analysis, gel filtration and mass spectrometry. Protein was assayed by the Bio-Rad procedure with bovine serum

albumin as standard, and the Laemmli method (Sambrook *et al.*, 1989) was used for SDS-PAGE. Native molecular masses were determined by gel filtration with a Protein-Pak 300SW column (7.8 × 300 mm) equilibrated with 0.2 M NaH₂PO₄ (pH 6.7) containing 0.2 M Na₂SO₄; DTT (1 mM) was added to the buffer when reducing conditions were required. Thyroglobulin, catalase, lactate dehydrogenase, bovine serum albumin, ovalbumin, chymotrypsin, ribonuclease, aprotinin and myoglobin were the standards used for calibration. N-terminal amino acid sequences were determined by Edman degradation using an Applied Biosystems protein sequencer. Accurate molecular mass measurements were made by electrospray and MALDI-TOF mass spectrometries.

Construction of a *yeiL-lacZ* transcriptional fusion and β -galactosidase assay. A *yeiL-lacZ* transcriptional fusion was constructed by PCR-amplification of the *yeiL* promoter region (1017 bp of upstream non-coding region and 25 bp of *yeiL* coding region) with two primers containing embedded *Eco*RI and *Bam*HI restriction sites: S470 (5'-TTCCGGAATTC-TTGCCAAGAACGCCAG-3') and S471 (5'-TAGGCGG-ATCCAATCCTTAAACGCGGAT-3'). The product was cloned into pGEM-T Easy (Promega), generating pGS1145, from which the 1046 bp *Eco*RI-*Bam*HI promoter fragment was transferred to pRS415 (Simons *et al.*, 1987) to generate pGS1224. The *yeiL-lacZ* promoter fusion was then transferred to λ RZ5 by homologous recombination to yield λ G271, and monolysogenic derivatives of various hosts were selected and screened with λ h80del9c, λ c190c17 and λ vir. β -Galactosidase specific activities were assayed according to Miller (1972) using at least two independent cultures.

RNA extraction and primer extension analysis. The hot acid phenol procedure (Aiba *et al.*, 1981) with rapid cooling was used to extract total RNA from aerobic stationary-phase cultures grown in peptone broth plus 0.2% glucose. Primer extension analysis was performed with 100 µg RNA, 10 pmol primer and AMV reverse transcriptase (50 U; NEB) by a procedure that allowed continuous incorporation of [α -³²P]dCTP (Cunningham *et al.*, 1997). The primers were: S557 (5'-TCACTCATCAATTGCTGCTT-3') for *yeiL*; and both S499 (5'-TCGCCGCCATCATTATAG-3') and S558 (5'-ATCCAGAATAATTTTCTCTTTTCCA-3') for *yeiK*. The extension products were fractionated on 6% acrylamide/7 M urea gels alongside sequence ladders derived from pGS1109 and the corresponding primers (Cunningham *et al.*, 1997).

RESULTS

The *yeiL* gene encodes a paralogue of CRP and FNR

The YeiL protein contains 219 amino acid residues, and has a total molecular mass of 25 294 Da and a predicted pI of 8.23. Sequence comparisons with representative members of each subgroup of the CRP-FNR family showed that YeiL is: 22% identical (42% similar) to the FNR of *E. coli*; 19% identical (45% similar) to the FLP of *L. casei*; 19% identical (44% similar) to the FixK of *Rhizobium meliloti*; and 17% identical (45% similar) to the CRP of *E. coli*.

A multiple sequence alignment of YeiL, CRP and FNR (Fig. 2) confirmed their close relationships and indicated that most of the secondary structural elements of CRP are retained by YeiL. The alignment shows that the glycine and proline residues flanking some of the β -strands of the β -roll in CRP are conserved in YeiL. The

