The BLUF-EAL protein YcgF acts as a direct anti-repressor in a blue-light response of Escherichia coli

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The blue light using FAD (BLUF)-EAL protein YcgF is a known blue-light sensor of Escherichia coli, but its direct regulatory output and physiological function have remained unknown. Here, we demonstrate that unlike other EAL domain proteins, YcgF does not degrade the signaling molecule c-di-GMP, but directly binds to and releases the MerR-like repressor YcgE from its operator DNA upon blue-light irradiation. As a consequence, a distinct regulon of eight small proteins (of 71–126 amino acids) is strongly induced. These include YmgA and YmgB, which, via the RcsC/RcsD/RcsB two-component phosphorelay system, activate production of the biofilm matrix substance colanic acid as well as acid resistance genes and the biofilm-associated bdm gene and down-regulate adhesive curli fimbriae. Thus, small proteins under YcgF/YcgE control seem to act as “connectors” that provide additional signal input into a two-component signaling pathway. Moreover, we found ycgF and ycgE expression to be strongly activated at low temperature, and we elucidate how blue light, cold, and starvation signals are integrated in the expression and activity of the YcgF/YcgE/small protein signaling pathway. In conclusion, this pathway may modulate biofilm formation via the two-component network when E. coli has to survive in an extrahost aquatic environment.

[Keywords: Biofilm; connectors; cyclic-di-GMP; GGDEF; RcsB; RpoS]

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GGDEF and EAL domain proteins have been recognized recently to act as diguanylate cyclases (DGC) and phosphodiesterases (PDE), respectively, that “make and break” the ubiquitous bacterial signaling molecule bis-(3′,5′)-cyclic-guanosine monophosphate (c-di-GMP). In general, c-di-GMP interferes with motility and promotes biofilm formation; i.e., it acts as a key signal in switching between the planktonic/single-cellular and sedentary/multicellular “life-styles” (for recent reviews, see Jenal and Malone 2006; Romling and Amikam 2006; Ryan et al. 2006). In pathogenic bacteria, c-di-GMP can also regulate virulence genes (Cotter and Stibitz 2007; Tamayo et al. 2007). The GGDEF and EAL amino acid motifs are part of the respective active centers and therefore are essential for the activities of these enzymes (Malone et al. 2007; Wassmann et al. 2007; Rao et al. 2008). Many bacterial species possess up to several dozen GGDEF/EAL domain proteins. In these proteins, the two domains can occur either alone (“GGDEF-only” or “EAL-only”) or in combination in single polypeptides (“GGDEF + EAL”). The majority of these proteins also carry N-terminal sensory input domains that can control the activities of the GGDEF and/or EAL domains.

Intriguingly, in a certain fraction of these proteins, the GGDEF and/or EAL motifs as well as additional amino acids relevant for enzymatic function are not conserved. This is found not only in GGDEF + EAL proteins, where one domain can be degenerate and the other then determines the enzymatic activity, but also in proteins where no intact GGDEF or EAL domains remain. These proteins should not have DGC or PDE activities, but act in different ways. Among the 29 GGDEF/EAL proteins of Escherichia coli, there are four such proteins: YeaI (a GGDEF-only protein), YhdA (featuring GGDEF and EAL domains, but both degenerate), and the two EAL-only proteins, YcgF and YdiV. The functions of Yeal, YcgF, and YdiV have remained unknown. YhdA promotes the turnover of two small RNAs, CsrB and CsrC, which by sequestering the RNA-binding protein CsrA can reduce the expression of the flagellar master regulator FlhDC at the post-transcriptional level (Suzuki et al. 2006).

In the present study, we unravel the molecular and physiological function of the YcgF protein, in which the degenerate EAL domain is preceded by an N-terminal blue-light-sensing BLUF domain (“blue light using FAD”) (Gomelsky and Klug 2002; Purcell and Crosson 2008).
biophysical studies, conformational changes upon blue-light irradiation have been observed in the BLUF domain as well as in the entire YcgF protein, and the protein is believed to dimerize upon photo-excitation (Rajagopal et al. 2004; Hasegawa et al. 2006; Nakasone et al. 2007; Schroeder et al. 2008). However, the molecular output function controlled by the BLUF domain as well as the physiological context, where all this plays a role for *E. coli*, have remained elusive.

