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 (YfgFGE) and EAL (YfgFE) domains possessed cyclic di-GMP-specific phosphodiesterase activity, but lacked diguanylate cyclase activity. However, the catalytically inactive GGDEF domain was required for YfgFGE dimerization and enhanced cyclic di-GMP phosphodiesterase activity in the presence of physiological concentrations of Mg2+. The cyclic di-GMP phosphodiesterase activity of YfgFGE and YfgFE 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.

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; Unden 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 is observed 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 TTGATNNNATC (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 adenyl cyclase (Pei & Grishin, 2001) and was first recognized in the PleD protein of Caulobacter crescentus (Hecht & Newton, 1995). PleD 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 fulfill 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 PeD 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, cellulosyl 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 (Sonhammer et al., 1998), all of which produced similar predicted topologies. The one shown is from TMHMM.](image-url)
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 pR25 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 P1vir-mediated transduction (Sambrook & Russell, 2001).

Site-directed mutagenesis of the consensus FNR site (TTGATATAATCATCAA 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 pGSS2239 (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 YfgFGE and YfgFE 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 YfgFGE construct encoded amino acids 319–747; the YfgFE construct encoded amino acids 481–747.

Electrophoretic mobility shift assays (EMSA). 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 (Te6) (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 (Cm²). 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 P1vir-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 r-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 work

Ap<sup>R</sup>, ampicillin resistant; Cm<sup>R</sup>, chloramphenicol resistant; Km<sup>R</sup>, kanamycin resistant; Tet<sup>R</sup>, tetracycline resistant.

<table>
<thead>
<tr>
<th>Strain, plasmid or oligonucleotide</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli strains</strong></td>
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<td>W3110</td>
<td>Prototroph</td>
<td>Laboratory collection</td>
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<td>MG1655</td>
<td>Prototroph</td>
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<td>MG1655 yfgF (JRG6069)</td>
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<td>W3110 yfgF mutant; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Source of cat cassette for linear transformation; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pGS1633</td>
<td>PygfI–lacZ fusion in pRS415 (192 bp promoter fragment, −108 to +84 relative to the transcript start); Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Partridge et al. (2007a)</td>
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<td>pRS415 lacyZ-based promoter-fusion vector; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Simons et al. (1987)</td>
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<td>pET32a Expression vector; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>JP13 RACE PCR DNA amplification primer: CCACACCCGGAAGAATATGCG</td>
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<td>JP14 RACE PCR DNA amplification primer: GAAAGCGGAAGCCCGCACCC</td>
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<td>MMW4 Forward primer with engineered EcoRI site for creating a yfgF low-copy-number plasmid: TTTGGAATTCTGTAATAATGATGAATTAACATG</td>
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<td>MMW46 Reverse primer with engineered HindIII site for creating a yfgF low-copy-number plasmid: TTTTACGATTTTGCAGGCAATGGGCTTGC</td>
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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 OD$_{600}$ 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 OD$_{600}$. 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 OD$_{600}$. Adhesion units were calculated by dividing the OD$_{600}$ of crystal violet-stained adhered cells by the OD$_{600}$ for the planktonic cells from the same cultures.

Motility assays were done using motility broth (5 g tryptone 1−1 and 5 g NaCl 1−1) and agar (5 g tryptone 1−1, 5 g NaCl 1−1 and 3 g agar 1−1). 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 benzanilide]. 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 18 000 g for 15 min. The supernatant was transferred to a clean Eppendorf tube and desiccated. 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$_{E_{	ext{c}}}$ and YfgF$_{E_{	ext{c}}}$ 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 (TTGATNNNNATCAA) (Eiglmeyer 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 was added (pGS1830) (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 %
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.
Differential regulation of \textit{yfgF} during the \textit{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 \textit{yfgF} was measured over the course of the anaerobic \textit{E. coli} K-12 growth cycle at 37 and 28 °C using MC1000 (lac) carrying a single-copy chromosomal \textit{yfgF–lacZ} fusion. Aerobic and anaerobic expression of \textit{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 \textit{yfgF–lacZ} expression in anaerobic cultures of an \textit{rpoS} (stationary phase alternative sigma factor) mutant suggested that the enhanced stationary phase expression of \textit{yfgF} was not dependent on RpoS (results not shown). In an \textit{fnr} mutant, anaerobic \textit{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 \textit{fnr} mutant under anaerobic conditions causing relatively high \textit{yfgF} expression during exponential phase. In addition to the increase in \textit{yfgF} expression seen in stationary phase, \textit{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 \textit{yfgF} expression: (i) FNR-dependent anaerobic activation and (ii) activation at lower growth rates.