		^^^^ α_A ^^^^-- β_1 --	-- β_2 --				
CRP		VLGKPTDPTLEWFLSHCHTHKYP-SK-STLIHQGE		34			
YeiL		MSESFAKDCFLTDVSADTRLHFH-LAR-DYIVQEGQ		34			
FNR	MIPEKRIIRRIQSGGCAIH	CQDCSISQLCIPFTLNEHELDQLDNI-IERKKPIQKGQTLFKAGD		63			
	--- β_3 ---	-- β_4 --	--- β_5 ---	-- β_6 --	----- β_7 -----	----- β_8 -----	
CRP	KAETLYYIVK	GSVAVLIKDEEG	KEMILSYLNQ	QDFIGELGLFEEGQERS	AWVRAKTACEVAEISYK		100
YeiL	QPSWLFYLTR	GRARLYATLAN	GRVSLIDFFA	APCFITGETELIDKDHEPRA-VQAIEE	CWCLALPMK		99
FNR	ELKSLYAIRSG	TIKSYTITEQ	QCDEQITGFHLAC	DLVGFDAL-GSGHPS-FAQALETSMVCEIPFE			127
	^^^ α_B ^^^	^^^^^^^^^^ α_C ^^^^^^^^^^		^^^^^^ α_D ^^^^^^	-- β_9 --	-- β_{10} --	
CRP	KFRQLIQVNP	DILMRLSAQMAR	RLQVTS	SEKVGNLAF	LVDVTGRIAQTLLNLAKQ-PDAMTHPDGMQI		167
YeiL	HYRPLLND	TLFLRKLCV	TLSHKNYRNIVSLTQ	NQSFPLVNRLAAFIL	-----LSQEGDLY		155
FNR	TLDDLSGKMP	NLRQQMRLMSGEIKG	QDMILLSSKKNAEER	LAAFTYNLSRRFAQRGFS	SPREFRL		193
	^^^^ α_E ^^^	^^^^^^ α_F ^^^^^^	-- β_{11} --	-- β_{12} --			
CRP	KITRQEIGQIVGCS	RETVGRIL	KMLEDQNLISAHGKTIVVYGR				209
YeiL	HEKHTQAAEY	VLGVSYRHLLYVLAQFI	HDGLLIKSKGYLIKNRKQLSGLALEMDPENKFS	GMMQ			219
FNR	TMTRGDIGNY	LGLTVETIS	RLLGRFQKSGMLAVKGYIT	TIENN	DALAQLAGHTRNVA		250

Fig. 2. Multiple alignment of the *E. coli* CRP, YeiL and FNR amino acid sequences generated by the GCG program PILEUP. The regions of greatest sequence conservation identified by DIAGON (Staden, 1982) are denoted by lines above the CRP sequence and below the FNR sequence. Secondary structural elements in CRP (Schultz *et al.*, 1991) are indicated above the alignment, and conserved glycine and proline residues that flank β -strands are boxed. The residues involved in cAMP-binding by CRP and the cysteine residues in YeiL and FNR are shown in bold. The DNA-recognition motifs in the relevant α -helices (α_F) of CRP and FNR are shown in bold and underlined.

regions of greatest sequence similarity ($P \leq 0.001$) identified by pairwise DIAGON comparisons (Staden, 1982) between YeiL and either CRP or FNR are also indicated in Fig. 2. These are different in each case but they represent major structural features such as the sensory domain (β -roll) and dimer interface (α_C) of CRP and the DNA-recognition helix (α_F) of FNR. In addition, the presence of a characteristic helix-turn-helix structure (α_E - α_F) was strongly predicted by the GCG program HELIX-TURN-HELIX. However, the motifs associated with the DNA-binding specificities of FNR (E--SR) and CRP (RE--G) in α_F (Guest *et al.*, 1996) are not conserved in YeiL, suggesting that YeiL recognizes different sites to those recognized by both FNR and CRP.

In terms of signal recognition, only one of the six residues involved in cAMP-binding in CRP is conserved in YeiL (Fig. 2), suggesting that cAMP is not a coeffect for YeiL. YeiL also lacks the cysteine-rich N-terminal extension that provides three of the four ligands for the sensory iron-sulphur cluster of FNR. However, YeiL contains five cysteine residues: one is near the N-terminus (C9) and four are centrally located (C68-X₂₂-C91-X₁-C93-X₂₂-C116). Of these, C91 and C93 are predicted to reside in the same β -strand (β_8) as the fourth iron ligand (C122) of FNR (Fig. 2). It is also relevant to note that the two cysteine residues (C5 and C102) participating in reversible intra-subunit dithiol-disulphide formation in FLP are likewise predicted to reside near the N-terminus and in β_8 , respectively (Gostick *et al.*, 1998, 1999).

The cysteine residues in YeiL were modelled at the corresponding positions in the X-ray structure of the

