Escherichia coli K-12 YfgF is an anaerobic cyclic di-GMP phosphodiesterase with roles in cell surface remodelling and the oxidative stress response

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The *Escherichia coli* K-12 *yfgF* gene encodes a protein with domains associated with cyclic di-GMP signalling: GGDEF (associated with diguanylate cyclase activity) and EAL (associated with cyclic di-GMP phosphodiesterase activity). Here, it is shown that *yfgF* is expressed under anaerobic conditions from a class II FNR (regulator of fumarate and nitrate reduction)-dependent promoter. Anaerobic expression of *yfgF* is greatest in stationary phase, and in cultures grown at 28 °C, suggesting that low growth rates promote *yfgF* expression. Mutation of *yfgF* resulted in altered cell surface properties and enhanced sensitivity when anaerobic cultures were exposed to peroxides. The purified YfgF GGDEF-EAL (YfgF_{GE}) and EAL (YfgF_E) domains possessed cyclic di-GMP-specific phosphodiesterase activity, but lacked diguanylate cyclase activity. However, the catalytically inactive GGDEF domain was required for YfgF_{GE} dimerization and enhanced cyclic di-GMP phosphodiesterase activity in the presence of physiological concentrations of Mg²⁺. The cyclic di-GMP phosphodiesterase activity of YfgF_{GE} and YfgF_E was inhibited by the product of the reaction, 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG). Thus, it is shown that the *yfgF* gene encodes an anaerobic cyclic di-GMP phosphodiesterase that is involved in remodelling the cell surface of *E. coli* K-12 and in the response to peroxide shock, with implications for integrating three global regulatory networks, i.e. oxygen regulation, cyclic di-GMP signalling and the oxidative stress response.

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INTRODUCTION

The *Escherichia coli* FNR (regulator of fumarate and nitrate reduction) protein is an oxygen-responsive global transcription factor (Constantinidou *et al.*, 2006; Green *et al.*, 2009; Green & Paget, 2004; Kang *et al.*, 2005; Partridge *et al.*, 2006, 2007b; Salmon *et al.*, 2003; Uden *et al.*, 2002). Under aerobic conditions FNR is an inactive monomeric apo-protein (Achebach *et al.*, 2005). However, under anaerobic conditions a dimeric form of FNR with two [4Fe-4S] clusters per dimer that is capable of enhanced site-specific DNA binding is predominant (Jervis & Green, 2007; Lazazzera *et al.*, 1996). Dimeric FNR binds at DNA sequences related to the consensus TTGATNNNNATCAA (Eiglmeier *et al.*, 1989). Once bound, FNR regulates transcription of target genes either by recruiting RNA polymerase (RNAP) or by inhibiting the formation of productive promoter–RNAP

interactions (Browning *et al.*, 2003). There are two basic types of FNR-dependent promoter that differ in the nature of the regulator–RNAP contacts that are formed. Class I promoters have FNR sites located close to –61.5, –71.5, –82.5 or –92.5, relative to the transcript start (Wing *et al.*, 1995), which allows only one protein–protein contact between the C-terminal domain of the RNAP α -subunit and activating region 1 (AR1) of FNR. In contrast, class II promoters have an FNR site centred close to –41.5 (Browning *et al.*, 2003), which allows the establishment of multiple contacts between RNAP and three activating regions (AR1, AR2 and AR3) of FNR.

FNR primarily functions to regulate anaerobic metabolism in *E. coli* and other bacteria by co-ordinating changes in gene expression that optimize growth potential in different niches. Over 130 genes are known to be either directly or indirectly under the control of FNR in *E. coli* K-12 (Constantinidou *et al.*, 2006; Kang *et al.*, 2005; Partridge *et al.*, 2006, 2007b; Salmon *et al.*, 2003), and the full extent of the FNR regulatory network is still being determined.

Recently, the *E. coli* K-12 *yfgF* gene has been identified as an FNR target (Partridge *et al.*, 2007a). The *yfgF* gene

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Abbreviations: EMSA, electrophoretic mobility shift assay; FNR, regulator of fumarate and nitrate reduction; pGpG, 5'-phosphoguanylyl-(3'-5')-guanosine; RACE, random amplification of cDNA ends.

encodes a protein of 747 amino acids and contains three previously recognized structural domains (Fig. 1). The N-terminal region is a membrane-associated sensor 1 (MASE1) domain with nine predicted transmembrane helices (Nikolskaya *et al.*, 2003). The precise mechanism of signal perception by MASE1 domains is unknown, although they have been linked with several cellular functions, including oxygen sensing in *Desulfovibrio vulgaris* (Xiong *et al.*, 2000).

The central section of the YfgF primary structure is occupied by a GGDEF domain, named after its conserved amino acid sequence motif (GG[D/E]EF). The GGDEF domain has similarity to adenylyl cyclase (Pei & Grishin, 2001) and was first recognized in the PelD protein of *Caulobacter crescentus* (Hecht & Newton, 1995). PelD catalyses cyclic di-GMP synthesis from two molecules of GTP and its activity is regulated by phosphorylation (Paul *et al.*, 2004, 2007). It is now recognized that the GGDEF motif is widespread in prokaryotes; for example, there are 19 GGDEF proteins in *E. coli* K-12 (Galperin *et al.*, 2001). However, although the overall conservation of this region in YfgF supports its assignment as a GGDEF domain, the key sequence motif (GG[D/E]EF) is replaced by SGNDL, suggesting that the GGDEF domain of YfgF will lack diguanylate cyclase activity. Catalytically inactive GGDEF domains are not uncommon and have been shown to fulfil a variety of cellular functions. For example, *E. coli* CsrD has degenerate GGDEF and EAL domains and binds the small RNAs *csrB* and *csrC*, which regulate biofilm formation, flagella function and carbon storage, to promote their degradation by RNase E (Suzuki *et al.*, 2006). *C. crescentus* PopA is a cyclic di-GMP-binding inactive GGDEF protein that targets the replication initiation inhibitor CtrA to the old cell pole for degradation by ClpXP, thereby contributing to cell cycle control (Duerig *et al.*, 2009). The *C. crescentus* PdeA protein contains both EAL and GGDEF domains and the activity of the EAL domain is modulated by GTP binding to the catalytically inactive GGDEF domain (Christen *et al.*, 2005). *Pseudomonas aeruginosa*

PelD is predicted to retain some of the secondary structural features of GGDEF proteins and binds cyclic di-GMP to control exopolysaccharide production (Lee *et al.*, 2007). Thus, the probability that YfgF lacks diguanylate cyclase activity does not preclude a significant role for the degenerate GGDEF domain.

The C-terminal region of YfgF contains an EAL domain, named after its conserved amino acid residues (EAL and DDFGTG motifs). EAL domains were first discovered in tandem with GGDEF domains (Tal *et al.*, 1998), and it was speculated that EAL domains might be cyclic di-GMP phosphodiesterases. This was supported by the conservation of several acidic residues that may constitute a phosphodiesterase active site (Galperin *et al.*, 2001) and the direct demonstration of phosphodiesterase activity of EAL domains, e.g. *C. crescentus* PdeA and *E. coli* YahA (Christen *et al.*, 2005; Schmidt *et al.*, 2005). The functional relationship between GGDEF and EAL domains was confirmed by Simm *et al.* (2004), who suggested that these domains regulate the concentration of cyclic di-GMP in the bacterial cell, which in turn might act as a secondary messenger in regulating a variety of cellular functions. The EAL domain of YfgF differs from the established consensus motif at only one position. Thus, YfgF has a perfect match to the DDFGTG motif and EIL in place of the EAL motif; however, the mismatched residue has been shown to be non-essential/less conserved (Schmidt *et al.*, 2005), and therefore YfgF may function as a cyclic di-GMP phosphodiesterase.

