doi:10.1016/j.jmb.2009.03.015 J. Mol. Biol. (2009) 388, 262–270

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Globins Synthesize the Second Messenger Bis-(3′–5′)-Cyclic Diguanosine Monophosphate in Bacteria

Xuehua Wan[1], Jason R. Tuckerman[2], Jennifer A. Saito[1], Tracey Allen K. Freitas[1], James S. Newhouse[3], Judith R. Denery[4], Michael Y. Galperin[5], Gonzalo Gonzalez[2], Marie-Alda Gilles-Gonzalez[2] and Maqsudul Alam[1,3,6*]

[1]*Department of Microbiology, University of Hawaii, 2538 McCarthy Mall, Snyder Hall 207, Honolulu, HI 96822, USA*

[2]*Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA*

[3]*Maui High Performance Computing Center, 550 Lipoa Parkway, Kihei, Maui, HI 96753, USA*

[4]*Department of Molecular Biosciences and Bioengineering, University of Hawaii, 1955 East–West Road, Honolulu, HI 96822, USA*

[5]*National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, MSC 3830, 8600 Rockville Pike, Bethesda, MD 20894, USA*

[6]*Advanced Studies in Genomics, Proteomics, and Bioinformatics, University of Hawaii, 2565 McCarthy Mall, Honolulu, HI 96822, USA*

Globin-coupled sensors are heme-binding signal transducers in Bacteria and Archaea in which an N-terminal globin controls the activity of a variable C-terminal domain. Here, we report that *Bpe*GReg, a globin-coupled diguanylate cyclase from the whooping cough pathogen *Bordetella pertussis*, synthesizes the second messenger bis-(3′–5′)-cyclic diguanosine monophosphate (c-di-GMP) upon oxygen binding. Expression of *Bpe*GReg in *Salmonella typhimurium* enhances biofilm formation, while knockout of the *Bpe*GReg gene of *B. pertussis* results in decreased biofilm formation. These results represent the first identification a signal ligand for any diguanylate cyclase and provide definitive experimental evidence that a globin-coupled sensor regulates c-di-GMP synthesis and biofilm formation. We propose that the synthesis of c-di-GMP by globin sensors is a widespread phenomenon in bacteria.

*Corresponding author. E-mail address: alam@hawaii.edu.

Present address: J. R. Denery, Department of Chemistry and Worm Institute for Research and Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

Abbreviations used: *Bpe*GReg, *Bordetella pertussis* globin-coupled regulator; c-di-GMP, bis-(3′–5′)-cyclic diguanosine monophosphate; DGC, diguanylate cyclase; EPS, exopolysaccharide; GCDC, globin-coupled diguanylate cyclase; LC, liquid chromatography; MS, mass spectrometry; TLC, thin-layer chromatography; PDE, phosphodiesterase; rdar, red, dry, and rough; SEM, scanning electron microscopy; *Av*GReg, *A. vinelandii* globin-coupled regulator; *Cv*GReg, *C. violaceum* globin-coupled regulator; HPLC, high-performance liquid chromatography.

Received 20 January 2009;
received in revised form
28 February 2009;
accepted 9 March 2009
Available online
13 March 2009

Edited by J. Karn

Keywords: globin; oxygen sensor; c-di-GMP; diguanylate cyclase; biofilm

Introduction

Globins are heme-containing proteins found in all three domains of life—Eukarya, Archaea, and Bacteria.[1] The discovery and examination of microbial globins showed that globins participate in many more roles than O_2 metabolism in cells, including sensing of gaseous ligands. The first globin-coupled sensors discovered were the HemAT aerotactic transducers that couple a globin-based O_2-sensing domain to a chemotaxis methyl-carrier protein domain.[2–4] The globin-coupled sensor proteins form a broad family of sensors with variable transmitter modules, including the GGDEF domain.[3,4] GGDEF domains are widespread in bacteria and possess diguanylate cyclase (DGC) activity.[5–10] DGCs synthesize bis-(3′–5′)-cyclic diguanosine monophosphate (c-di-GMP), a global second messenger implicated in regulating bacterial motility, exopolysaccharide (EPS) production, and biofilm formation at both translational and post-translational levels.[8,11–14] Given that biofilm formation is important for virulence of many bacterial pathogens,[9,15–17] including *Bordetella pertussis*,[18] these data suggest a role of O_2 sensing in regulating pathogenicity.

Results and Discussion

Our *in silico* analysis of microbial genome sequences identified *Bpe*GReg (*B. pertussis g*lobin-coupled *reg*ulator) from the whooping cough pathogen *B. pertussis* as a potential globin-coupled DGC (GCDC) (Fig. S1). The conserved domain search initially identified two domains in *Bpe*GReg (475 residues): a globin domain (residues 1–155) and a GGDEF domain (residues 297–475) (Fig. 1a). A hybrid threading/homology modeling technique identified the highest scoring template for the region spanning residues 167 to 289 as the receiver domain of response regulator PhoB from *Escherichia coli*.[19] Thus, *Bpe*GReg consists of three domains: an N-terminal globin domain, a middle domain, and a C-terminal DGC domain. We created a homology model of *Bpe*GReg based on the crystal structures of HemAT-*Bs* from *Bacillus subtilis* and PleD from *Caulobacter crescentus* (Fig. 1a). In this model, *Bpe*GReg is a dimer, with an active site in each monomer for binding to one GTP molecule and an inhibitory site in each monomer[20,21] for binding to a pair of intercalated c-di-GMP molecules. We therefore postulated that *Bpe*GReg is a heme-binding protein that can synthesize c-di-GMP and influence biofilm formation based on O_2 tension.