cAMP-CRP-DNA complex (Schultz *et al.*, 1991) as shown in Fig. 3(a). This hypothetical structure suggested that a disulphide bond could be formed between the cysteine residues (C116) at the dimer interfaces of two subunits. The measured distance between the β carbon atoms (C_β) of C116_A and C116_B in the model was 4.76 Å, which is close to the values found in typical protein disulphides, 3.7 to 4.3 Å (1 Å = 0.1 nm). The model also suggested that three of the cysteine residues (C68, C91 and C93) could potentially serve as ligands for the assembly of an iron-sulphur cluster in each monomer (Fig. 3a). The inter-molecular distances between their C_β s were measured as 10.6 Å (C68-C91), 5 Å (C91-C93) and 10.2 Å (C93-C68). Then, by using the three-dimensional-database search program ASSAM (Artymiuk *et al.*, 1994), it was found that this arrangement of three cysteine residues is most closely matched by that associated with the [4Fe-4S] cluster of porcine mitochondrial aconitase (Robbins & Stout, 1989), where the corresponding inter-molecular distances are 8 Å (C358-C421), 4.76 Å (C421-C424) and 8 Å (C424-C358). The relevant cysteine residues of YeiL and aconitase are superimposed in Fig. 3(b) and it is predicted that such a [4Fe-4S] cluster could be accommodated by YeiL. The fourth iron ligand could be provided by one of the remaining cysteine residues (C9 and C116, from the same or an adjacent subunit), by a hydrogen-bonded water molecule (as found in aconitase), or by some other residue. From these structural predictions it is conceivable that the proposed transcription regulatory activity of YeiL might be modulated by a sensory iron-sulphur cluster (as in FNR), by reversible disulphide formation, or by a combination of both mechanisms. Intra-molecular disulphide bridges could

Fig. 3. Hypothetical model of a YeiL dimer and spatial organization of a potential iron-sulphur cluster. (a) model of a YeiL dimer based on the three-dimensional structure of the cAMP-CRP-DNA complex (Schultz *et al.*, 1991), drawn with MOLSCRIPT (Kraulis, 1991). The cysteine residues are shown with space-filling sulphur atoms (light spheres) and distances were measured between the pairs of C β atoms connected by lines. (b) Spatial positioning of cysteine sulphur atoms co-ordinating the iron atoms of a hypothetical iron-sulphur cluster in YeiL (dark spheres) compared to a [4Fe-4S] cluster in mitochondrial aconitase (light spheres).

be formed between the cysteine residue near the N-terminus and a centrally located cysteine residue (as in FLP) or between another pair(s) of cysteine residues. Alternatively, inter-molecular disulphide bonds could be formed between independent subunits at the dimer interface, e.g. between C116a and C116b (Fig. 3a), or at other sites.

Overproduction and purification of YeiL

The 657 bp *yeiL* coding region was PCR-amplified from chromosomal DNA and cloned into a variety of expression vectors in order to overproduce and purify the YeiL protein. Most of the strategies, including IPTG-induced expression of intact YeiL and several

Fig. 4. Overproduction and purification of the MBP-YeiL fusion protein. Coomassie-blue-stained SDS-PAGE gels showing: lane 1, protein standards; lane 2, suspension of induced bacteria (10 µg protein); lane 3, soluble cell-free extract (15 µg protein); lane 4, amylose-affinity-purified MBP-YeiL (5 µg protein). The positions of the fusion protein, GroEL, a degradation product (MBP-X), and the standards (ovotransferrin, 78 kDa; bovine serum albumin, 67 kDa; ovalbumin, 42.7 kDa; and carbonic anhydrase, 30 kDa), are indicated.

fusion proteins (GST-YeiL, His₆-YeiL and YeiL-His₆), were unsuccessful despite achieving high yields of amplified protein. This was largely because most of the amplified protein was insoluble even after low-temperature expression. Although the insoluble fraction could be solubilized by detergents and chaotropic ions (but not reducing agents), it invariably reprecipitated upon controlled removal of these agents, in the presence or absence of reductants. Furthermore, the small amount of soluble material in cell-free extracts was heavily contaminated by chaperone proteins. However, a soluble fusion protein was obtained by cloning the *yeiL* coding region downstream of the gene encoding a truncated maltose-binding protein (MBP) in pMal-c2 to generate pGS1226. The truncated MBP lacks a signal sequence; hence the MBP-YeiL fusion remained in the cytoplasm. In typical experiments, IPTG-induced cultures of CAG626(pGS1226) overproduced a protein having a subunit mass of ~66 000 Da (predicted mass 68 417 Da including the linker); 90% of this protein remained in the soluble fraction, where it represented ~8% of total soluble protein, as estimated by quantitative densitometry of Coomassie-stained gels (Fig. 4). It was purified to ≥85% homogeneity in a single step by amylose affinity chromatography (Fig. 4) in final yields of 4–7 mg MBP-YeiL fusion protein per litre of culture. N-terminal sequence analysis confirmed that the ~66 000 Da component is an MBP fusion, and it also showed that the major contaminants are GroEL (the ~60 000 Da band) and MBP-X (the ~43 000 Da band),

Fig. 5. Gel filtration analysis of MBP-YeiL. (a) Elution profiles of purified MBP-YeiL (100 µg) obtained with no DTT in the sample or elution buffer (upper panel) and with 4 mM DTT in the sample and 1 mM DTT in the elution buffer (lower panel). The molecular mass scale was derived from the elution volumes of standard proteins. (b) Silver-stained SDS-PAGE gel of the fractions marked by letters in (a); the absorbance marked F was due to DTT. The positions of known components and standards are indicated.

presumed to be a degradation product of MBP-YeiL because the host strain lacks intact MBP, predicted mass 42 000 Da (Fig. 4). Factor Xa-mediated cleavage of YeiL from the fusion was very poor, so MBP-YeiL was used

in all subsequent studies. It was argued that the MBP moiety would not interfere with the biochemical characterization because it lacks cysteine and because it has been used successfully in characterizing a synthetic iron-sulphur protein (Benson *et al.*, 1998). As isolated the MBP-YeiL protein was extremely labile and prone to precipitation but could be stored for up to 10 d at 4 °C in the buffer containing 1 mM DTT.