Here, we demonstrate that YcgF does not have PDE activity, but in a process that requires its EAL domain, it is a directly interacting antagonist for a repressor, YcgE. Blue-light-induced YcgF-mediated release of YcgE from its target operators results in the expression of a distinct set of small regulatory proteins, which, via the Rcs phosphorelay system, affects the production of colanic acid—i.e., a biofilm matrix component—and adhesive curli fimbriae. Moreover, we show that expression of YcgF and YcgE as well as their target genes are strongly induced by low temperature. Thus, YcgF is the first EAL protein shown to act by direct protein–protein interaction, and in doing so, in response to blue light, it acts as the sensory input component of a novel signal transduction pathway of a complex blue light and cold stress response that controls biofilm formation in an extrahost environment.

Results

Identification of target genes of the MerR-like regulator YcgE

On the *E. coli* chromosome, the ycgF gene is followed by ycgE, which encodes a MerR-like regulator of unknown function. Whether the two genes constitute an operon was not clear at the beginning of this study [see Materials and Methods for further details]. What drew our attention to YcgE was the finding that not only does it contain a MerR-like N-terminal domain, but also that the complete protein shows clear homology with MlrA, which is an activator essential for the expression of the curli regulator CsgD [Brown et al. 2001]. csgD transcription is also regulated by YdaM and YciR, i.e., a DGC (GGDEF-only) and a PDE (with GGDEF and EAL domains). In other words, here was a precedent for a MerR-like protein and an EAL protein cooperating in transcription initiation [Weber et al. 2006]. We therefore suspected that YcgE and YcgF might act in a common pathway that controls the expression of an unknown set of genes.

As a first step, we identified YcgE-regulated genes, as this would allow us to develop a convenient reporter assay for the function of YcgE and, possibly, YcgF. Genome-wide transcriptional profiling on microarrays revealed a small set of eight genes that are derepressed in a ycgE knockout mutant [in cells grown in LB at 28°C (Supplementary Table S1): Whereas two major acid resistance genes (gadB, hdeB) showed only moderate derepression [threelfold to fourfold], six other genes of unknown molecular functions (ycgZ, yliL, ymgA, ymgB, ymgC, and ynaK) were rather strongly activated in the ycgE mutant [sixfold to 22-fold].

This latter group of genes is remarkable in several aspects. First, all six genes encode small proteins of 78–90 amino acids with unknown molecular functions [see also in Table 1]. Second, ycgZ, ymgA, and ymgB are under the control of the general stress σ factor σS [RpoS] [Weber et al. 2005], and their arrangement on the *E. coli* chromosome suggested that they might constitute an operon. ymgC is the next gene downstream from this region, but it did not show significant control by σS [Weber et al. 2005]. However, Northern blot analysis with probes complementary to ycgZ and ymgC indicated that all four genes are expressed in a single polycistronic mRNA of ~1400 nucleotides [nt] (our unpublished results; see also

**Table 1. Genes differentially expressed in MC4100 (ycgF<sup>+</sup>) and its otherwise isogenic ycgF<sup>−</sup>kan derivative as determined by genome-wide transcriptional profiling on microarrays**

| Gene<sup>b</sup> | b-number | Protein size [amino acids/kilodalton] | YcgF dependence [ratio: ycgF<sup>−</sup>/ycgF<sup>+</sup>] | σS<sup>c</sup> dependence [ratio: ypoS<sup>−</sup>/ypoS<sup>+</sup>] | LB/OD4 | NaCl | pH 5 |
|-----------------|----------|--------------------------------------|---------------------------------|---------------------------------|-------|------|------|
| ybgS            | b0753    | 126/12.8                             | 3.68                            | 8.43                            | 38.08 | 16.12 |      |
| yilL            | b0816    | 89/10.0                              | 40.56                           | 5.11                            | 5.53  | 4.00  |      |
| ycgZ            | b1164    | 78/8.7                               | 12.66                           | 4.98                            | 5.89  | 4.63  |      |
| ymgA            | b1165    | 90/10.3                              | 60.20                           | 4.46                            | 4.63  |      |      |
| ymgB            | b1166    | 88/9.6                               | 85.90                           | 3.30                            | 3.84  |      |      |
| ymgC            | b1167    | 82/9.7                               | 70.70                           | 27.74                           |      |      |      |
| ynaK            | b1365    | 87/9.7                               | 8.66                            | 2.73                            | 4.64  | 4.10  |      |
| bdm (yddX)      | b1481    | 71/7.9                               |                                 |                                 |      |      |      |