To characterize *Bpe*GReg, we purified recombinant His-tagged *Bpe*GReg by metal-affinity chromatography. The recombinant protein had the expected molecular mass of 53.7 kDa (Fig. 1b) and showed characteristic heme protein absorption spectra in the near-UV and visible regions (Fig. 1c–e and Fig. S2a and b). Addition of ligands to the ferrous *Bpe*GReg caused the Soret absorption band to shift from 431 nm (deoxy, unliganded Fe^{II} state) to 416 nm for the O_2-bound state, 422 nm for the carbon monoxide-bound state, and 420 nm for the nitric oxide-bound state. We further measured the O_2, CO, and NO binding affinities and kinetics of *Bpe*GReg (Table 1). *Bpe*GReg binds O_2 and CO ($K_d = 0.64$ and 0.055 μM, respectively) with affinities comparable with those of sperm whale myoglobin (Table 1). The association rate constant for NO binding to *Bpe*GReg was 16 $\mu M^{-1}\ s^{-1}$, a value similar to that reported for sperm whale myoglobin (Table 1). We concluded that *Bpe*GReg is a heme-binding protein.

To test whether *Bpe*GReg functions as a globin-regulated DGC, we measured the conversion of GTP to c-di-GMP using two independent methods: reversed-phase liquid chromatography (LC) coupled with mass spectrometry (MS) and a thin-layer chromatography (TLC) radioactive assay using [α-^{32}P]GTP as the substrate. The data showed that ferrous *Bpe*GReg can synthesize c-di-GMP from GTP (Fig. 2). We then measured the production of c-di-GMP by different states of *Bpe*GReg, unliganded and liganded. The ferrous O_2-bound form of *Bpe*GReg produced the highest amount of c-di-GMP per mole of protein compared with the unliganded, CO-bound, and NO-bound forms (Fig. 2c). We concluded that *Bpe*GReg is an O_2-switched DGC that cycles between a low-activity unliganded ferrous state and a highly active O_2-bound state.

Our *Bpe*GReg model predicted that c-di-GMP can bind to inhibitory sites, causing feedback inhibition of the enzyme activity as initially noted for PleD.[20] To test the feedback inhibition of *Bpe*GReg, we coupled the cyclase reaction with a phosphodiesterase (PDE) that continuously linearized the cyclic nucleotide product to pGpG. Comparison of the reaction with and that without PDE clearly indicated product inhibition (Fig. 3a). The reaction quickly slowed without removal of the c-di-GMP product and stopped long before the GTP substrate was exhausted. By contrast, the reaction was linear and proceeded to completion when it was coupled to PDE (Fig. 3a and d). Furthermore, the addition of 20 μM c-di-GMP at the start of a *Bpe*GReg-catalyzed reaction strongly inhibited the reaction (Fig. 3b). The use of a

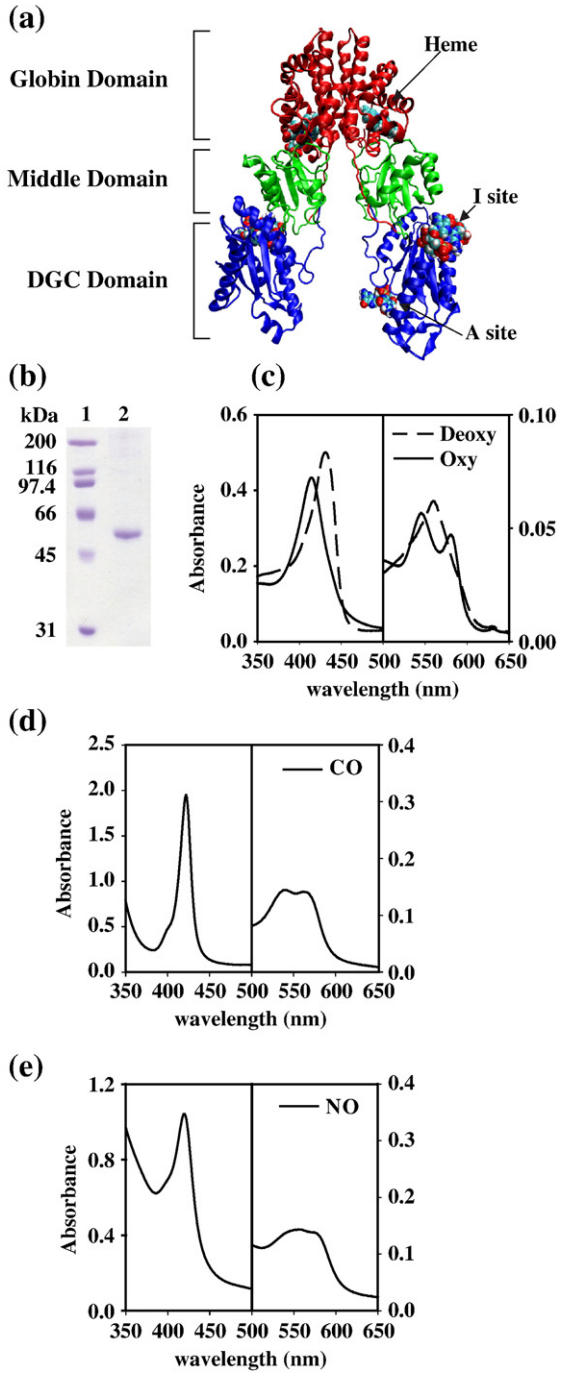

Fig. 1. Identification of *Bpe*GReg from *B. pertussis* as a globin. (a) Model of a possible complete *Bpe*GReg dimer in ribbon representation with globin (red), middle (green), and DGC (blue) domains. (b) Coomassie-stained SDS-PAGE of purified *Bpe*GReg. Lane 1, molecular weight markers; lane 2, $BpeGReg_{6\times\text{-His}}$. (c–e) Absorption spectra of *Bpe*GReg in ferrous state and bound to various ligands. All measurements were done at 25 °C and pH 7.5 in 100 mM sodium phosphate buffers.

coupled assay allowed us to measure the reaction rates of the liganded and unliganded forms of *Bpe*GReg without the complications of feedback inhibition. Thus, we determined that the O_2-bound *Bpe*GReg produces c-di-GMP at an initial rate of 2.5 min^{-1}, a rate 10 times faster than that of the unliganded ferrous form (Fig. 3c–f). In conclusion, O_2 enhances the DGC activity of *Bpe*GReg, but accumulation of the c-di-GMP product inhibits it.