Biochemical characterization of the MBP-YeiL fusion protein

Gel filtration analysis indicated that freshly prepared native fusion protein exists as a high-molecular-mass oligomer or aggregate of >300 000 Da (Fig. 5a). SDS-PAGE and N-terminal sequence analysis showed that the purified protein contained GroEL and some lower-molecular-mass components that are probably degradation products of the unstable MBP-YeiL protein (Fig. 5b, lane A). However, when analysed in the presence of DTT (4 mM in the sample and 1 mM in the elution buffer) only a small fraction of the fusion protein (~10%) was associated with GroEL and the rest eluted as a monomer which was still very prone to degradation (Fig. 5a and b, lanes B and C versus D and E): no dimeric MBP-YeiL was detected. Mass spectrometry served mainly to illustrate the instability of the fusion protein because much of the protein appeared to be degraded to products of 44 646 and 57 322 Da and the corresponding homo- and hetero-dimers (89 300, 102 000 and 114 600 Da). Nevertheless, molecules of 68 700 Da (MALDI-TOF) and 68 478 Da (electrospray), representing monomeric MBP-YeiL (predicted 68 417 Da), were detected. The extra mass could be due to protein modification or remnants of degraded iron-sulphur clusters, as observed previously with FNR (Green *et al.*, 1993). Assuming that the MBP moiety is stable, the sizes of the major degradation products indicated that they retain either one cysteine residue (C9) or all five cysteine residues (C9, C68, C91, C93 and C116). This suggests that C9 and C116 may be located on the surface of the YeiL moiety, where intra- and inter-molecular disulphide bridges lead to the formation of stable monomeric and dimeric degradation products. The presence of the latter further suggests that C9 and C116 might participate in the reversible formation of disulphide-linked YeiL dimers. It was also inferred that the oligomeric form of MBP-YeiL detected by gel filtration (Fig. 5a) might be a non-covalent aggregate of either monomers or disulphide-bonded dimers (because it is disaggregated by DTT).

Purified MBP-YeiL contained substoichiometric amounts of iron and acid-labile sulphide (Table 2). This suggested that at least a fraction of the protein retained an iron-sulphur cluster or degradation products therefrom, as is frequently observed with iron-sulphur proteins. After anaerobic reconstitution of potential iron-sulphur clusters, the iron content increased to approximately four per monomer but less sulphide was incorporated (Table 2). The procedure for detecting

Table 2. Biochemical analysis of MBP-YeiL

The iron and acid-labile sulphide contents were assayed before and after anaerobic NifS-mediated reconstitution and desalting; the net contents of free thiol groups and disulphide bonds were determined with samples of the native protein dialysed versus DTT-free buffer. The quoted values were derived from at least two independent preparations and have been adjusted to allow for the 15 % contamination by GroEL.

MBP-YeiL	Chemical composition (mol per mol of MBP-YeiL monomer)				
	Fe	Acid-labile S	Fe/S ratio	Free SH	Disulphide
Native (as prepared)	0.28–0.36	0.12–0.25	1.7–2.2	2.9–3.4	0.96–0.98
Reconstituted	3.80–4.40	2.59–3.29	1.3–1.5	ND	ND

ND, Not determined.

acid-labile sulphide (Beinert, 1983) requires disruption of the protein without precipitation, which is more difficult if the Fe–S cluster is stable. The results would therefore be consistent with MBP-YeiL acquiring one relatively stable [4Fe–4S] cluster per monomer. Anaerobic NifS-catalysed reconstitution for 16 h in a sealed cuvette was accompanied by the acquisition of a straw-brown appearance and an increase in absorbance at 420 nm, giving a spectrum that is typical of iron–sulphur proteins. The molar absorption coefficient (ϵ_{420}) of reconstituted MBP-YeiL was $19750 \text{ M}^{-1} \text{ cm}^{-1}$ (allowing for the 85 % purity of the samples analysed). This is somewhat higher than the value of $17200 \text{ M}^{-1} \text{ cm}^{-1}$ for the model compound $[\text{Fe}_4\text{S}_4(\text{S-Et})_4]^{2-}$, but it is much higher than the ϵ_{420} values of typical $[2\text{Fe-2S}]^{2+}$ clusters, $9000\text{--}11000 \text{ M}^{-1} \text{ cm}^{-1}$ (Holm & Ibers, 1977).