<sup>a</sup>The two strains were grown in LB medium at 37°C to an OD578 of 0.7, then shifted to 16°C and exposed to blue light for three additional hours before samples for RNA preparation were taken. For details of microarray analysis, see the Materials and Methods.  
<sup>b</sup>Gens are ordered according to b-numbers (reflecting chromosomal position).  
<sup>c</sup>Data were taken from Weber et al. [2005], where ypoS<sup>−</sup>/ypoS<sup>+</sup> ratios were determined under three different conditions that result in the accumulation of σS<sup>c</sup>: [1] entry into stationary phase in LB (LB/OD4), [2] 20 min after the addition of 0.3 M NaCl at an OD<sub>578</sub> of 0.3 to cultures growing in M9/glycerol [NaCl], and [3] 40 min after a shift to pH 5 at an OD<sub>578</sub> of 0.4 of cultures growing in LB (pH 5). (−) Ratio is <2.00, i.e., no significant σS<sup>c</sup> dependence. The full ypoS<sup>−</sup>/ypoS<sup>+</sup> data sets can also be found on http://www.biologie.fu-berlin.de/en/microbio/mibi-hengge/scidata/index.html.
in the absence of activated to some extent by vegetative RNA polymerase the ycgE (YcgFI193L) suggesting that the single ycgZ mutant, eliminating the BLUF-EAL protein YcgF acts in a regulatory pathway upstream of YcgE.

In order to study the regulatory roles of YcgE and YcgF, a single-copy ycgZ::lacZ reporter fusion was constructed [containing the entire intergenic control region present upstream and integrated at att(λ) in the chromosome]. In an otherwise wild-type background, this reporter fusion showed weak expression that was slightly stimulated during entry into stationary phase (Fig. 1A). Consistent with microarray data (Supplemental Table S1, Weber et al. 2005), ycgZ expression was strongly derepressed in the ycgE mutant and positively σ^5-regulated both in ycgE^+ and ycgE mutant strains (Fig. 1A). In the ycgE mutant, eliminating σ^5 only partially reduced expression, suggesting that the single ycgZ promoter can also be activated to some extent by vegetative RNA polymerase in the absence of σ^5 [our unpublished data]. In the ycgF mutant, ycgZ expression was reduced (Fig. 1B). In the absence of YcgE, however, the ycgF mutation no longer affected ycgZ expression, which was similarly elevated as in the mutant defective in ycgE alone (Fig. 1A,B). These data indicate that YcgF plays an activating role in the control of ycgZ and that it acts upstream of YcgE in a common pathway. This was further confirmed with a YcgF-overexpressing plasmid, which activated ycgZ expression to the same extent as the mutation in ycgE, and this effect was not additive to that of the ycgE mutation (Fig. 1C). Taken together, these data suggest that the BLUF-EAL protein YcgF is a direct or indirect antagonist for the transcriptional regulator YcgE.