It has been shown that in motile bacterial cells, high levels of c-di-GMP suppress motility in favor of EPS production and biofilm formation.[8,25] *Salmonella typhimurium* strain ATCC 14028 produces an rdar (red, dry, and rough) colony morphology on Congo red agar (as indicators of cellulose or biofilm formation) at 28 °C but not at 37 °C.[26] However, when the DGC AdrA is overexpressed in this strain at 37 °C (leading to high levels of c-di-GMP), the temperature regulation is overcome and the rdar morphotype develops.[25,27] Accordingly, we tested whether the expression of *Bpe*GReg in *S. typhimurium* at 37 °C would affect motility, EPS production, and biofilm formation by this bacterium. We found that in cells expressing *Bpe*GReg, motility was suppressed (Fig. 4a) and the rdar morphotype was observed (Fig. 4b). Cells carrying only the vector did not develop the rdar morphotype (Fig. 4b). In parallel, we examined the colonies by scanning electron microscopy (SEM). The SEM micrographs showed that the cells expressing *Bpe*GReg produced layers of biofilm in contrast to the control strain (Fig. 4c). An alternative biofilm assay (liquid culture in glass tubes) showed that *Bpe*GReg also enhances biofilm formation in liquid culture (Fig. 4d). These physiological data demonstrate that *Bpe*GReg, when expressed in *S. typhimurium*, inhibits motility and enhances EPS production and biofilm formation.

We next investigated the function of the middle domain (Fig. 1a). Bioinformatic analysis of this domain identified one highly conserved residue, His225 (Fig. S1). Mutation of His225 to alanine resulted in a protein with normal globin absorption spectra (Fig. S4). Neither the unliganded state nor the O_2-bound state produced any detectable c-di-GMP, however. In addition, physiological assays showed failure of the *Bpe*GReg H225A mutant to confer an rdar morphotype or support biofilm formation (Fig. 4a–d). These data suggest that the middle domain might be required for proper folding of the enzymatic domain but not the heme-binding domain.

To examine whether *Bpe*GReg is involved in biofilm formation in its native *B. pertussis* host, we constructed a *bpeGReg* knockout mutant of *B. pertussis* strain ATCC 9340. A transcriptional fusion suicide vector pFUS2[28] was used to inactivate the target *bpeGReg* gene. Compared with wild-type *B. pertussis* 9340, the knockout strain formed less biofilm (Fig. 4e). The residual biofilm formation could be due to the fact that, besides *Bpe*GReg, the *B. pertussis* genome encodes four other predicted DGCs and four c-di-GMP PDEs.[7] These proteins likely respond to physiological signals other than O_2 and probably modulate a variety of processes via the second messenger c-di-GMP, including biofilm and virulence factor production.

It has been reported that *B. pertussis* does not grow anaerobically but will grow in O_2 tensions as low as 6% of atmospheric O_2.[29] Thus, the lowest concentration of dissolved O_2 to allow growth, about 78 μM,

Table 1. Ligand-binding parameters of *Bpe*GReg compared with HemAT-*Bs* and sperm whale myoglobin

	O_2			CO			NO
	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)	k_{on} (μM^{-1} s^{-1})
*Bpe*GReg	7.0	4.5	0.64	1.03	0.056	0.055	16
				0.12		0.45	
HemAT-*Bs*[a]	19	1900	100	0.34	0.067	0.20	
		87	4.6				
SWMb[b]	17	15	0.88	0.51	0.019	0.037	22

Binding parameters for *Bpe*GReg were measured at 25 °C in 100 mM NaPi, pH 7.5. The association of CO to *Bpe*GReg was bimodal and manifested two on-rate constants.

[a] HemAT-*Bs* data are from Zhang *et al*.[22] The dissociation of O_2 from HemAT-*Bs* was bimodal and manifested two rate constants.

[b] Sperm whale myoglobin (SWMb) O_2 and CO binding data are from Springer *et al*.;[23] NO binding data are from Brucker *et al*.[24]

would be expected to keep *Bpe*GReg ($K_d = 0.64$ μM) in an O_2-bound active state. Considering that *B. pertussis* colonizes the upper respiratory tract, *Bpe*GReg could serve as a key O_2 sensor for directing *B. pertussis* to colonize or not.

We provided experimental evidence that a globin-based O_2 sensor regulates DGC activity and controls bacterial biofilm formation. Our working hypothesis for *Bpe*GReg is that O_2 binding to the globin domain will reorient the *Bpe*GReg dimer so as to enhance *Bpe*GReg activity. Alternatively, it is also possible that O_2 binding to the globin domain of *Bpe*GReg will enhance its dimerization to an active enzyme, analogously to the phosphorylation-dependent dimerization needed for the activation of PleD.[30] GCDCs are found in a variety of bacteria besides *B. pertussis*, including several other pathogens and a number of free-living soil and marine organisms (Fig. S1). Analysis of GCDCs from *Azotobacter vinelandii* and *Chromobacterium violaceum* shows that, like *Bpe*GReg, they also regulate biofilm formation and motility (Fig. S5), indicating that O_2 sensing by globins to enhance biofilm production is a widespread phenomenon that was previously overlooked.

Materials and Methods

Sequence alignment and homology modeling

The multiple sequence alignment of selected GCDCs was created in ClustalX and manually refined in DNAStar before final presentation using Adobe Illustrator. Homology modeling was performed using the Prime package (Schrödinger). A threaded model of the middle domain (residues 156–266) of *Bpe*GReg was produced using Threader 3.5 from University College London. The alignment of the middle domain with Protein Data Bank entry 1b00 was modeled using MODELLER Release 8v2 from the Andrej Sali laboratory at the University of California, San Francisco (San Francisco, CA). Analyses of hydrophobicity and hydrophilicity in the active and inhibitory sites in the GGDEF domain were calculated and illustrated with Site Map 2.0 (Schrödinger).