The stability of the putative iron–sulphur cluster was investigated by exposing anaerobically reconstituted and desalted MBP-YeiL to air in a spectrophotometer cuvette while recording the UV–visible spectrum. The absorbance at 420 nm declined slowly by 10 % over a period of 100 min and there was a corresponding increase at 330 nm, after which the solution became turbid due to protein precipitation. Similar experiments with reconstituted FNR have shown that protein precipitation starts after 10 min exposure to air as a consequence of cluster degradation (Lazazzera *et al.*, 1996). The increase in absorbance at 330 nm is frequently seen during the degradation (and acquisition) of the iron–sulphur clusters of FNR and related proteins, e.g. the FNR of *Azotobacter vinelandii* (Wu *et al.*, 2000), but the reason is not known. Gel filtration analysis after reconstitution showed that 40 % of the protein was released from the oligomeric form but it was rapidly degraded and again no dimers were detected.

As isolated, MBP-YeiL contained approximately three free thiol groups and one disulphide bond per monomer (Table 2). This is consistent with its being a multimeric aggregate linked by one inter-molecular disulphide bond per monomer. However, because three or four thiols might be used for assembling the iron–sulphur cluster,

and because disulphide-bond formation could occur between thiols within and between monomers (including iron-liganding thiols after cluster disassembly), it is impossible to deduce the precise disposition of the five cysteine residues in YeiL. Nevertheless, YeiL clearly has the potential to assemble an iron–sulphur cluster and to form intra- and inter-molecular disulphide bonds, as predicted. By analogy with FNR and FLP the DNA-binding form of YeiL might be dimeric and its activity might likewise be modulated by a sensory iron–sulphur cluster, by reversible dithiol–disulphide formation, or by both mechanisms.

Construction and phenotypic characterization of a *yeiL* mutant

The *yeiL* gene was disrupted by replacing a segment of the coding region by a *kan^R* cassette (*in vitro*), then transferred to the *E. coli* chromosome (Oden *et al.*, 1990), and ultimately to W3110 to generate JRG3827 (*yeiL::kan^R*). The growth patterns of the *yeiL* mutant (JRG3827) and its isogenic parent (W3110) were compared under aerobic, microaerobic and anaerobic ($\pm$ nitrate) conditions at 37 °C in LB broth ($\pm$ glucose) and in minimal medium E containing different carbon sources. This showed that the growth yields (OD_{600}) achieved by the parental strain were consistently 10–15 % higher than those of the mutant during aerobic growth in rich media and minimal media containing glucose, maltose or succinate. No differences in growth rates or other features of the growth cycle were observed between the mutant and parental strains. No complementation of the growth defect was observed with pGS1109 because this high-copy *yeiL⁺* plasmid adversely affected the growth of both mutant and wild-type transformants, particularly in minimal media. In contrast, the growth yields (OD_{600} after fourfold dilution) observed in LB medium for mutant and wild-type transformants containing a low-copy plasmid (pGS1376) increased by 10–14 % and 25–30 % (respectively) relative to the untransformed host strains. However, the deleterious effects again became apparent in minimal media, where complementation varied with substrate from very weak

Fig. 6. Growth phase and factor-dependence of *yeiL-lacZ* expression. Cultures of λ *yeiL-lacZ* (λ G271) monolysogens were grown at 37 °C from 1% stationary-phase inocula in peptone broth (50 ml, supplemented with 0.2% glucose) by shaking aerobically at 200 r.p.m. for 16 h (unless specified times are indicated). (a) Expression of the *yeiL-lacZ* transcriptional fusion during the growth cycle of JRG3885 (wild-type): $\square$, growth (OD_{600}); $\bullet$, β -galactosidase specific activity (Miller units). (b) Effects of regulator mutations on stationary-phase *yeiL-lacZ* expression (Miller units): mean values for four independent cultures are shown with standard deviations. The monolysogens and mean OD_{600} values of 16 h cultures were: wild-type (JRG3885, 2.86); *yeiL* (JRG3887, 2.2); *rpoS* (JRG3889, 2.5); *ihfA* (JRG3891, 2.4); *lrp* (JRG3893, 2.1); *fnr* (JRG3886, 2.78); *fnr yeiL* (JRG3888, 2.4); *fnr rpoS* (JRG3890, 2.6); *fnr ihfA* (JRG3892, 2.2); *fnr lrp* (JRG3894, 1.6).

(succinate) to insignificant (glucose) and detrimental (maltose).