Cyclic di-GMP was discovered in prokaryotes by Benziman and colleagues (Ross *et al.*, 1987), and has been shown to be involved in a variety of cellular functions such as cell surface remodelling, cellulose synthesis, virulence, motility and biofilm formation (Hisert *et al.*, 2005; Jenal, 2004; Jenal & Malone, 2006; Römling & Amikam, 2006; Ross *et al.*, 1987; Ryjenkov *et al.*, 2006; Tischler & Camilli, 2004, 2005).

Recently, overexpression of *yfgF*, the subject of the work reported here, has been shown to partially suppress the

Fig. 1. Representation (not to scale) of the domain structure of the *E. coli* K-12 YfgF protein. The relative positions of the MASE1, GGDEF and EAL domains are shown. The numbers indicate the positions of amino acids that start and finish the predicted intracellular and extracellular loops of the MASE1 domain and the limits of the GGDEF and EAL domains within the 747 aa YfgF protein. The amino acid sequences equivalent to the GGDEF and EAL motifs present in YfgF are shown in parentheses. Membrane topology was predicted using three programs: TopPred (Claros & von Heijne, 1994), TMPred (Hoffmann & Stoffel, 1993) and TMHMM (Sonnhammer *et al.*, 1998), all of which produced similar predicted topologies. The one shown is from TMHMM.

effects of a *yhjH* mutation (Girgis *et al.*, 2007). Mutation of *yhjH*, which encodes a cyclic di-GMP phosphodiesterase (Pesavento *et al.*, 2008), impairs the motility of *E. coli*. Overproduction of *yfgF* restores motility to the *yhjH* mutant (Girgis *et al.*, 2007). Thus, it has been suggested that mutation of *yhjH* increases the intracellular concentration of cyclic di-GMP, resulting in reduced motility, and that overproduction of YfgF compensates for the absence of YhjH by increasing the capacity of the bacteria to degrade cyclic di-GMP (Girgis *et al.*, 2007). However, in *Salmonella*, *yfgF* is able to restore motility to a strain that lacks the capacity to synthesize cyclic di-GMP, suggesting that the effects of YfgF on motility are mediated independently of cyclic di-GMP (Solano *et al.*, 2009). Thus, YfgF has a role in regulating motility in enteric bacteria, but whether this is mediated through cyclic di-GMP and indeed whether YfgF has any capacity to either synthesize or hydrolyse cyclic di-GMP had not been established. Here, it is shown that *yfgF* is expressed from an FNR-dependent class II promoter under anaerobic conditions, and encodes a cyclic di-GMP phosphodiesterase that is involved in remodelling the cell surface of *E. coli* K-12 and in counteracting the effects of exposure of anaerobic cultures to peroxide stress.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed in Table 1. Cultures of *E. coli* were grown in Lennox (L) broth (Lennox, 1955) supplemented with appropriate antibiotics (ampicillin, 100 µg ml⁻¹; chloramphenicol, 20 µg ml⁻¹; and tetracycline, 25 µg ml⁻¹). For β -galactosidase assays, L broth containing appropriate antibiotics was inoculated (1:50) from overnight cultures and incubated under anaerobic (sealed bottles filled to the neck), aerobic (10 ml medium in 250 ml conical flask shaken at 250 r.p.m.) or microaerobic conditions [10 ml medium in 250 ml conical flask shaken at 250 r.p.m. under an atmosphere of 2.5% oxygen in a MACO VA500 workstation (DW Scientific)] conditions at 37 °C, except where indicated. β -Galactosidase activities were measured according to Miller (1972).

Nucleic acid methods. DNA was isolated and manipulated by conventional methods (Sambrook & Russell, 2001). The *yfgF* promoter region was amplified from the chromosome of *E. coli* MC1000 by PCR using appropriate oligonucleotides designed to contain *EcoRI* and *BamHI* restriction sites. The DNA sequence of the amplified fragment was verified after ligation into *EcoRI*- and *BamHI*-digested pRS415 (Simons *et al.*, 1987). The inserts were then transferred to λ RZ5 and the resulting (*yfgF*-*lacZ*) fusions were introduced in single copy into the λ attachment site of the indicated *E. coli* strains (Table 1). Lysogeny status was verified using the method of Powell *et al.* (1994). Further transfer of the promoter fusions into other *E. coli* strains was achieved using bacteriophage P1_{vir}-mediated transduction (Sambrook & Russell, 2001).

Site-directed mutagenesis of the consensus FNR site (TTGATAT-ATATCAA to ATCATATATATGAT) was achieved using the oligonucleotides shown in Table 1. DNA sequencing after ligation into pRS415 was used to verify the authenticity of the altered promoter.

For 5' random amplification of cDNA ends (RACE)-PCR transcript mapping, RNA was isolated from anaerobic cultures of *E. coli* W3110 using Qiagen RNeasy mini kits according to the manufacturer's

instructions. The transcript start was identified using 2 µg *E. coli* RNA per RACE reaction according to the manufacturer's instructions (Roche). The initial cDNA template was generated using oligonucleotide primer JP12 (Table 1). The cDNA was polyadenylated and then amplified using JP12 and the oligo dT-anchor primer (Roche). Further amplification was achieved using two internal primers, JP13 and JP14 (Table 1), and the oligo dT-anchor primer. The final product of ~200 bp (as judged by agarose gel electrophoresis) was subjected to DNA sequencing.

The plasmid pGS2239 (a pBAD/HisB derivative containing the *yfgF* ORF) was constructed by amplifying the *yfgF* ORF from the chromosome of *E. coli* W3110 by PCR using appropriate oligonucleotides designed to contain *EcoRI* and *HindIII* restriction sites, which was then ligated into *EcoRI*- and *HindIII*-digested pBAD/HisB (MMW46 and MMW47).

The pET32a constructs for YfgF_{GE} and YfgF_E protein purification were created by amplifying the appropriate sections of the *yfgF* gene from the chromosome of *E. coli* W3110 by PCR using oligonucleotides (MMW1, MMW2, MMW3 and MMW5) designed to contain *NcoI* and *HindIII* restriction sites and ligation into *NcoI*- and *HindIII*-digested pET32a. The YfgF_{GE} construct encoded amino acids 319–747; the YfgF_E construct encoded amino acids 481–747.

Electrophoretic mobility shift assays (EMSAs). Binding of FNR to the *yfgF* promoter was investigated using EMSA and FNR protein FNR-D154A (FNR*) isolated as described by Meng *et al.* (1997). Radiolabelled *yfgF* promoter DNA (–108 to +84) was incubated with 8 µM FNR*, an FNR protein that retains the ability to bind DNA under aerobic conditions (Ziegelhoffer & Kiley, 1995) together with Tris/HCl (20 mM), glycerol (5%, v/v), KCl (100 mM), BSA (0.1 mg ml⁻¹) and DTT (1 mM) for 5 min before separating the FNR*–DNA complexes from DNA on Tris/borate/EDTA-buffered polyacrylamide gels. After electrophoresis the gels were transferred to filter paper (3MM, Whatman) and dried for autoradiography.