Bacterial strains and culture conditions

E. coli TOP10 cells (Invitrogen) were used for routine cloning. Rosetta2(DE3)pLysS cells (Novagen) were used for expression of the His-tagged proteins. *S. typhimurium* ATCC 14028, donated by Dr. Susan Ayin (University of Hawaii, Manoa, HI), was used for physiological studies. *S. typhimurium* was cultured in LB medium without salt. *B. pertussis* ATCC 9340 was grown at 37 °C on Bordet–Gengou agar plates with 15% defibrinated sheep blood or Stainer–Scholte broth supplemented with heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (Sigma). When appropriate, the antibiotics used were ampicillin (100 μg/ml), chloramphenicol (34 μg/ml), and gentamycin (20 μg/ml). Genomic DNAs from *B. pertussis* ATCC 9797D, *A. vinelandii* ATCC 12518, and *C. violaceum* ATCC 12472 were purchased from American Type Culture Collection. Genomic DNA from *B. pertussis* 9340 was extracted using a GNOME DNA Isolation Kit (QBiogene).

Plasmid construction

For protein expression and purification, *Bpe*GReg (GenBank accession no. NP_882025) was engineered with an N-terminal hexahistidine tag by PCR (primers listed in Table S1). The PCR product was cloned into the pCR4Blunt-TOPO vector (Invitrogen) and subcloned into the pET-3a expression vector (Novagen). *A. vinelandii* globin-coupled regulator (*Av*GReg; GenBank accession no. ZP_00415257) and *C. violaceum* globin-coupled regulator (*Cv*GReg; GenBank accession no. NP_899909) were engineered with C-terminal hexahistidine tags and cloned into pET-25b and pET-27b, respectively. For expression in *S. typhimurium*, non-tagged versions of *Bpe*GReg, *Av*GReg, and *Cv*GReg were cloned into the pTrc99A vector (Table S1). The QuikChange Site-Directed Mutagenesis protocol (Stratagene) was used to construct the H225A mutant (Table S1).

A 600-bp region (nucleotides 289–888) of the *bpeGReg* gene was amplified by PCR (Table S1) and cloned into the suicide vector pFUS2 (provided by Dr. Camille Locht) to inactivate *Bpe*GReg in *B. pertussis* 9340. Gene inactivation was performed by one-step homologous recombination[28] and confirmed by PCR.

Expression and purification

His-tagged *Bpe*GReg, *Av*GReg, and *Cv*GReg were overexpressed in *E. coli* Rosetta2(DE3)pLysS cells induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside at room temperature for 6–8 h and purified by Co^{2+}-affinity chromatography according to Piatibratov *et al*.[31] Purified proteins were analyzed by SDS-PAGE, and the concentration was determined by the Bradford protein assay (BioRad) using a bovine serum albumin standard.[32] The purified proteins were stored at −70 °C until their use.