Studies with a *yeiL-lacZ* transcriptional fusion

Insights into the temporal expression and regulation of the *yeiL* gene were sought by constructing monolysogens containing λ G271 prophages in which the *yeiL* promoter region is fused to a *lacZ* reporter (see Methods). During aerobic growth in rich media *yeiL* expression increased 6.5-fold in stationary phase relative to late exponential phase (Fig. 6a) and the degree of induction relative to early exponential phase may have been even greater if exponential phase inocula had been used. The reason for the reproducible two-stage increase is not known. Similar high levels of expression were observed in late-

stationary-phase cultures relative to exponential-phase cultures grown in glucose (0.2%), glycerol (40 mM) and succinate (40 mM) minimal media (data not shown). However, the growth-phase-dependent response was not observed under anaerobic fermentative conditions, where the stationary-phase activities of the wild-type monolysogen (JRG3885) were about fourfold lower than those of the comparable aerobic cultures shown in Fig. 6(a) (data not shown).

Studies with monolysogens lacking YeiL or various transcription factors indicated that in the stationary phase aerobic *yeiL* expression is positively autoregulated by YeiL, and significantly dependent on RpoS (σ^S) and Lrp but not IHF (Fig. 6b). In contrast, there was a ~ 1.9 -fold increase in stationary-phase *yeiL* expression in the *fnr* mutant (JRG3886) under both aerobic conditions (Fig. 6) and anaerobic fermentative conditions (data not shown), suggesting that *yeiL* transcription is directly or indirectly repressed by FNR. The fourfold decrease in stationary-phase *yeiL* expression observed under anaerobic conditions with the parental strain (JRG3885) was also observed with the *fnr* mutant (JRG3886), which suggests that YeiL may function primarily during the aerobic stationary phase. The requirements for YeiL, RpoS and Lrp for optimal *yeiL* expression were retained in the absence of FNR (Fig. 6b). Expression of the *yeiL-lacZ* fusion also became partly dependent on IHF in the *fnr* background, suggesting that FNR and IHF may exert opposing negative and positive effects at overlapping binding sites in the *yeiL* promoter region.

Identification of transcription start sites for the *yeiL* and *yeiK* genes

Primer extension analysis with RNA from cultures of two *fnr* mutants, RK5279(pGS1109) and RK5279(λ G271) (also designated JRG3886), chosen for their enhanced *yeiL* expression and sampled at different times in early stationary phase (6–10 h), revealed a single extension product, which was most abundant in the 8 h sample (Fig. 7a). This corresponded to a potential *yeiL* transcription start site located 65 bp upstream of the translation initiator codon and 7 bp downstream of a potential σ^{70} or σ^S promoter containing plausible –35 (TTGAAT) and –10 (TATGCT) hexamers (Fig. 7b). The –35 hexamer is overlapped by a potential FNR site, which closely matches the FNR-site consensus (TTGAT-N₄-ATCAA) and its location is consistent with the observed FNR-mediated repression of *yeiL* (Fig. 7c). There is a potential Lrp site (YAGHAWATTWDCTR; Y = T or C; H = A, C or T; W = A or T; D = G, A or T; R = A or G; Cui *et al.*, 1995) centred at –120 bp just inside the *yeiK* coding region, and there are two sequences matching the IHF-site consensus (WATCAANNNTTTR; Freundlich *et al.*, 1992), which are consistent with the observed effects of Lrp and IHF on *yeiL* gene expression (Fig. 7c). There are two overlapping regions of partial dyad symmetry centred at –62 (TTTTCTacagcaaAGAAA) and –64.5 (TTaTtTTTtCTacAGcAAAgAaAA), one of

Fig. 7. Primer extension mapping of potential *yeiL* and *yeiK* transcriptional start sites with RNA from cultures of RK5279(pGS1109) (*fnr yeiL*⁺). (a) Extension product of *yeiL* transcript in RNA from an 8 h culture, aligned with a sequence ladder generated from pGS1109 and the same primer, S557. (b) Extension product of *yeiK* transcript in RNA from a 6 h culture and a sequence ladder generated from pGS1109 with the same primer, S558. (c) Nucleotide sequence of the *yeiL* and *yeiK* promoter region showing the presumed transcription start sites (+1, →), putative -35 and -10 hexamers (underlined or overlined); potential binding sites for FNR, Lrp and IHF (boxed); regions of partial dyad symmetry (converging arrows); ribosome-binding sites (RBS, consensus bases italicized), and translation initiators (fMet).

which could conceivably represent a YeiL-binding site associated with its positive autoregulatory effect on *yeiL* gene expression.