Construction of a *yfgF* mutant. A disruption in the *yfgF* gene of *E. coli* W3110 was obtained by linear transformation based on the method of Yu *et al.* (2000). Oligonucleotides containing 3' end sequences complementary to the first or last 20 bp of the chloramphenicol-resistance cassette of plasmid pACYC184 (Martinez *et al.*, 1988) and 5' end sequences flanking *yfgF* were constructed (Table 1). Linear DNA carrying the resistance cassette and flanking regions was generated by PCR. *E. coli* strain W3110 containing plasmid pTP223 (Tet^R) (Poteete & Fenton, 1984), which carries the λ red recombinase genes under the control of an IPTG-inducible promoter, were grown overnight at 37 °C, diluted (1:100) in L broth containing tetracycline (25 µg ml⁻¹) and IPTG (2 mM), and grown to OD₆₀₀ ~0.3. Electrocompetent cells were prepared and transformed with approximately 5 µg PCR product then recovered in 1 ml L broth for 1 h before plating on selective medium (Cm^R). Resulting colonies were immediately cured of pTP223 and mutations selected for by PCR and DNA sequencing. Further transfer of the mutations into clean *E. coli* W3110 genetic backgrounds was achieved using bacteriophage P1_{vir}-mediated transduction (Sambrook & Russell, 2001).

Phenotypic assays. The sensitivity of the *yfgF* mutants (*E. coli* W3110 and *E. coli* MG1655 derivatives) to a range of stress reagents was determined using disc diffusion assays. Sterile filter paper discs loaded with 10 µl of 10% solutions of *t*-butyl hydroperoxide, hydrogen peroxide or cumene hydroperoxide were placed in the centre of L agar plates seeded with the test bacteria. The plates were incubated under anaerobic conditions for 16 h at 37 °C before determination of the area of the zones of growth inhibition around the filter discs.

To measure the kinetics of peroxide killing, bacterial cultures were grown under anaerobic conditions to OD₆₀₀ 0.3 in sealed 13 × 100 mm

Table 1. Bacterial strains, plasmids and oligonucleotides used in this workAp^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Km^R, kanamycin resistant; Tet^R, tetracycline resistant.

Strain, plasmid or oligonucleotide	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
W3110	Prototroph	Laboratory collection
MG1655	Prototroph	Kang <i>et al.</i> (2004)
MG1655 <i>yfgF</i> (JRG6069)	MG1655 <i>yfgF</i> ::Tn5KAN-I-SceI; Km ^R	Kang <i>et al.</i> (2004)
W3110 <i>yfgF</i> (JRG5355)	W3110 <i>yfgF</i> mutant; Cm ^R	This work
MC1000	$\Delta(lacIPOZYA)X74$ <i>galU galK</i> $\Delta(araABC-leu)$	Spiro & Guest (1987)
JRG1728	MC1000 $\Delta(tyrR-fnr-rac-trg)17$ <i>zdd-230</i> -Tn9; Cm ^R	Spiro & Guest (1987)
JRG5070	MC1000/ λ RZ5(<i>P_{yfgF}-lacZ</i>) from pGS1633; Ap ^R	This work
JRG5071	JRG1728/ λ RZ5(<i>P_{yfgF}-lacZ</i>) from pGS1633; Ap ^R	This work
JRG5676	MC1000 <i>yfgF</i> mutant; Cm ^R	This work
Plasmids		
pACYC184	Source of <i>cat</i> cassette for linear transformation; Cm ^R	Martinez <i>et al.</i> (1988)
pGS1633	<i>P_{yfgF}-lacZ</i> fusion in pRS415 (192 bp promoter fragment, -108 to +84 relative to the transcript start); Ap ^R	Partridge <i>et al.</i> (2007a)
pRS415	<i>lacYZA</i> -based promoter-fusion vector; Ap ^R	Simons <i>et al.</i> (1987)
pTP223	<i>lam</i> , <i>bet</i> and <i>exo</i> genes of phage lambda, under the control of the <i>lac</i> promoter; Tet ^R	Poteete & Fenton (1984)
pGS1830	<i>P_{yfgF}-lacZ</i> fusion in pRS415 with the FNR-binding site altered from TTGAT-N ₄ -ATCAA to ATCAT-N ₄ -ATGAT; Ap ^R	This work
pET32a	Expression vector; Ap ^R	Novagen
pGS2064	pET32a derivative for expression of the EAL domain (amino acids 482–748) of YfgF; Ap ^R	This work
pGS2077	pET32a derivative for expression of the GGDEF-EAL domain of YfgF (amino acids 320–748); Ap ^R	This work
pBAD/HisB	Low-copy-number cloning vector; Ap ^R	Invitrogen
pGS2239	pBAD/HisB derivative containing the <i>yfgF</i> ORF under the control of the <i>araC</i> promoter; Ap ^R	This work
Oligonucleotides		
JP3	Forward primer for amplification and cloning of the <i>yfgF</i> promoter region with engineered <i>EcoRI</i> site: TTTGAATTCTGTAATAATGATGAATTAACATG	This work
JP4	Reverse primer for amplification and cloning of the <i>yfgF</i> promoter region with engineered <i>BamHI</i> site: TTTTGGATCCGAGAAATCCCCACATCC	This work
JP18	Mutagenesis of the <i>yfgF</i> FNR site: CTAAGTGTCATATGAAAGTACCAAAT-CATATATATGATACAAAATAACCC	This work
JP19	Mutagenesis of the <i>yfgF</i> FNR site: GGGTTATTTTGTATCATATATATGATT-TGGTACTTTCATATGACACTTAG	This work
JP12	RACE PCR cDNA primer: GCATATTTGCGGACAAATCC	This work
JP13	RACE PCR DNA amplification primer: CCACACCGGAAGAAATATGCG	This work
JP14	RACE PCR DNA amplification primer: GAACAGCGGAAGCCCCACC	This work
JP24	Forward primer for amplification of the <i>cat</i> cassette: GCACTGATGATCAGCATGAT-GATGTTTTTCAGCTGGGCGAAGCCACTGGAGCACCTCAA	This work
JP25	Reverse primer for amplification of the <i>cat</i> cassette: CCAACAGAGAATGATAA-TGATAATAAAATTAGCCGTTAATGATAGCGACGGGAGAGCCTGAGCAAA	This work
MMW1	Forward primer with engineered <i>NcoI</i> site for creating EAL expression plasmid: TTTTCCATGGGGGCAATGTATTGCAACGGGAATT	This work
MMW2	Reverse primer with engineered <i>HindIII</i> site for creating EAL expression plasmid: TTTTAAGCTTTCAGGCACTTTCGCGAATGGGTTCG	This work
MMW3	Forward primer with engineered <i>NcoI</i> site for creating GGDEF expression plasmid: TTTTCCATGGCGGTAGTCAGGCGCATTACGCGCT	This work
MMW5	Reverse primer with engineered <i>HindIII</i> site for creating GGDEF expression plasmid including stop codon: TTTTAAGCTTCTAGCGACGCTGCATATTTCCGGGG	This work
MMW46	Forward primer with engineered <i>EcoRI</i> site for creating a <i>yfgF</i> low-copy-number plasmid: TTTTGAATTCATGAAACTGAATGCAACTTATATAA	This work
MMW47	Reverse primer with engineered <i>HindIII</i> site for creating a <i>yfgF</i> low-copy-number plasmid: TTTTAAGCTTTCAGGCACTTTCGCGAATGGGTTCGA	This work

glass tubes before being challenged by addition of *t*-butyl hydroperoxide (0.4 mM), hydrogen peroxide (2 mM) or cumene hydroperoxide (0.2 mM). Samples were removed at 20 min intervals for serial dilution. The c.f.u. on L agar plates was calculated after 16 h incubation at 37 °C.