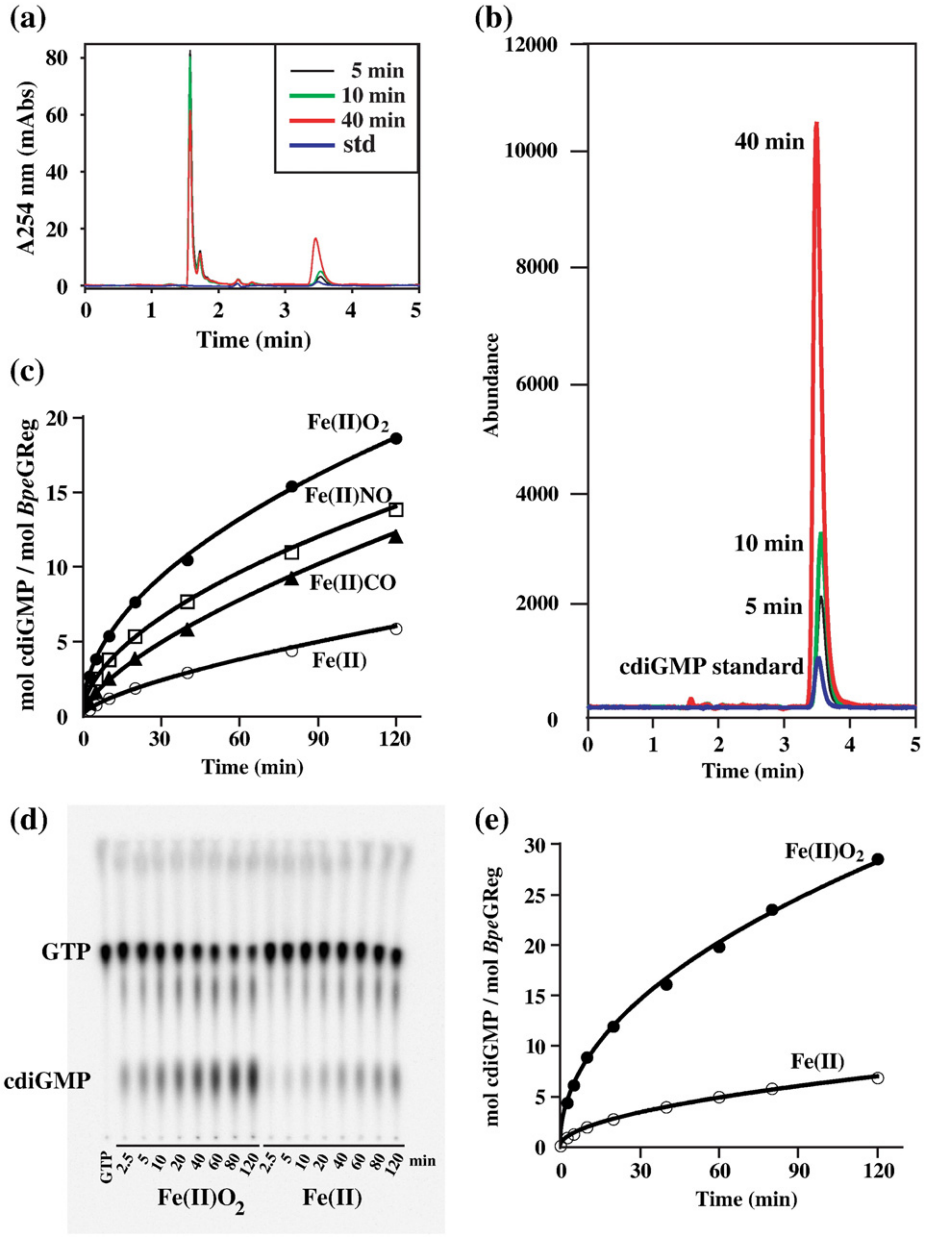

Fig. 2. Identification and quantification of c-di-GMP production *in vitro* by different liganded states of *Bpe*GReg. All the experiments shown here measure the conversion of GTP (100–500 μM) to c-di-GMP. (a) Activity of *Bpe*GReg at 5 min (black), 10 min (green), and 40 min (red) as assayed by reversed-phase HPLC. The GTP elutes at 1.8 min, whereas the c-di-GMP elutes at 3.6 min; a c-di-GMP standard is shown (blue). (b) Mass spectra coupled to the HPLC shown in panel (a); the c-di-GMP is detected with single-ion mode (negative) at an m/z of 689. (c) Influence of heme ligands on *Bpe*GReg activity; activity data for the deoxy, O_2-bound, CO-bound, and NO-bound states are based on HPLC–MS and TLC assays that gave essentially identical results. Levels of c-di-GMP were measured for reactions stopped at 2.5, 5, 10, 20, 40, 80, and 120 min. (d) Influence of O_2 on *Bpe*GReg activity; levels of c-di-GMP were measured by TLC for reactions stopped at 2.5, 5, 10, 20, 40, 60, 80, and 120 min. The reactions correspond to the curves shown in panel (e). The differences between the respective oxy and deoxy curves in panels (c) and (e) are due to the different degrees of feedback inhibition in the starting preparations of enzyme when the DGC assay is not coupled to a PDE (see Fig. 3).

Absorption spectra and ligand-binding parameters

Unless otherwise noted, all determinations of UV-Vis absorption and ligand binding were for 2–5 μM protein in 0.10 M sodium phosphate, pH 7.5, at 25 °C. Absorption spectra were monitored with a Cary 4000 UV-Vis spectrophotometer (Varian). Laser-flash photolysis and stopped-flow measurements were done with an LKS.60 laser kinetic spectrometer fitted with a PiStar stopped-flow drive unit (Applied Photophysics). For sample excitation, the LKS.60 spectrometer was coupled to a Quantel Brilliant B Nd:YAG laser with second-harmonic generation. Ligand-binding kinetics were followed for 2–5 μM protein at a wavelength of maximum difference between the starting and final