The transcription start site of the adjacent and divergent *yeiK* gene (Fig. 1) was identified by primer extension using the RNA samples from RK5279(pGS1109) and two different primers. Again, only one extension product was detected (Fig. 7b) and this corresponded to a potential *yeiK* start site located 66 bp upstream of the YeiK initiator codon (Fig. 7c). There is a potential -10 hexamer with an extended -10 motif (TGnTAAGAT) located 7 bp upstream of the start site. The most plausible -35 hexamer (TTAGCA) is located only 15 bp upstream of the -10 hexamer, which is rather close for a typical σ^{70} promoter, but the poor -35 site may be compensated by the extended -10 sequence. Thus it appears that *yeiL* and *yeiK*, the putative nucleoside hydrolase gene, are divergently transcribed from promoters in the same small segment of DNA. Transcription from one promoter may thus affect expression from the other. This is consistent with the primer extension experiments, which indicated that *yeiK* transcripts are most abundant in 6 h RNA samples, where the *yeiL* transcripts are far less abundant.

Starvation response of the *yeiL* mutant

A role for YeiL in starvation survival was sought because the *yeiL* gene was more highly expressed in the post-exponential phase. The viabilities of the *yeiL::kan*^R mutant (JRG3827) and parent (W3110) were accordingly compared in M9 minimal medium under non-limiting and under carbon-, phosphorus- and nitrogen-limiting conditions. Under carbon- and phosphate-limiting conditions the viabilities of both strains declined in parallel over 7 d (data not shown). However, when medium containing 0.2 mM NH₄Cl (1% of the normal concentration) was inoculated with bacteria that had been pre-grown for 16 h in nitrogen-sufficient medium, modest but significant and reproducible differences in viability were observed (Fig. 8). After starting at comparable values, mutant viability fell to 10% during the second day compared to 75% for the parental strain. After 3 d, mutant viability stabilized between 2% and 1% while parental viability continued to fall from 21% to 2% (Fig. 8). In comparable studies with a JRG3827 transformant containing the low-copy *yeiL*⁺ plasmid (pGS1376), viability was partially restored from 10% to 63% (day 2) and 2% to 12% (day 3). It was therefore concluded that YeiL performs a direct or indirect role in

Fig. 8. Effects of *yeiL* mutation on the nitrogen-starvation response. The viabilities (% survival) of W3110 (●) and the *yeiL::kan^R* mutant JRG3827 (□) grown in N-deficient M9 medium are based on daily viable counts (c.f.u. ml⁻¹, determined in triplicate) expressed as percentages of the comparable day 1 values. The data shown are typical of three independent experiments.

maintaining mid-term, but not long-term, stationary-phase viability under N-starvation.

DISCUSSION

The *yeiL* gene of *E. coli* was predicted to encode a new and distinct member of the CRP–FNR family of transcriptional regulators that might have an iron–sulphur centre and a reversible intra- or inter-molecular disulphide bond. Analysis of the purified MBP–YeiL fusion protein indicated that it is most probably a protein aggregate containing one inter-molecular disulphide bond (C9–C116 from different subunits) and three free thiols (C68, C91 and C93) per monomer (Fig. 3a). Under reducing conditions this would permit the observed assembly of a [4Fe–4S] cluster liganded by the three free thiols and one other thiol (possibly C9), and the potential release of monomers. The monomers could in turn engage either in reversible inter-molecular disulphide formation between the C116 thiols to generate covalently linked dimers, or in intra-molecular disulphide formation (depending on the state of the iron–sulphur cluster) to generate non-covalent dimers. Compared to FNR (Green *et al.*, 1996; Khoroshilova *et al.*, 1997) the iron–sulphur cluster acquired by MBP–YeiL had a much greater aerobic stability but the protein was itself less stable. This suggests that YeiL is not an oxygen-responsive regulator. Nevertheless, if the acquisition of the iron–sulphur is not simply fortuitous or for purely structural purposes, cluster assembly and the potential for reversible disulphide and dimer formation could provide functional switches for DNA-binding and transcription regulation analogous to those operating in FNR and FLP. Further elucidation of the molecular structure of YeiL and the regulatory switch was precluded by the insolubility and instability of the protein.

The problems experienced in amplifying YeiL as a soluble protein or fusion protein, the inherent instability of isolated MBP–YeiL, and the presence of GroEL as the main contaminant, may be functionally significant. They might indicate that considerable chaperone activity is needed to promote correct assembly of YeiL when overproduced. On the other hand they could mean that YeiL resembles the heat-shock sigma factor (σ^{32}) in being associated with chaperones (DnaJ, DnaK, GrpE) which promote instability in unstressed bacteria, or in becoming resistant to proteolysis and hence functional when dissociated from chaperones in a stressed host (Gamer *et al.*, 1996; Straus *et al.*, 1990). σ^{32} is also responsible for the induction of heat-shock proteins including GroEL under starvation conditions (VanBogelen & Neidhardt, 1990), so the association of MBP–YeiL with GroEL may serve as a post-translational mechanism for controlling the concentration of functional YeiL during starvation. Alternatively, YeiL might resemble the temperature-sensing TlpA protein of *Salmonella typhimurium*, which is a DNA-binding regulator that can shift between unfolded monomeric and folded oligomeric states in response to changes in temperature by a process that involves inter-molecular disulphide formation (Hurme *et al.*, 1997). However, the occurrence of the oligomeric form of TlpA at normal physiological concentrations is uncertain and the same might apply to the multimeric form of YeiL.