Sedimentation assays were performed on bacterial cultures (*E. coli* W3110 and *E. coli* MG1655, and the corresponding *yfgF* mutants) grown in L broth under anaerobic conditions for 16 h at 37 °C in 13 × 100 mm glass tubes. These cultures ($n=3$) were then left to stand for 24 h at 20 °C to allow sedimentation. The LIVE/DEAD stain kit (Molecular Probes) was used to determine the viability of the sedimented bacteria according to the manufacturer's instructions. Light microscopy (Leica microscope, model DMLB, ×100 magnification) was used to visualize the aggregation of sedimented cells. The relative rates of sedimentation of the parent and *yfgF* mutant were measured spectrophotometrically by monitoring the turbidity of anaerobic stationary phase cultures in sealed cuvettes at OD₆₀₀ for 10 h at 30 min intervals.

Biofilm assays were done using 96-well plates with L broth-only controls essentially as described by Antoniani *et al.* (2010). Wells were inoculated with 200 µl of a 1:10 dilution of an overnight culture of *E. coli* W3110 or the corresponding *yfgF* mutant, and then incubated for 16 h under anaerobic conditions at 37 °C. The growth of the cultures was measured at OD₆₀₀. The planktonic cells were removed and the remaining biofilm was stained for 5 min with 200 µl 1% (w/v) crystal violet solution. Excess stain was removed by three washes with deionized water before the plate was air-dried. To quantify the extent of staining, 200 µl ethanol:acetone (4:1) was added to each well, and after incubating for 20 min the amount of biofilm was measured by determining OD₆₀₀. Adhesion units were calculated by dividing the OD₆₀₀ of crystal violet-stained adhered cells by the OD₆₀₀ for the planktonic cells from the same cultures.

Motility assays were done using motility broth (5 g tryptone l⁻¹ and 5 g NaCl l⁻¹) and agar (5 g tryptone l⁻¹, 5 g NaCl l⁻¹ and 3 g agar l⁻¹). The required strains were grown for 48 h and then diluted threefold in motility broth. An aliquot (9 µl) was added to a motility agar plate and allowed to air-dry ($n=3$). The plates were placed in anaerobic jars and incubated for 18 h, after which the diameters of the resulting motility haloes were measured (Harshey & Matsuyama, 1994).

Protein purification. Expression of His-tagged protein constructs was induced in the exponential growth phase by addition of 100 µg IPTG ml⁻¹ at 25 °C. The bacteria were collected by centrifugation after 45 min incubation at 25 °C. The cell pellets were resuspended in breakage buffer [25 mM HEPES, pH 7.5, containing 5% (v/v) glycerol, 0.5 M NaCl and 10 mM benzamide]. All subsequent steps were carried out at 4 °C. The bacterial suspensions were lysed in a French pressure cell at 37 MPa and clarified by centrifugation at 27 000 g for 15 min. The soluble fraction was filtered and a 5 ml aliquot was loaded onto a HiTrap Chelating column (1 ml) charged with Ni²⁺, equilibrated with binding buffer A (10 mM HEPES, pH 7.5, containing 0.2 M NaCl). The target protein was eluted with a linear gradient (0–100%, 20 ml) of elution buffer B (10 mM HEPES, pH 7.5, containing 0.125 M NaCl and 0.5 M imidazole). The fractions containing the target protein were identified by SDS–polyacrylamide electrophoresis and were pooled before dialysis for a minimum of three times for 2 h against 10 mM HEPES, pH 7.5, containing 0.125 M NaCl. The purity of the samples was analysed by SDS–polyacrylamide electrophoresis and Coomassie blue staining. Protein concentrations were estimated using the Bio-Rad protein assay (Bio-Rad).

Diguanylate cyclase and phosphodiesterase assays. Substrate (ATP, GTP, cGMP, cAMP or cyclic di-GMP, 100 µM final

concentration) was added to 1 µM protein with 10 mM Mg²⁺ or Mn²⁺ as indicated in 10 mM HEPES, pH 7.5, containing 0.125 M NaCl and incubated at 37 °C for up to 3 h. To determine the rate of phosphodiesterase activity with cyclic di-GMP as the substrate the reactions were assembled as above with various concentrations of Mn²⁺ and Mg²⁺, samples were taken at intervals and the reaction was stopped by boiling. HPLC (Waters 100 pump, Waters 490E multiwave detector and Waters 745B data module) was used to analyse the reactions with a Jupiter 4 µ Proteo column (Phenomenex) equilibrated with 100 mM KH₂PO₄, 4 mM tetrabutyl ammonium hydrogen sulphate, pH 4.9. A gradient programme was used to separate the nucleotides as described in Ryjenkov *et al.* (2005).

Measurement of intracellular cyclic di-GMP concentration.

Approximately 150 mg (wet weight) bacterial cells was collected from anaerobic cultures and resuspended in 50 µl double-distilled and filtered water. After heating at 95 °C for 5 min, 500 µl ice-cold ethanol was added and the sample was centrifuged for 5 min at 18 000 g. The supernatant was transferred to a clean Eppendorf tube and desiccated. The pellet was resuspended in 11 µl double-distilled and filtered water. The suspension was clarified by centrifugation and then analysed by HPLC (see above).

Analysis of oligomeric state by cross-linking and gel filtration.

Chemical cross-linking was achieved using 0.01% (v/v) glutaraldehyde and 750 µg protein ml⁻¹ in 10 mM HEPES, pH 7.5, containing 0.125 M NaCl (total reaction volume 20 µl). The reaction was stopped after 15 min by the addition of an equal volume of 1 M Tris/HCl, pH 8.0.

For estimation of molecular mass by gel filtration, YfgF_{GE} and YfgF_E were dialysed against phosphate buffer (50 mM, pH 7.0) containing 0.25 M NaCl. The proteins were then applied to a calibrated Superdex 200 HR column equilibrated with the same phosphate buffer and eluted at a flow rate of 0.5 ml min⁻¹. The standards were: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (43 kDa), myoglobin (17 kDa) and vitamin B12 (1.4 kDa).

RESULTS

Expression of the *E. coli* K-12 *yfgF* gene is driven from a class II FNR-dependent promoter

The *E. coli* FNR protein recognizes and binds DNA sequences related to an established consensus (TTGATNN-NNATCAA) (Eiglmeier *et al.*, 1989). A previous study to identify new members of the FNR regulon by searching the *E. coli* K-12 genome sequence for sites similar to this consensus using the search pattern tool in the Colibri database (<http://genolist.pasteur.fr/Colibri>) identified *yfgF* as a potential target (Partridge *et al.*, 2007a). Preliminary transcriptional analysis using a multi-copy *yfgF-lacZ* fusion plasmid (pGS1633) showed FNR-dependent anaerobic activation of *yfgF* expression in exponential phase cultures (Fig. 2a, lanes 1–4; Partridge *et al.*, 2007a). The FNR-dependent anaerobic activation observed with pGS1633 was abolished when a derivative with an impaired FNR site (pGS1830) was analysed (Fig. 2a, lanes 5–8). These observations were confirmed using a chromosomal *yfgF-lacZ* fusion. Expression of single-copy *yfgF-lacZ* in MC1000 was approximately sixfold greater under anaerobic conditions than under aerobic conditions (Fig. 2b, compare columns 1 and 7, and 3 and 9). Under microaerobic conditions (2.5%