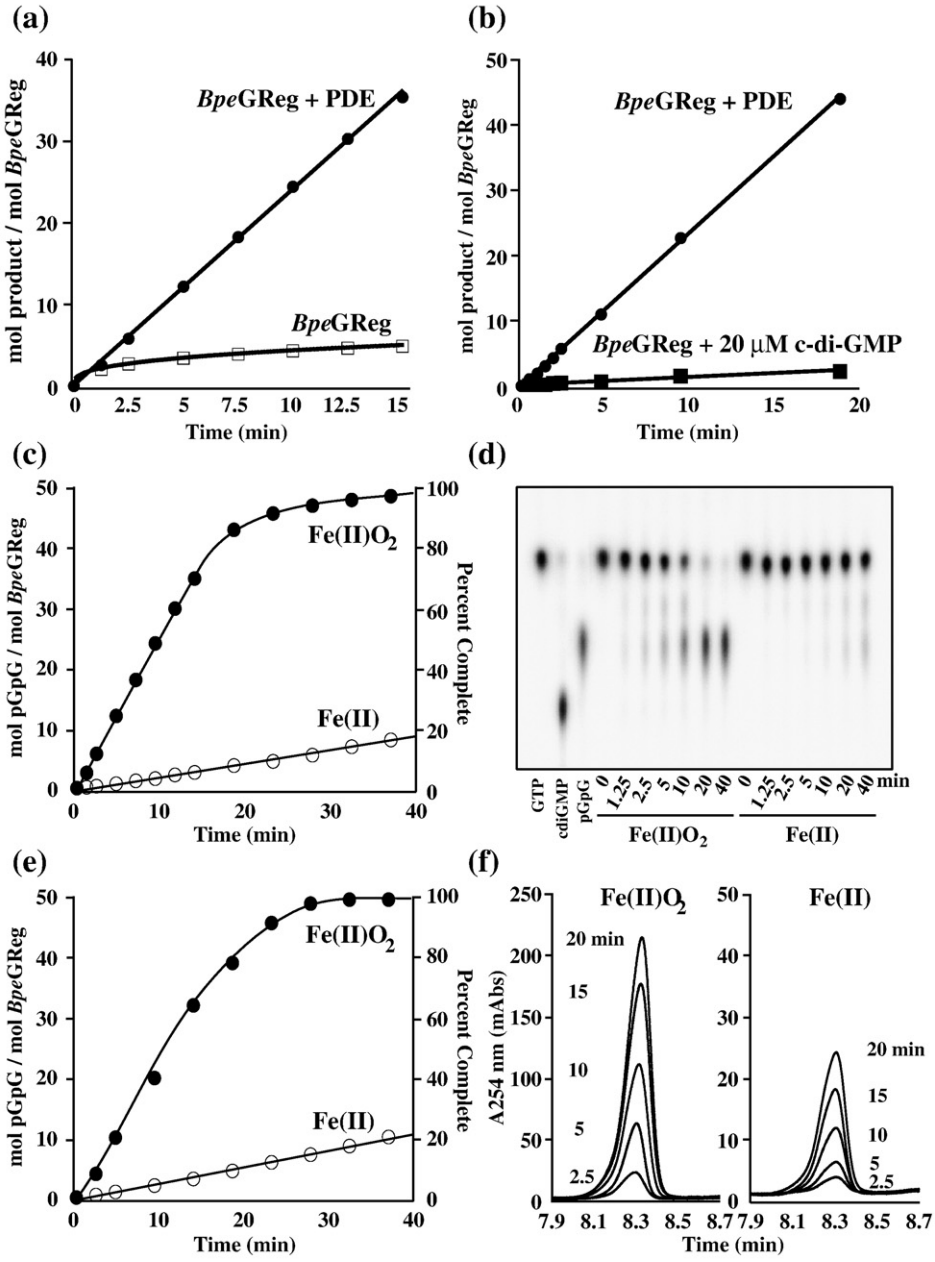

Fig. 3. Activation of *Bpe*GReg by O_2 and feedback inhibition of the enzyme by c-di-GMP. Rates of conversion of 500 μM GTP to c-di-GMP were followed either by measuring c-di-GMP directly or by measuring formation of pGpG from a PDE-coupled reaction. (a) Activity of 5 μM oxy-*Bpe*GReg without coupling to a PDE or with coupling to a PDE. (b) Activity of 1 μM oxy-*Bpe*GReg in a reaction started in the presence of 20 μM c-di-GMP or coupled to a PDE. (c) Activity of 5 μM *Bpe*GReg in air [Fe(II)O_2] or under anaerobic conditions [Fe(II)]. Note the 13-fold drop in the activity in the absence of O_2, and note that the reaction is essentially complete within 30 min in air. (d) TLC showing representative time points from the reactions shown in panel (c); the first three lanes are shown as references, with the R_f values being 0.58 for GTP, 0.18 for c-di-GMP, and 0.34 for pGpG. (e) Repeat of the reactions shown in (c) for analysis by HPLC. (f) HPLC traces of representative pGpG peaks from the reactions shown in panel (e); the peaks are from the 2.5-, 5.0-, 10-, 15-, and 20-min time points.

species. Each rate constant was calculated from a linear plot of k_{obs} *versus* ligand concentration including at least four ligand concentrations.

The *Bpe*GReg O_2 association rate constant [k_{on} (O_2)] was measured by laser-flash photolysis at 436 or 414 nm using 64–1024 μM O_2. The *Bpe*GReg O_2 dissociation rate constant [k_{off} (O_2)] was measured by stopped flow at 436 nm by mixing 2–5 μM oxy-*Bpe*GReg with 1 mM sodium dithionite. The *Bpe*GReg CO association rate constant [k_{on} (CO)] was measured by laser-flash photolysis at 419 nm using 30–480 μM CO. The *Bpe*GReg CO dissociation rate constant [k_{off} (CO)] was measured by ligand displacement in the stopped flow at 423 nm by mixing 3.5 μM carbonmonoxy-*Bpe*GReg (in 20 μM CO) with 500 μM nitric oxide. The *Bpe*GReg NO association rate constant [k_{on} (NO)] was measured by laser-flash photolysis at 435 nm using 15–60 μM nitric oxide.

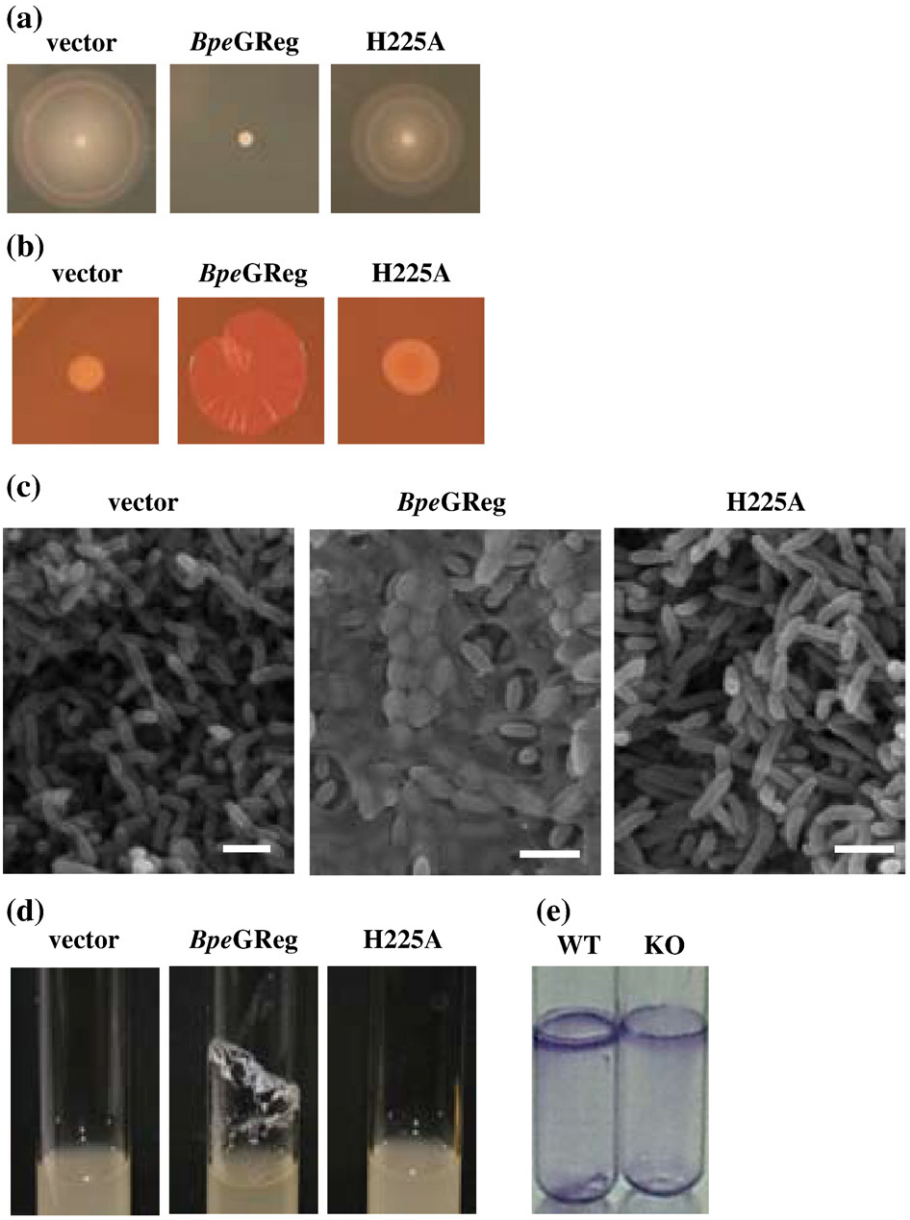

Fig. 4. Phenotypic effects of *Bpe*GReg and its H225A mutant. (a–d) Phenotypes of *S. typhimurium* cells either harboring an empty vector or expressing *Bpe*GReg and the H225A mutant. (a) Swimming motility on 0.3% tryptone agar plates. (b) rdar morphotype development on Congo red plates. (c) SEM micrographs of the colonies shown in panel (b). Scale bars represent 2 μm. (d) Biofilm formation in liquid culture. (e) Biofilm assay of wild-type (WT) *B. pertussis* and the *Bpe*GReg knockout (KO) strain. Adherent cells were stained with crystal violet.