YcgF does not have phosphodiesterase activity, but acts as a direct antagonist for YcgE

How does YcgF antagonize the action of YcgE? In the degenerate EAL domain of YcgF, all four amino acids required to bind c-di-GMP and three amino acids crucial for Mg^2+ binding and catalysis [Rao et al. 2008] are not conserved [Supplemental Fig. S1], thus making it highly unlikely that YcgF acts via c-di-GMP. Nevertheless, we wanted to clarify whether the EAL domain of YcgF may be required to bind c-di-GMP and three amino acids crucial for Mg^2+ binding and catalysis (Rao et al. 2008) are not conserved [Supplemental Fig. S1], thus making it highly unlikely that YcgF acts via c-di-GMP. Nevertheless, we wanted to clarify whether the EAL domain of YcgF may act as a direct antagonist for YcgE. Derivatives of MC4100 carrying ycgZ::lacZ and ycgE and ycgF mutations as indicated were grown in LB at 37°C. OD_578 (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve. [B] A ycgF mutation reduces ycgZ expression in the presence but not in the absence of YcgE. Derivatives of MC4100 carrying ycgZ::lacZ and ycgE and ycgF mutations as indicated were grown and analyzed as described for A. [C] A ycgF-carrying plasmid activates ycgZ expression, provided the repressor YcgE is present. Derivatives of MC4100 carrying ycgZ::lacZ as well as mutations in ycgF and ycgE as indicated were transformed with a YcgF-overproducing plasmid [pQE30Xa-ycgF] or the vector alone [pQE30Xa], and β-galactosidase activities were determined after 24 h growth in LB/ampicilline at 37°C.
motif alone is not sufficient to restore such activity, as several important amino acids at other key positions of the EAL domain are not present [Supplemental Fig. S1]. In addition, both wild-type YcgF as well as the “improved EAL” variant were also unable to bind c-di-GMP [Supplemental Fig. S2B]. These data indicate that c-di-GMP is not involved in the activity of YcgF. Moreover, we observed that overexpression of the DGC YaiC or the PDE YhjH did not alter the expression of the target gene ycgZ (Supplemental Fig. S3; note that overexpression of YaiC and YhjH did have the expected effects on motility as detailed in the figure legend). In addition, knockout mutations in all genes encoding proteins with intact GGDEF motifs [i.e., known or putative DGCGs] did not affect ycgZ::lacZ expression [data not shown]. Altogether, this means that despite YcgF being an EAL protein, c-di-GMP does not play a role in the YcgF/YcgE regulatory pathway.

We therefore tested whether YcgF and YcgE interact directly. Full-size YcgE as well as its N-terminal MerR-like domain (YcgE-NTD) and the C-terminal domain (YcgE-CTD) were cloned and purified with a TRX-His6-S tag at their N-terminal ends, which allows specific binding to S-protein agarose. In affinity chromatography (“pull-down”) experiments, putative interaction of these proteins with purified full-size YcgF and the YcgF-Bluf domain alone was tested (the C-terminal YcgF-EAL domain alone was extremely prone to aggregation and could not be isolated in soluble form). Not only did the full-size proteins YcgE and YcgE interact [Fig. 2A], but specifically the N-terminal MerR-like domain of YcgE was able to bind to YcgF [Fig. 2B]. As no interaction was observed with the N-terminal YcgF-Bluf domain alone, we conclude that the C-terminal EAL domain of YcgF [either alone or in cooperation with the BLUF domain] is essential for this direct interaction with the MerR-like YcgE-NTD. The “improved EAL” variant of YcgF also bound to YcgE as well as to YcgE-NTD [data not shown].