Fig. 2. Anaerobic expression of *yfgF* is dependent on the transcription factor FNR. (a) Cultures of MC1000 (*lac*; open bars) and JRG1728 (*lac fnr*; filled bars) carrying multicopy (pGS1633 and pGS1830) *yfgF-lacZ* fusions were grown under aerobic and anaerobic conditions at 37 °C in L broth to early exponential phase (2 h growth). Expression of *yfgF* was estimated by measuring β -galactosidase activities. The culture conditions are indicated beneath each column. (b) Cultures of MC1000 (*lac*; open bars) and JRG1728 (*lac fnr*; filled bars) carrying single-copy *yfgF-lacZ* fusions were grown under aerobic (+), microaerobic (2.5 % oxygen atmosphere; μ) and anaerobic (-) conditions at the indicated temperatures in L broth to the early exponential (E; 2 h growth) or stationary (S; 8 h growth) phase. Expression of *yfgF* was estimated by measuring β -galactosidase activities. The conditions are indicated beneath each column. All values are mean $\pm$ SD ($n=9$).

oxygen atmosphere) *yfgF* expression was only slightly reduced compared with that observed under anaerobic conditions (Fig. 2b, compare columns 5 and 7). In an *fnr* mutant (JRG1728), *yfgF* expression was lower than that observed with the parental strain (MC1000) under all oxygen tensions tested (Fig. 2b). In early exponential phase cultures, *yfgF-lacZ* expression was approximately sixfold higher under anaerobic conditions compared with aerobic conditions in the *fnr* mutant, even though the absolute expression levels were much lower than those of the parent (Fig. 2b, columns 2 and 8). However, this enhancement was only approximately twofold in stationary phase cultures (Fig. 2b, columns 4 and 10), suggesting that if there is oxygen-responsive control of *yfgF* expression beyond FNR it

exerts a relatively minor influence. Direct evidence of FNR binding to the *yfgF* promoter was obtained by EMSA with an FNR protein (FNR-D154A, designated FNR*) that retains some DNA-binding activity in the presence of air (Ziegelhoffer & Kiley, 1995). The EMSA assay showed that FNR* was able to bind at the native *yfgF* promoter region, but not at the same promoter fragment carrying the FNR site mutations present in pGS1830 that abolished the FNR response *in vivo* (Fig. 3a). The *yfgF* transcript start was mapped by 5' RACE-PCR and was found to be located 41.5 bp downstream of the centre of the FNR site (Fig. 3b, c). Thus, it has been shown that expression of the *E. coli* K-12 *yfgF* gene is driven from a class II FNR-dependent promoter.

Fig. 3. Expression of *yfgF* is controlled by a class II FNR-dependent promoter. (a) Binding of FNR to the *yfgF* promoter. Radiolabelled *yfgF* promoter fragments [native promoter, lanes 1 and 2; promoter with the FNR site mutated (TTGATATATATCAA→ATCATATAT-ATGAT), lanes 3 and 4] were incubated with (+) or without (-) 8 μ M FNR* (FNR-D154A) for 10 min before separating *yfgF*-FNR* complexes from free (*yfgF*) DNA in Tris/borate/EDTA-buffered polyacrylamide gels and visualization by autoradiography. (b) DNA sequence data obtained from RACE-PCR. The DNA sequence of the *yfgF* transcript is underlined and the polyA tail is shown in bold type. (c) Nucleotide sequence of the *yfgF* promoter. The transcript start is the large upper-case 'C' marked +1. The consensus FNR site centred at -41.5 is boxed and the core inverted repeat is highlighted in bold type.

Differential regulation of *yfgF* during the *E. coli* growth cycle at 37 and 28 °C

Cyclic di-GMP signalling has been implicated in the regulation of genes, such as those involved in motility and adhesion, that are transiently expressed during the growth cycle or are expressed at lower growth temperatures. Therefore, expression of *yfgF* was measured over the course of the anaerobic *E. coli* K-12 growth cycle at 37 and 28 °C using MC1000 (*lac*) carrying a single-copy chromosomal *yfgF-lacZ* fusion. Aerobic and anaerobic expression of *yfgF* increased approximately twofold in stationary phase (8 h) compared with early exponential phase (2 h) at 37 °C (Fig. 2b, columns 1, 3, 7 and 9). Measurements of *yfgF-lacZ* expression in anaerobic cultures of an *rpoS* (stationary phase alternative sigma factor) mutant suggested that the enhanced stationary phase expression of *yfgF* was not dependent on RpoS (results not shown). In an *fnr* mutant, anaerobic *yfgF* expression was similar in exponential and stationary phase cultures grown at 37 °C (Fig. 2b, columns 8 and 10), whereas under aerobic conditions the twofold stationary phase enhancement was retained (Fig. 2b, columns 2 and 4). This is attributed to the lower growth rate of the *fnr* mutant under anaerobic conditions causing relatively high *yfgF* expression during exponential phase. In addition to the increase in *yfgF* expression seen in stationary phase, *yfgF* expression was also increased by approximately twofold at 28 °C compared with 37 °C for both mid-exponential and stationary phase anaerobic cultures, and again this might be caused by the lower growth rate at 28 °C compared with 37 °C (Fig. 2b, compare columns 7 and 11, and 9 and 12). Thus, the data are consistent with two levels of control acting on *yfgF* expression: (i) FNR-dependent anaerobic activation and (ii) activation at lower growth rates.

Properties of *E. coli* K-12 *yfgF* mutants

The primary structure of YfgF and the regulation of *yfgF* expression suggested a role in anaerobic cyclic di-GMP signalling pathways. Cyclic di-GMP signalling is involved in controlling biofilm formation, sedimentation and motility (Römling & Amikam, 2006). Measurement of anaerobic biofilm formation at 37 °C (0.34 ± 0.12 adhesion units for *E. coli* W3110 *yfgF* mutant and 0.13 ± 0.043 adhesion units for the parent) indicated that biofilm formation was enhanced for the *yfgF* mutant. However, the *yfgF* mutant surprisingly exhibited a decreased tendency to sediment under anaerobic conditions (Fig. 4a, b). Consistent with the greater tendency of the parent strain to sediment, light microscopy revealed the presence of aggregates of bacterial cells in the anaerobic sediments of the parental strain but not in those of the *yfgF* mutant (Fig. 4c). LIVE/DEAD staining of bacteria recovered from the parent strain and mutant sediments showed that both contained similar numbers of live bacteria (parent $88.7\% \pm 1.82$; *yfgF* $86.6\% \pm 5.85$; $n=9$), and stationary phase survival was unaffected by mutation of *yfgF* (data not shown). These data imply that YfgF plays a role in reprogramming anaerobic gene expression to favour attachment to surfaces (biofilm formation) over self-self interactions, resulting in aggregation and sedimentation.