Enzymatic DGC assays

Deoxy-*Bpe*GReg was produced by reduction of the purified protein with 10 mM DTT in an anaerobic chamber (Coy). Oxy-*Bpe*GReg was prepared by diluting deoxy-*Bpe*GReg into air-saturated reaction buffer. Generally, reactions contained 1–5 μM *Bpe*GReg in 20 mM sodium phosphate, pH 7.5, 10 mM $MgCl_2$, and 2 mM DTT at 23 °C. For the DGC–PDE coupling assays, *Ec*Dos was added to a 3-fold molar excess to the *Bpe*GReg. Reactions were started by adding 500 μM GTP ([α-^{32}P], Perkin-Elmer). Aliquots were removed from the reaction at the indicated times and mixed with one-fourth volume of 0.50 M ethylenediaminetetraacetic acid, pH 8.0. The stopped reactions were then heated at 100 °C for 5 min, and precipitated proteins were removed by centrifugation at 10,000 rpm for 10 min. The supernatant was analyzed by TLC, high-performance LC (HPLC), or LC–MS. For TLC analysis, 2 μl of the supernatant was spotted onto polyethyleneimine–cellulose F TLC plates (Merck KGaA). The plates were developed in a 1.5:1 KH_2PO_4 (1.5 M, pH 3.6)/$(NH_4)_2SO_4$ (4 M, pH 3.6) solution. Under these conditions, GTP migrates with R_f=0.58, c-di-GMP migrates with R_f=0.18, and pGpG migrates with R_f=0.34. Data acquisition was by phosphorimaging with a storage phosphor screen (Kodak K-HD) and a Typhoon 9200 variable mode imager (Amersham Pharmacia Biotech). Data analysis was with Image Quant 5.2 software (Molecular Dynamics). Known amounts of [α-^{32}P]GTP were used for standardization. For the HPLC analysis shown in Fig. 3f, 10 μl of the stopped reactions was injected onto an

Adsorbosphere Nucleotide–Nucleoside reversed-phase C18 HPLC column (Alltech, 250×4.6 mm) equipped with an Adsorbosphere Nucleotide–Nucleoside guard column (Alltech/Grace, 7.5×4.6 mm). The mobile phase consisted of (a) 0.15 M NaH_2PO_4, pH 5.2, and (b) 40% acetonitrile (balance A eluent). A linear gradient from 0% to 35% B for 10 min at 1 ml/min was used to separate GTP, c-di-GMP, and pGpG. Known quantities of all three molecules were used for standardization. For the LC–MS analyses shown in Fig. 2a and b, the samples were injected into a Dionex C-18 column (150×4.6 mm) in an Agilent 1100 series LC–MS system and separated with a gradient from 2% acetonitrile/98% trifluoroacetic acid (vol/vol) to 95% aceotonitrile/5% trifluoroacetic acid at a flow rate of 1 ml/min. Nucleotides were detected at a wavelength of 254 nm for LC, as shown in Fig. 2a; c-di-GMP was detected in negative single-ion monitoring mode at an m/z of 689 with MS, as shown in Fig. 2b.

Phenotypic assays

To detect cellulose biosynthesis, *S. typhimurium* was grown on LB without salt plates containing Congo red (40 μg/ml) for 40 h at 37 °C. Swimming motility was assayed on 0.3% agar plates (1% tryptone, 0.5% NaCl, and 1 μM thiamine) at 28 °C for 6 h. Biofilm formation was observed in glass tubes for *S. typhimurium* and *B. pertussis*. *S. typhimurium* strains were diluted to an OD_{600} of 0.01 in LB medium without salt. Five milliliters was aliquoted into glass tubes and sealed with parafilm. The tubes were incubated at 37 °C with shaking (250 rpm) for 36 h. Adherence to the glass and cell clumping were visually compared. *B. pertussis* strains were incubated at 37 °C without shaking for 7 days, and adherent cells were stained with 0.1% crystal violet. *S. typhimurium* colonies grown on Congo red plates were observed by SEM. Sections of colonies were fixed with 4% glutaraldehyde and 0.05% ruthenium red in 0.1 M cacodylate buffer, pH 7.4, post-fixed with 1% osmium tetroxide, dehydrated in ethanol, and critical point dried. Samples were coated with gold/palladium and examined with a Hitachi S-800 field emission scanning electron microscope at 15 kV.

Acknowledgements

This work was supported by the National Science Foundation (grant no. MCB0446431) and the U.S. Army Telemedicine and Advanced Technology Research Center (award no. W81XWH0520013) (M.A.); by the National Institutes of Health Intramural Research Program at the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (M.Y.G.); and by the National Science Foundation (grant no. 620531) and Welch Foundation (grant no. I-1575) (M.A.G.G.). We thank Dr. C. Locht for providing the pFUS2 vector; Dr. G. Erdem, J. F. Teiber, and G. L. Kramer for providing laboratory facilities; Dr. D. Raze and C. Mizumoto for technical assistance; Dr. H. Wahab and N. Bahiyahn for initial homology modeling; Dr. A. C. Whelen for providing the clinical strain of *B. pertussis*; Dr. E. H. S. Sousa for discussions; and Dr. G. Hazelbauer, Dr. C. Appleby, Dr. P. Patek, and Dr. D. Hunt for critical reading of the manuscript.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.03.015