Clues about the role of YeiL came from studying the consequences of inactivating the *yeiL* gene and the use of a *yeiL-lacZ* reporter fusion to investigate *yeiL* gene expression under different physiological conditions and in different hosts. Studies with the *yeiL::kan^R* mutant (JRG3287) revealed a small but significant and reproducible deficiency in aerobic growth yield in several media and a remarkable enhancement of growth yield in rich medium by low-copy (but not high-copy) *yeiL⁺* plasmids, especially in the wild-type host. Although the apparent increase in growth efficiency was not observed under all growth conditions it deserves further investigation as a mechanism for enhancing biotechnological productivity. The mutant also exhibited a specific deficiency in nitrogen-starvation survival, which was partially complemented by supplying *yeiL⁺* *in trans*. It is not clear how the two features of the mutant phenotype are related. A role in the aerobic stationary phase was inferred from the very significant post-exponential increase in *yeiL* gene expression in rich (Fig. 6a) and minimal media. This view was strengthened by finding that *yeiL* expression is significantly dependent on RpoS (σ^S), the stationary-phase sigma factor (Kolter *et al.*, 1993), which is also essential for starvation survival (Lange & Hengge-Aronis, 1991). The requirements for Lrp and IHF (especially in the absence of FNR) are consistent with their greater abundance in slow-growing or stationary-phase organisms (Landgraf *et al.*, 1996; Goosen & van de Putte, 1995). No rationale can be offered at present for the positive autoregulatory effect of YeiL on *yeiL* gene expression, nor for the aerobic repression by FNR, be it direct or indirect.

However, the fact that expression of the *rpoS*, *lrp* and *ihf* genes is stimulated by ppGpp (Aviv *et al.*, 1994; Lange *et al.*, 1995) and that many genes induced by carbon, phosphorus and nitrogen starvation are regulated by the stringent response (Nyström, 1995; Rao *et al.*, 1998), is consistent with YeiL being involved in the response to nutrient starvation.

Although the nitrogen-starvation-survival defect may not be a direct consequence of *yeiL* inactivation, it may be significant that *yeiL* is located within a cluster of genes concerned with nucleoside metabolism. Indeed, *yeiL* and the adjacent *yeiK* gene, encoding a potential purine salvage pathway enzyme, are divergently transcribed from overlapping promoters, suggesting that YeiL might regulate *yeiK* expression as well as autoregulate its own synthesis. Salvage pathways recycle nucleosides and nucleobases for nucleic acid synthesis but these intermediates can also serve as carbon and nitrogen sources. It is therefore conceivable that YeiL might control expression of the salvage pathways or in some other way repress the recycling of nucleobases to nucleic acids and enhance their use as general nitrogen sources, during nitrogen-limited growth. Lack of YeiL might exacerbate the nitrogen deficiency and thus be responsible for the modest nitrogen-starvation-survival defect of the *yeiL* mutant. The toxic effects of excess YeiL might likewise be due (at least in part) to repression of the salvage pathways. There is no clear connection between the presence of potential iron-sulphur or disulphide-dithiol redox sensors in YeiL, and post-exponential growth and nitrogen metabolism. Regulator defects often generate pleiotropic phenotypes and it is hoped that further studies will reveal the basis of the relatively diverse and subtle changes observed here. The curious and as yet unexplained fourfold stationary-phase activation/deactivation of overproduced apoaconitase A observed by Prodromou *et al.* (1991) may be related insofar as it indicates that iron-sulphur cluster assembly/disassembly is occurring when YeiL is being expressed and/or activated.

This work has laid a foundation for future investigations aimed at defining the precise role of the third member of the CRP family in *E. coli*. Further studies will be needed to elucidate exactly how extra YeiL enhances growth yield whereas excess YeiL is toxic, and also how YeiL regulates *yeiL* and *yeiK* gene expression. It will also be important to identify other potential members of the YeiL regulon and to find ways of stabilizing the free protein in order to define the nature of the DNA-binding form, the nucleotide sequence to which it binds, and the physiological signal that is recognized, as well as to establish whether the iron-sulphur cluster and disulphide-dithiol formation participate in DNA-binding or signal recognition.

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