The response of the *E. coli* W3110 *yfgF* mutant to exposure to a range of stress reagents in disc diffusion assays indicated that the *yfgF* mutant was more sensitive than the parent strain to hydrogen peroxide, *t*-butyl hydroperoxide and cumene hydroperoxide (Table 2). This enhanced sensitivity was confirmed in killing assays when cultures grown under anaerobic conditions were challenged with these stress reagents (Table 2, Fig. 4d). Exposure of the *yfgF*

Fig. 4. Phenotypes of the *yfgF* mutant. (a) Sedimentation in static cultures of *E. coli* W3110 and MG1655 (Wt), and independent *E. coli* W3110 and MG1655 *yfgF* mutants (*yfgF*). Cultures were grown in L broth under anaerobic conditions at 37 °C and then left to stand at 20 °C for 24 h. Representative cultures are shown. (b) The rate of sedimentation is lower in *yfgF* mutants. Cultures were grown as described above before being transferred to anaerobic sealed cuvettes. The rates of bacterial sedimentation were measured spectrophotometrically by monitoring turbidity at 600 nm for 10 h at 30 min intervals. Line 1, MG1655 *yfgF* mutant containing pBAD/HisB plasmid; line 2, W3110 *yfgF* mutant; line 3, MG1655 *yfgF* mutant containing pGS2239 (*yfgF* expression plasmid); line 4, W3110. The plots show mean $\pm$ SD ($n=3$). (c) Representative light micrographs showing enhanced aggregation of bacterial cells in the *E. coli* W3110 (Wt) cultures compared with the *E. coli* W3110 *yfgF* mutant (*yfgF*) ($\times 100$ magnification; bar, 20 μ m). (d) Sensitivity of the *E. coli* W3110 *yfgF* mutant to peroxide stress in liquid culture. Cultures of *E. coli* W3110 (wild-type; open circles) and *E. coli* W3110 *yfgF* (*yfgF*; filled circles) were grown under anaerobic conditions to OD₆₀₀ 0.3 before exposure to *t*-butyl hydroperoxide (0.4 mM final concentration). At $t=0$, $t=20$, $t=40$ and $t=60$ min, the c.f.u. in the treated cultures was determined. The data presented are mean $\pm$ SD (triplicate assays of three independent cultures).

mutant to other stress reagents (sodium nitroprusside, S-nitrosoglutathione, hydrochloric acid and sodium hypochlorite) in disc diffusion assays revealed no differences in sensitivity compared with the parental strain (data not shown), suggesting a specific role for YfgF in the peroxide stress response.

The phenotypic tests described above are consistent with YfgF playing a role in cyclic di-GMP signalling under anaerobic conditions in *E. coli* K-12. However, attempts to complement the *E. coli* W3110 *yfgF* mutation by supplying the wild-type *yfgF* gene *in trans* on a multi-copy plasmid (pGS2239) were unsuccessful. Placing *yfgF* under the control of the pBAD promoter allowed a range of YfgF expression levels by titration of cultures with increasing amounts of arabinose (0, 0.0002, 0.002, 0.02 and 0.2 %). This showed that concentrations of arabinose of greater than 0.002 % inhibited growth, suggesting that overproduction of YfgF is toxic (data not shown). Therefore, *yfgF* expression was minimized in the complementation experiments by using arabinose at a final concentration of 0.0002 %. These

expression conditions were sufficient to complement the motility defect of an *E. coli* MG1655 *yhjH* mutant under both aerobic and anaerobic conditions (results not shown), confirming that YfgF was expressed as a functional protein. Furthermore, it was confirmed that YfgF was expressed from pGS2239 by Western blotting using the anti-Xpress antibody to probe for the Xpress epitope in the recombinant YfgF protein. Nevertheless, even this limited expression of *yfgF* from pGS2239 in either the *yfgF* mutant or the parental strain (*E. coli* W3110) resulted in phenotypes resembling those of the *yfgF* mutant (i.e. reduced sedimentation and enhanced biofilm production), and thus pGS2239 failed to complement the *yfgF* mutant.

The inability to complement the *yfgF* phenotype using the *yfgF* expression plasmid pGS2239 prompted the analysis of an independent *yfgF* mutant (*E. coli* MG1655 *yfgF*; Table 1) to confirm that the phenotypes of the *E. coli* W3110 *yfgF* mutant were caused by disruption of *yfgF*. Like the *E. coli* W3110 *yfgF* mutant, the *E. coli* MG1655 *yfgF* mutant also exhibited increased sensitivity to *t*-butyl hydroperoxide

Table 2. Sensitivity of the *E. coli yfgF* mutant to peroxides

For the disc diffusion assays, an aliquot of bacterial culture (OD₆₀₀ 0.4) was mixed with 3 ml soft-top agar (0.65 %) and poured onto L agar plates. Once the agar had set, sterile filter discs were placed in the centre of each plate and 10 µl of the indicated stress reagent (10 % solutions) was applied to the discs. The plates were incubated under anaerobic conditions for 16 h at 37 °C. Zones of inhibition were measured and the clearance areas calculated. Data are mean ± SD of three independent cultures each assayed in triplicate. For survival in liquid cultures the strains were grown to OD₆₀₀ 0.3 before exposure to the stress reagent (hydrogen peroxide, 2 mM final concentration; *t*-butyl hydroperoxide, 0.4 mM final concentration; cumene hydroperoxide, 0.2 mM final concentration). Culture viability was estimated by measuring c.f.u. remaining after 60 min exposure to the stress reagent. The data are mean ± SD for three independent cultures each assayed in triplicate.

Strain	Stress reagent		
	Hydrogen peroxide	<i>t</i> -Butyl hydroperoxide	Cumene hydroperoxide
Zone of growth inhibition (mm²)			
Parent (W3110)	304 ± 14	955 ± 78	785 ± 112
<i>yfgF</i> mutant (JRG5355)	435 ± 45	1088 ± 108	1453 ± 55
Parent (MG1655)	–	1431 ± 119	–
<i>yfgF</i> mutant (JRG6069)	–	1841 ± 228	–
Culture viability (percentage of initial c.f.u.)			
Parent (W3110)	59 ± 10	84 ± 16	94 ± 9
<i>yfgF</i> mutant (JRG5355)	0.7 ± 0	5 ± 0.1	72 ± 5

(Table 2) and decreased sedimentation (Fig. 4a), when compared with the parent *E. coli* MG1655 strain. However, in contrast to the *E. coli* W3110 *yfgF* mutant, the sedimentation phenotype of the MG1655 *yfgF* mutant was partially complemented by pGS2239 (Fig. 4b). Thus, disruption of *yfgF* in both *E. coli* W3110 and *E. coli* MG1655 resulted in the same phenotypes, suggesting that YfgF plays a role in modifying *E. coli* K-12 cell surface features and resistance to peroxide stress, but that complementation of the *yfgF* mutation *in trans* was problematic, perhaps because the activity/amount of YfgF in the cell has to be tightly regulated.

Finally, as stated above, Girgis *et al.* (2007) showed that a *yfgF* expression plasmid was able to rescue the motility defect of an *E. coli* MG1655 *yhjH* mutant, suggesting a possible role for YfgF in motility. Therefore, the motilities of *E. coli* MG1655, *E. coli* MC1000 and the corresponding *yfgF* mutants were measured under anaerobic conditions and were found to be similar for all the strains (data not shown), indicating that deletion of *yfgF* does not affect motility under these conditions.