This direct interaction suggested that YcgF may modulate the activity of the MerR-like domain of YcgE; i.e., DNA binding. We therefore tested YcgE binding to the ycgZ promoter region (ycgZp) and a potential effect of YcgF thereon in electrophoretic mobility shift assays. YcgE was found to bind specifically to the ycgZp DNA fragment; i.e., YcgE plays a direct role in ycgZ transcriptional repression [Fig. 3; Supplemental Fig. S4]. YcgF, in contrast, did not show any binding, when added alone to the ycgZp DNA fragment [Fig. 3]. When YcgF and YcgE were both present in the assay, YcgE was released from the DNA in a YcgF dose-dependent manner [Fig. 3A]. This release was dependent on blue-light irradiation and was modulated by the order of addition of the relevant components. When YcgE and ycgZp DNA were preincubated before the addition of YcgF and blue-light irradiation [Fig. 3A], more YcgF was required to chase YcgE from the DNA than when YcgE and YcgF were preincubated and blue-light-irradiated before the addition of DNA [Fig. 3B], and nearly stoichiometric amounts of YcgF were sufficient to completely release YcgE, when the latter procedure was further modified to include a second irradiation after DNA addition [Fig. 3C]. These data suggest high affinities and low off-rates of the interaction reactions involved. This is also consistent with no rebinding of YcgE to ycgZp DNA, when samples treated as in Figure 3A were kept in the dark for 60 min before running them on the gel [data not shown], and the slow reversion to the ground state in the photocycle of YcgF [Schroeder et al. 2008]. In the absence of blue-light irradiation, even a relatively large excess of YcgF did not release YcgE from its operator. This means either that YcgF, despite possible binding to DNA-bound YcgE, does not induce a conformational change in YcgE required for its release from the operator site, or that YcgF does not efficiently bind to DNA-bound YcgE. The latter would be in contrast to YcgF binding to free YcgE in the absence of DNA [as in the interaction assay shown in Fig. 2], suggesting that YcgF and ycgZp DNA compete for overlapping binding sites on the YcgE MerR-like domain.

We conclude that blue-light irradiation, which is sensed by its BLUF domain and transmitted by conformational changes [Hasegawa et al. 2006; Nakasone et al. 2007], enables YcgF to interact with operator-bound YcgE and to release it from the DNA. Moreover, this biochemical activity of YcgF is fully consistent with the genetically defined role of YcgF as an antagonist to the YcgE repressor in the control of ycgZ expression [Fig. 1].
The blue-light response initiated by the YcgF/YcgE pathway is important at low temperature

The YcgF/YcgE pathway responds to blue light as an input signal. Why and under what conditions is blue-light sensing relevant for \textit{E. coli}? Is blue light just an indicator for being in a certain environment, or does blue-light irradiation itself cause stress; i.e., can it be detrimental to cellular physiology? It is obvious that any blue-light effects should occur in an outside environment rather than inside a mammalian host. Consistently, in a study of expression patterns of all GGDEF/EAL genes in \textit{E. coli} (Sommerfeldt et al. 2009), it was observed that \textit{ycgF} belongs to a group of GGDEF/EAL genes that are more strongly expressed at 28°C than at 37°C. Moreover, \textit{ycgF} and \textit{ycgZ} are members of an \textit{E. coli} cold shock stimulon described by two microarray studies (Polissi et al. 2003; White-Ziegler et al. 2008). This prompted us to study the regulation of the entire YcgF/YcgE/\textit{ycgZ} system in closer detail (using single-copy \textit{lacZ} reporter fusions also to \textit{ycgF} and \textit{ycgE}; note that each of these two genes is expressed from its own transcriptional start site) (for details, see the Materials and Methods). We found that induction of \textit{ycgF} was even stronger when the temperature was reduced further below 28°C, both after steady-state growth [Fig. 4A] as well as after a shift from 37°C to 16°C [Fig. 4B]. \textit{ycgE}, too, was increasingly expressed at lower temperature [Fig. 4A,C], although not as strongly as \textit{ycgF} (the difference in expression at 16°C vs. 37°C was about sevenfold for \textit{ycgE}, but >30-fold for \textit{ycgF}). Thus, the YcgF/YcgE pathway seems especially important in an outside environment at low temperatures that support only slow growth of mesophilic bacteria like \textit{E. coli}.

Consistently, the target gene \textit{ycgZ} also was induced upon shift from 37°C to 16°C, and even more so if the culture was also exposed to blue-light irradiation in addition to the temperature downshift [Fig. 5]. Both the analysis of the \textit{ycgZ}:\textit{lacZ} fusion [Fig. 5A] as well as complete transcript analysis by Northern blotting [Fig. 5B] showed that the expression of \textit{ycgZ} (and the entire \textit{ycgZ}-\textit{ymgABC} mRNA) is completely dependent on YcgF.
The two weakly YcgE-repressed acid resistance proteins (71 and 126 amino acids, respectively) (Table 1). DMC4100 (wt) and its derivatives carrying mRNA as detected by Northern blot analysis. ycgZ-ymgABC determined along the growth curve. (b) Blue-light-irradiated or kept in the dark. OD578 (open symbols) were shifted to 16°C only and not 28°C. Again, the entire region. The transcript detected has a coding region; i.e., it corresponds to the full-size operon transcript.