References

1. Vinogradov, S. N., Hoogewijs, D., Bailly, X., Arredondo-Peter, R., Guertin, M., Gough, J. *et al.* (2005). Three globin lineages belonging to two structural classes in genomes from the three kingdoms of life. *Proc. Natl Acad. Sci. USA*, **102**, 11385–11389.
2. Hou, S., Larsen, R. W., Boudko, D., Riley, C. W., Karatan, E., Zimmer, M. *et al.* (2000). Myoglobin-like aerotaxis transducers in Archaea and Bacteria. *Nature*, **403**, 540–544.
3. Hou, S., Freitas, T., Larsen, R. W., Piatibratov, M., Sivozhelezov, V., Yamamoto, A. *et al.* (2001). Globin-coupled sensors: a class of heme-containing sensors in Archaea and Bacteria. *Proc. Natl Acad. Sci. USA*, **98**, 9353–9358.
4. Freitas, T. A., Hou, S. & Alam, M. (2003). The diversity of globin-coupled sensors. *FEBS Lett.* **552**, 99–104.
5. Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Ohana, P., Mayer, R. *et al.* (1987). Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature*, **325**, 279–281.
6. Galperin, M. Y., Nikolskaya, A. N. & Koonin, E. V. (2001). Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol. Lett.* **203**, 11–21.
7. Galperin, M. Y. (2005). A census of membrane-bound and intracellular signal transduction proteins in bacteria: bacterial IQ, extroverts and introverts. *BMC Microbiol.* **5**, 35.
8. Römling, U., Gomelsky, M. & Galperin, M. Y. (2005). c-di-GMP: the dawning of a novel bacterial signaling system. *Mol. Microbiol.* **57**, 629–639.
9. Camilli, A. & Bassler, B. L. (2006). Bacterial small-molecule signaling pathways. *Science*, **311**, 1113–1116.
10. Jenal, U. & Malone, J. (2006). Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu. Rev. Genet.* **40**, 385–407.
11. Sudarsan, N., Lee, E. R., Weinberg, Z., Moy, R. H., Kim, J. N., Link, K. H. & Breaker, R. R. (2008). Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science*, **321**, 411–413.
12. Ross, P., Mayer, R., Weinhouse, H., Amikam, D., Huggirat, Y., Benziman, M. *et al.* (1990). The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. *J. Biol. Chem.* **265**, 18933–18943.
13. Ryjenkov, D. A., Simm, R., Römling, U. & Gomelsky, M. (2006). The PilZ domain is a receptor for the second messenger c-di-GMP. *J. Biol. Chem.* **281**, 30310–30314.
14. Lee, V. T., Matewish, J. M., Kessler, J. L., Hyodo, M., Hayakawa, Y. & Lory, S. (2007). A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Mol. Microbiol.* **65**, 1474–1484.
15. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science*, **284**, 1318–1322.
16. Kolter, R. & Greenberg, E. P. (2006). Microbial sciences: the superficial life of microbes. *Nature*, **441**, 300–302.
17. Yildiz, F. H. (2007). Processes controlling the transmission of bacterial pathogens in the environment. *Res. Microbiol.* **158**, 195–202.

18. Sloan, G. P., Love, C. F., Sukumar, N., Mishra, M. & Deora, R. (2007). The *Bordetella* Bps polysaccharide is critical for biofilm development in the mouse respiratory tract. *J. Bacteriol.* **189**, 8270–8276.
19. Sola, M., Gomis-Ruth, F. X., Serrano, L., Gonzalez, A. & Coll, M. (1999). Three-dimensional crystal structure of the transcription factor PhoB receiver domain. *J. Mol. Biol.* **285**, 675–687.
20. Chan, C., Paul, R., Samoray, D., Amiot, N. C., Giese, B., Jenal, U. & Schirmer, T. (2004). Structural basis of activity and allosteric control of diguanylate cyclase. *Proc. Natl Acad. Sci. USA*, **101**, 17084–17089.
21. Christen, B., Christen, M., Paul, R., Schmid, F., Folcher, M., Jenoe, P. *et al.* (2006). Allosteric control of cyclic di-GMP signaling. *J. Biol. Chem.* **281**, 32015–32024.
22. Zhang, W., Olson, J. S. & Phillips, G. N., Jr. (2005). Biophysical and kinetic characterization of HemAT, an aerotaxis receptor from *Bacillus subtilis*. *Biophys. J.* **88**, 2801–2814.
23. Springer, B. A., Sligar, S. G., Olson, J. S. & Phillips, G. N., Jr. (1994). Mechanisms of ligand recognition in myoglobin. *Chem. Rev.* **94**, 699–714.
24. Brucker, E. A., Olson, J. S., Ikeda-Saito, M. & Phillips, G. N., Jr. (1998). Nitric oxide myoglobin: crystal structure and analysis of ligand geometry. *Proteins*, **30**, 352–356.
25. Simm, R., Morr, M., Kader, A., Nimtz, M. & Römling, U. (2004). GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol. Microbiol.* **53**, 1123–1134.
26. Gerstel, U. & Römling, U. (2003). The csgD promoter, a control unit for biofilm formation in *Salmonella typhimurium*. *Res. Microbiol.* **154**, 659–667.
27. Kader, A., Simm, R., Gerstel, U., Morr, M. & Römling, U. (2006). Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **60**, 602–616.
28. Antoine, R., Alonso, S., Raze, D., Coutte, L., Lesjean, S., Willery, E. *et al.* (2000). New virulence-activated and virulence-repressed genes identified by systematic gene inactivation and generation of transcriptional fusions in *Bordetella pertussis*. *J. Bacteriol.* **182**, 5902–5905.
29. Wood, G. E., Khelef, N., Guiso, N. & Friedman, R. L. (1998). Identification of Btr-regulated genes using a titration assay. Search for a role for this transcriptional regulator in the growth and virulence of *Bordetella pertussis*. *Gene*, **209**, 51–58.
30. Paul, R., Abel, S., Wassmann, P., Beck, A., Heerklotz, H. & Jenal, U. (2007). Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. *J. Biol. Chem.* **282**, 29170–29177.
31. Piatibratov, M., Hou, S., Brooun, A., Yang, J., Chen, H. & Alam, M. (2000). Expression and fast-flow purification of a polyhistidine-tagged myoglobin-like aerotaxis transducer. *Biochim. Biophys. Acta*, **1524**, 149–154.
32. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.