Properties of the GGDEF (YfgF_{GE}) and EAL (YfgF_E) domains of YfgF

Attempts to measure intracellular cyclic di-GMP concentrations in extracts from parent (*E. coli* W3110) and *yfgF* mutant bacteria grown under anaerobic conditions were unsuccessful (results not shown). This suggests that cyclic di-GMP concentrations are low under anaerobic conditions or that cyclic di-GMP turnover is rapid *in vivo*, and although there are phenotypic traits associated with deletion of the *yfgF* gene

(see above), it was not possible to correlate these with changes in the concentration of intracellular cyclic di-GMP. This observation and the report that YfgF has cyclic di-GMP-independent activity (Solano *et al.*, 2009) prompted us to seek direct evidence that YfgF does indeed possess diguanylate cyclase and/or phosphodiesterase activities. Expression plasmids containing *yfgF* subgenes encoding GGDEF-EAL (YfgF_{GE}) and EAL (YfgF_E) domains were created and the corresponding proteins isolated. Diguanylate or diadenylate cyclase activity was not detected using GTP or ATP as the substrate (data not shown). This observation is consistent with the very poor conservation of key amino acids within the GGDEF domain (see above). However, both YfgF_{GE} and YfgF_E possessed cyclic di-GMP-specific phosphodiesterase activity, which resulted in the production of 5'-phosphoguanlyl-(3'-5')-guanosine (pGpG) (Fig. 5a). The pH optimum was 7.5 and activity was enhanced upon addition of either 10 mM Mg²⁺ or Mn²⁺ (Fig. 5b, columns 1–3). Activation of YfgF_E by 10 mM Mg²⁺ was much lower than that observed for YfgF_{GE} (Fig. 5b, column 2). In the presence of 10 mM Mn²⁺ (Fig. 5b, column 3) both YfgF_{GE} and YfgF_E exhibited activities that were similar to, or greater than, those reported for other cyclic di-GMP phosphodiesterases (Tanaka *et al.*, 2007). In addition, the phosphodiesterase activity of YfgF_{GE} and YfgF_E was inhibited by Ca²⁺, as has been observed for some other cyclic di-GMP phosphodiesterases (results not shown; Christen *et al.*, 2005; Tamayo *et al.*, 2005). Activity was not detected with cGMP or cAMP (results not shown), showing that YfgF is a cyclic di-GMP-specific phosphodiesterase, and prolonged incubation (up to 30 min at 37 °C) did not result in pGpG degradation.

Fig. 5. *E. coli* YfgF is a cyclic di-GMP phosphodiesterase. (a) HPLC traces of the products of reactions of YfgF_{GE} (1 μ M) and YfgF_E (1 μ M) with cyclic di-GMP (100 μ M) and Mn²⁺ (10 mM) at 37 °C. The contents of reactions are indicated next to the traces. The trace marked 'YfgF_{GE} reaction mix plus pGpG' was obtained by adding pGpG (100 μ M) to the reaction mixture after cyclic di-GMP hydrolysis was complete and shows co-elution of the reaction product and pGpG. The final trace (pGpG) shows a pGpG standard elution profile. (b) The effect of divalent cations and pGpG on YfgF phosphodiesterase activity. The reactions contained YfgF_{GE} (1 μ M, open bars) or YfgF_E (1 μ M, filled bars), cyclic di-GMP (100 μ M) and the supplements indicated. Key: 1, no addition; 2, 10 mM Mg²⁺; 3, 10 mM Mn²⁺; 4, 0.37 mM Mn²⁺; 5, 30 mM Mg²⁺; 6, 0.37 mM Mn²⁺ and 30 mM Mg²⁺; 7, 10 mM Mn²⁺ and 30 mM Mg²⁺; 8, 10 mM Mn²⁺ after pre-incubation with 100 μ M pGpG; 9, as 8, but with 10 μ M pGpG; 10, as 9, but with 1 μ M pGpG. The values shown are mean $\pm$ SD ($n=6$, duplicate assays from three different protein preparations).

In the presence of physiological concentrations of Mn²⁺ (0.37 mM; Medicis *et al.*, 1986), both YfgF_{GE} and YfgF_E phosphodiesterase activities increased approximately five-fold compared with their activities in the absence of cations (Fig. 5b, compare columns 1 and 4). In the presence of physiological concentrations of Mg²⁺ (30 mM; Medicis *et al.*, 1986) the phosphodiesterase activities of YfgF_{GE} and YfgF_E were enhanced by ~50-fold and ~10-fold, respectively (Fig. 5b, compare columns 1 and 5). Thus, YfgF_{GE} was more responsive than YfgF_E to Mg²⁺. Peroxide shock has been shown to increase intracellular Mn²⁺ concentrations (Anjem *et al.*, 2009; Kehres *et al.*, 2000), and hence the phosphodiesterase activities of YfgF_{GE} and YfgF_E in the presence of physiological concentrations of Mg²⁺ (30 mM) plus low (0.37 mM) or elevated (10 mM) concentrations of Mn²⁺ were determined (Fig. 5b, columns 6 and 7). Under these conditions the activity of YfgF_{GE} was approximately twofold greater than that of YfgF_E (Fig. 5b, columns 6 and 7). However, in the presence of 10 mM Mn²⁺ alone there was no difference between the activities of YfgF_{GE} and YfgF_E (Fig. 5b, column 3). Thus, one role of the GGDEF domain appears to be to facilitate Mg²⁺-dependent YfgF phosphodiesterase activity, whilst still allowing YfgF activity to respond to enhanced Mn²⁺ concentrations.

Catalytically inactive GGDEF domains, such as that present in YfgF, have previously been shown to activate directly linked EAL domains by allosterically binding GTP (Christen *et al.*, 2005). Pre-incubating YfgF_{GE} and YfgF_E with guanine nucleotides (10–100 μ M GTP, GDP or GMP) did not affect cyclic di-GMP phosphodiesterase activity (data not shown). However, the phosphodiesterase activity of YfgF_{GE} and YfgF_E was severely inhibited following pre-incubation of the proteins with 100 μ M pGpG, but not by 10 or 1 μ M pGpG, before addition of 100 μ M cyclic di-GMP in the presence of 10 mM Mn²⁺ (Fig. 5b, compare columns 3 and 8), implying that pGpG is an effective inhibitor of YfgF phosphodiesterase activity only at high concentrations.

Oligomeric state of YfgF_{GE} and YfgF_E

Active GGDEF domains must dimerize to act as diguanylate cyclases (Chan *et al.*, 2004; Christen *et al.*, 2006), whereas monomeric EAL domains can be active as phosphodiesterases (Schmidt *et al.*, 2005). Gel filtration and chemical cross-linking showed that YfgF_{GE} was a dimer (M_r ~115 000), whereas YfgF_E was monomeric, M_r ~60 000 (Fig. 6). Thus it was concluded that YfgF_{GE} retains

Fig. 6. Oligomeric states of $YfgF_{GE}$ and $YfgF_E$. (a) Size-exclusion chromatography of $YfgF_{GE}$ and $YfgF_E$. The gel-filtration column was calibrated using the standards listed in Methods. $YfgF_{GE}$ eluted at 12.8 ml, equivalent to a molecular mass of 115 kDa (monomer size 67 kDa); $YfgF_E$ eluted at 13.8 ml, equivalent to a molecular mass of 65 kDa (monomer size 48 kDa). (b) Chemical cross-linking of $YfgF_{GE}$ and $YfgF_E$. $YfgF_{GE}$ (25 μ g) or $YfgF_E$ (25 μ g) was incubated with 10 mM Mn^{2+} and 0.01 % glutaraldehyde for 15 min. After quenching the reaction, samples were separated by SDS-PAGE and stained with Coomassie blue. Lanes: 1, protein standards (sizes are indicated in kDa); 2, $YfgF_{GE}$ without glutaraldehyde; 3, $YfgF_{GE}$ with glutaraldehyde; 4, $YfgF_E$ without glutaraldehyde; 5, $YfgF_E$ with glutaraldehyde. The locations of the $YfgF_{GE}$ monomer, $YfgF_{GE}$ dimer and $YfgF_E$ monomer are shown. The dashed arrow indicates the expected location of a $YfgF_E$ dimer.

the ability to dimerize *in vitro*. However, the role played by GGDEF domain-mediated dimerization of $YfgF$ is currently unknown, although, as shown above, the GGDEF domain is important for the ability of $YfgF$ phosphodiesterase activity to respond to Mg^{2+} in the presence of various concentrations of Mn^{2+} , and so this response may be linked to the ability to form $YfgF$ dimers.