An EAL protein as a direct anti-repressor

Figure 5. Blue light and temperature down-shift additively induce ycgZ expression in a YcgF-dependent manner. (A) MC4100 carrying ycgZ::lacZ as well as its ycgF mutant derivative were grown in LB to an OD_{578} of 0.7, where the cultures were shifted to 16°C and split into two aliquots that were either blue-light-irradiated or kept in the dark. OD_{578} (open symbols) and specific β-galactosidase activities (closed symbols) were determined along the growth curve. [B] Expression of the ycgZ-ymgABC mRNA as detected by Northern blot analysis. MC4100 [wt] and its derivatives carrying ycgF::cat, ycgE::kan, or ΔycgZ-ymgABC::cat were grown as described for A. RNA was prepared 3 h after the shift to 16°C and subjected to Northern blot analysis using a probe corresponding to the ycgZ coding region. The transcript detected has ~1400 nt and can also be detected with a probe corresponding to the ymgC coding region (data not shown); i.e., it corresponds to the full-size operon transcript.

even when cells were exposed to 16°C only and not irradiated. Thus, also in the absence of blue light, YcgF can and is in fact required to relieve repression by YcgE at low temperatures (Fig. 5B).

Having learned that the YcgF/YcgE system is particularly important in a cold environment, we wondered whether we had detected all target genes of YcgE in our initial microarray transcriptome experiments performed at 28°C (Supplemental Table S1). In addition, the question arose whether YcgF acts exclusively via YcgE or whether it may also have an additional effect on other regulatory pathways. We therefore compared the transcriptional profiles of ycgF+ and ycgF mutant strains at 16°C and in the presence of blue light. The list of genes differentially affected was again small and nearly identical to that observed for ycgE+/− in the initial experiment [Table 1]: Again, the entire ycgZ-ymgA-ymgB-ymgC operon as well yliL and ynaK were found. In addition, two new genes were detected, bdm and ybgS, which also encode small proteins [71 and 126 amino acids, respectively] [Table 1]. The two weakly YcgE-repressed acid resistance genes gadB and hdeB did not show significant differential expression on the ycgE+/− microarrays under these conditions. Finally, we noted that the ratios of differential expression of the six genes found in both microarray experiments were stronger in the ycgE+/− experiment at 16°C with blue-light irradiation, indicating that these conditions are the natural ones for strong activation of these genes.

Monitoring the effect of blue-light irradiation on growth rate at several temperatures revealed that blue light can be detrimental for growth specifically at very low temperature ≪23°C] (Supplemental Fig. S5). However, survival assays [i.e., the determination of colony-forming units] showed that blue-light irradiation at 16°C is not lethal for E. coli cells (Supplemental Fig. S6). The ycgF mutation did not further affect growth rate or survival, indicating that while the YcgF/YcgE system is activated by blue light in a cold environment, its downstream function is not to counteract any direct detrimental effects of blue light irradiation.

The YcgF/YcgE regulatory pathway affects biofilm and stress response functions via the Rcs phosphorelay system