Properties of \textit{E. coli} K-12 \textit{yfgF} mutants

The primary structure of YfgF and the regulation of \textit{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 \textit{E. coli} W3110 \textit{yfgF} mutant and 0.13 ± 0.043 adhesion units for the parent) indicated that biofilm formation was enhanced for the \textit{yfgF} mutant. However, the \textit{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 \textit{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% ± 1.82; \textit{yfgF} 86.6% ± 5.85; \textit{n} = 9), and stationary phase survival was unaffected by mutation of \textit{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 \textit{E. coli} W3110 \textit{yfgF} mutant to exposure to a range of stress reagents in disc diffusion assays indicated that the \textit{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 \textit{yfgF}
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 yjhH 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-butyll hydperoxide...
Table 2. Sensitivity of the E. coli yfgF mutant to peroxides

For the disc diffusion assays, an aliquot of bacterial culture (OD$_{600}$ 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 OD$_{600}$ 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Stress reagent</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Zone of growth inhibition (mm$^2$)</td>
<td></td>
</tr>
<tr>
<td>Parent (W3110)</td>
<td>304 ± 14</td>
</tr>
<tr>
<td>yfgF mutant (JRG5355)</td>
<td>435 ± 45</td>
</tr>
<tr>
<td>Parent (MG1655)</td>
<td>–</td>
</tr>
<tr>
<td>yfgF mutant (JRG6069)</td>
<td>–</td>
</tr>
<tr>
<td>Culture viability (percentage of initial c.f.u.)</td>
<td></td>
</tr>
<tr>
<td>Parent (W3110)</td>
<td>59 ± 10</td>
</tr>
<tr>
<td>yfgF mutant (JRG5355)</td>
<td>0.7 ± 0</td>
</tr>
</tbody>
</table>

(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 ygjH mutant, suggesting a possible role for YfgF in motility. Therefore, the motilities of E. coli MG1655, E. coli MG1655 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’-phosphoguanosine-3’,5’-diphosphate (pGpG) (Fig. 5a). The pH optimum was 7.5 and activity was enhanced upon addition of either 10 mM Mg$^{2+}$ or Mn$^{2+}$ (Fig. 5b, columns 1–3). Activation of YfgF$_{GE}$ by 10 mM Mg$^{2+}$ was much lower than that observed for YfgF$_{GE}$ (Fig. 5b, column 2). In the presence of 10 mM Mn$^{2+}$ (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 Ca$^{2+}$, 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.
In the presence of physiological concentrations of Mn$^{2+}$ (0.37 mM; Medicis et al., 1986), both YfgFGE and YfgFE 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 Mg$^{2+}$ (30 mM; Medicis et al., 1986) the phosphodiesterase activities of YfgFGE and YfgFE were enhanced by ~50-fold and ~10-fold, respectively (Fig. 5b, compare columns 1 and 5). Thus, YfgFGE was more responsive than YfgFE to Mg$^{2+}$. Peroxide shock has been shown to increase intracellular Mn$^{2+}$ concentrations (Anjem et al., 2009; Kehres et al., 2000), and hence the phosphodiesterase activities of YfgFGE and YfgFE in the presence of physiological concentrations of Mg$^{2+}$ (30 mM) plus low (0.37 mM) or elevated (10 mM) concentrations of Mn$^{2+}$ were determined (Fig. 5b, columns 6 and 7). Under these conditions the activity of YfgFGE was approximately twofold greater than that of YfgFE (Fig. 5b, columns 6 and 7). However, in the presence of 10 mM Mn$^{2+}$ alone there was no difference between the activities of YfgFGE and YfgFE (Fig. 5b, column 3). Thus, one role of the GGDEF domain appears to be to facilitate Mg$^{2+}$-dependent YfgF phosphodiesterase activity, whilst still allowing YfgF activity to respond to enhanced Mn$^{2+}$ 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 YfgFGE and YfgFE with guanine nucleotides (10–100 μM GTP, GDP or GMP) did not affect cyclic di-GMP phosphodiesterase activity (data not shown). However, the phosphodiesterase activity of YfgFGE and YfgFE was severely inhibited following pre-incubation of the proteins with 100 μM pGpG, but not by 10 or 1 μM pGpG, before addition of 100 μM cyclic di-GMP in the presence of 10 mM Mn$^{2+}$ (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 YfgFGE and YfgFE**

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 YfgFGE was a dimer ($M_r \approx 115\,000$), whereas YfgFE was monomeric, $M_r \approx 60\,000$ (Fig. 6). Thus it was concluded that YfgFGE retains...
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/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

**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
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 promotor 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.

ACKNOWLEDGEMENTS

The authors thank R. E. Roberts for technical assistance, A. J. G. Moir for assistance with HPLC, S. Gooch for contributions made during an undergraduate laboratory project, and the Biotechnology and Biological Sciences Research Council (UK) for supporting this work.

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Characterization of E. coli yfgF


Edited by: G. H. Thomas