DISCUSSION

Cyclic di-GMP is a widely used second messenger in bacteria, where it controls a range of functions, including biofilm formation, aggregation, adhesion, motility and virulence (Ryan *et al.*, 2006). Generally, proteins with GGDEF domains are diguanylate cyclases and are responsible for cyclic di-GMP synthesis (Ryjenkov *et al.*, 2005), and proteins with EAL domains are generally phosphodiesterases and are responsible for cyclic di-GMP degradation (Schmidt *et al.*, 2005). Here it is shown that *E. coli* K-12 $YfgF$, which possesses MASE1, GGDEF and EAL

domains, is a cyclic di-GMP-specific phosphodiesterase. The cyclic di-GMP phosphodiesterase activity is stimulated by Mg^{2+} and Mn^{2+} , but is inhibited by the product of the reaction, pGpG. The direct demonstration of $YfgF$ cyclic di-GMP phosphodiesterase activity, alongside the work of Solano *et al.* (2009), which showed that *Salmonella* $YfgF$, which is 73 % identical and 87 % similar over 733 amino acids, restores motility to a cyclic di-GMP-free *Salmonella* strain, suggests that $YfgF$ has both cyclic di-GMP-dependent and -independent activities.

Although the $YfgF$ GGDEF domain lacks diguanylate cyclase activity, it is responsible for dimerization of $YfgF$ and enhanced cyclic di-GMP phosphodiesterase activity in the presence of physiological concentrations of Mg^{2+} . The ability of a GGDEF domain to influence the activity of a linked EAL by modulating its responsiveness to cations has not been previously reported. However, complex responses to cations have been observed before; for example, the *Vibrio cholerae* cyclic di-GMP phosphodiesterase VieA is activated by both Mn^{2+} and Mg^{2+} separately, but is inhibited by the presence of the two cations together (Tamayo *et al.*, 2005). Intriguingly, it is known that exposure to peroxide induces Mn^{2+} uptake (Anjem *et al.*, 2009; Kehres *et al.*, 2000), and the sensitivity of the *yfgF* mutant to peroxide stress might be partially accounted for by the absence of Mn^{2+} -stimulated $YfgF$ phosphodiesterase activity in this strain. The finding that *yfgF*, a gene that is expressed under anaerobic conditions, is involved in the oxidative stress response is somewhat counterintuitive. However, $YfgF$ might be present under anaerobic conditions to manage the peroxide shock associated with anaerobic to aerobic transitions (Partridge *et al.*, 2006). In this case, peroxide-induced Mn^{2+} accumulation would enhance the phosphodiesterase activity of $YfgF$ that was synthesized under anaerobic conditions.

Cyclic di-GMP signalling networks in bacteria are complex (Pesavento & Hengge, 2009). One way of reducing this complexity is the temporal separation that might be achieved by differential regulation of transcription. Sommerfeldt *et al.* (2009) investigated the expression profile of 12 GGDEF, nine EAL and seven GGDEF and EAL domain-encoding genes in *E. coli* K-12 under aerobic conditions. The aerobically expressed genes could be classified into different groups based on whether they were predominantly expressed in the exponential or stationary phase of the growth cycle and whether expression was greatest at 37 or 28 °C. Only two putative diguanylate cyclases (YliF and YneF) and three putative cyclic di-GMP phosphodiesterases ($YfgF$, YcgG and YliE) exhibited no expression under aerobic conditions. It is shown here that *yfgF* is expressed under anaerobic conditions and that the growth phase- and temperature-responsive expression pattern of *yfgF* differs from the aerobic expression patterns of other GGDEF/EAL-encoding genes reported by Sommerfeldt *et al.* (2009) in that *yfgF* is predominantly, but not exclusively, expressed in stationary phase at 28 °C. The FNR-dependent regulation of *yfgF* expression under anaerobic conditions thus isolates $YfgF$ activity from that of other

E. coli phosphodiesterases, with the possible exception of the EAL domain protein YjcC, which has a plausible FNR site (TaGAannnnATCAA; matches to the consensus in upper-case type) centred 80 bp upstream of the *yjcC* start codon, and directly links cyclic di-GMP metabolism to the oxygen-sensing FNR family of transcription factors. However, oxygen sensing and cyclic di-GMP signalling have already been linked through the *E. coli* DosCP system (Tuckerman *et al.*, 2009). Both DosC (diguanylate cyclase) and DosP (phosphodiesterase) possess oxygen-binding haem groups and it has been shown that oxygen binding to reduced haem of isolated DosP enhances phosphodiesterase activity (Chang *et al.*, 2001; Delgado-Nixon *et al.*, 2000; Sasakura *et al.*, 2002; Tanaka *et al.*, 2007). Thus, *E. coli* K-12 has at least two phosphodiesterases that respond to oxygen availability, the first (DosP) responds to oxygen directly and is active under aerobic conditions, the second (YfgF) responds to oxygen indirectly, via FNR-mediated transcription regulation, and is active under anaerobic conditions. Furthermore, the diguanylate cyclase activity of DosC is dependent on the presence of DosP (Tuckerman *et al.*, 2009). Thus, the product of the DosCP complex is pGpG, which has been shown here to be a strong inhibitor, and therefore a potential regulator, of YfgF activity.

Disruption of the *E. coli* K-12 *yfgF* gene causes phenotypic changes that overlap those previously associated with dysregulation of cyclic di-GMP signalling pathways. However, attempts to complement the W3110 *yfgF* mutant were frustrated by finding that overproduction of YfgF, even in the parental strain, resulted in a *yfgF* mutant phenotype. Similar observations have been reported for a *Pseudomonas aeruginosa* PA2567 mutant (Kulasakara *et al.*, 2006). PA2567 encodes an EAL domain protein and both the mutant and the overproducing strain exhibited decreased biofilm formation. However, it was possible to partially complement the sedimentation phenotype of the MG1655 *yfgF* mutant (Fig. 4b). *E. coli* W3110 and MG1655 are closely related and the behaviour of the *yfgF* mutants of these two strains suggests that removing or overproducing components of the cyclic di-GMP signalling network can have complex and even counterintuitive outcomes, and hence that the activity of YfgF is tightly regulated.

In conclusion, this work shows that *E. coli* K-12 possesses a gene (*yfgF*) that is expressed under anaerobic conditions from a class II FNR-dependent promoter and encodes a cyclic di-GMP phosphodiesterase. The product of the reaction, pGpG, inhibits the cyclic di-GMP phosphodiesterase activity of YfgF *in vitro*, and thus pGpG might play a regulatory role in cyclic di-GMP signalling pathways. Phenotypic studies show that YfgF acts in remodelling the cell envelope and in responding to stress when anaerobic cultures are exposed to peroxide. Thus, this work has established YfgF as a link between three major global regulatory networks, the oxygen responsive FNR regulon, the oxidative stress response and cyclic di-GMP signalling, with implications for understanding the mechanisms that co-ordinate the response to changes in oxygen availability in *E. coli* K-12.

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