What are the molecular and physiological functions of the eight small proteins under YcgF/YcgE control? When we cloned the ycgZ-ymgAB region on a standard multicopy plasmid we noticed that transformants were mucoid, i.e., they produced more capsule material. In order to study this effect in more detail, we cloned all four genes separately as well as the entire operon under the control of the IPTG-inducible tac promoter [tacP] on a low-copy-number plasmid [pCAB18, which carries the p15A origin]. We found that even with basal levels of expression (no inducer added), YmgB increased the production of capsule material already at 28°C. At 16°C, not only Ymb, but also YmgA produced such an effect [Fig. 6A]. At 16°C, also the wild-type strain carrying the vector only had a moderate mucoid appearance that seemed to be less pronounced with the ycgZ-carrying plasmid, suggesting that YcgZ may have an effect opposite to that of YmgA/ Ymb. Mucoidy was due to the extracellular polysaccharide colanic acid, as it was abolished in a genetic background defective for the response regulator RcsB [Fig. 6A], which is essential for the expression of the enzymes that synthesize this biofilm matrix component [Majdalani and Gottesman 2005]. That capsule genes were not significantly differentially regulated in our microarrays under these conditions. How-
plasmid. Down-regulation of curli expression was also dependent on the presence of RcsB (Fig. 6C). Moreover, the presence of the ymgB-encoding plasmid stimulated the expression of the acid resistance gene gadB two- to threefold during entry into stationary phase in an RcsB-dependent manner [assayed with a single-copy gadB::lacZ fusion] (data not shown). This is consistent with gadB showing up as a moderately YcgE-repressed gene in our initial microarray analysis [Supplemental Table S1]. Also, microarray-based transcriptome analysis [our unpublished data] confirmed that the presence of pYmgB strongly reduced the expression of the curli csg genes and activated numerous acid resistance genes as well as the small protein genes bdm and ybgS. Moreover, among the additional genes affected, many were previously identified as RcsB-controlled [Hagiwara et al. 2003], but the rcsC, rcsD, and rcsB genes themselves were not differentially regulated [data not shown].

Taken together, these data indicate that induction of YmgB modulates the activity [but not expression] of the Rcs two-component signaling pathway, that YmgA is a back-up component with similar activity that further boosts the response at very low temperature, and that YcgZ may partially [or conditionally] counteract the effects of YmgA/YmgB. Thus, the Rcs phosphorelay system with its multiple outputs seems a major indirect target of the YcgF/YcgE pathway. In order to further corroborate this conclusion, we wanted to see whether such effects are elicited not only by YmgA/YmgB expressed from a plasmid, but also by the knockout mutation of ycgE [the phenotype of which corresponds to the effect of full activation of YcgF by blue light] [see Fig. 5B]. As a downstream target, we tested the expression of bdm [as a single-copy lacZ reporter fusion], as this gene is known to be under direct control of RcsB, which binds in the bdm promoter region and does not require RcsA for activation [Francez-Charlot et al. 2005]. Consistent with the microarray data [Table 1; Supplemental Table S1], the

Figure 6. Physiological effects of the small proteins induced by the YcgF/YcgE signaling pathway. (A) Effects of the small proteins YcgZ, YmgA, YmgB, and YmgC on colony phenotype. MC4100 and its rcsB::kan derivative were transformed with pCAB18-derived plasmids expressing the small proteins as indicated and grown on LB/ampicillin plates at 16°C (50 h) or 28°C (24 h). (B) Effects of the small proteins YcgZ, YmgA, YmgB, and YmgC on curli fimbriae expression. MC4100 carrying csgB::lacZ fusion as well as the pCAB18-derived plasmids expressing the small proteins as indicated were grown in LB/ampicillin at 16°C and 28°C (50 and 24 h, respectively), and specific β-galactosidase activities were determined. (C) Inhibition of curli expression by YmgB requires RcsB. MC4100 carrying csgB::lacZ fusion as well as the pCAB18-derived plasmids expressing the small proteins as indicated were grown in LB/ampicillin at 16°C and 28°C (50 and 24 h, respectively), and specific β-galactosidase activities were determined. (D) A ycgE mutation affects bdm expression via YmgB and RcsB. MC4100 carrying bdm::lacZ and its derivatives with single or double mutations in ycgE, ymgB, and rcsB as indicated were used [note that the ymgB::kan insertion shown as well as the nonpolar ymgB flip-out deletion showed the same effect]. In C and D, cells were grown in LB/ampicillin at 28°C and OD578 (open symbols), and specific β-galactosidase activities [closed symbols] were determined along the growth curve.
ycgE mutation enhanced bdm::lacZ expression, and this effect required the presence of RcsB [i.e., rcsB single and rcsB ycgE double knockout mutant showed the same low expression of bdm; Fig. 6D]. In addition, mutation of ymgB slightly reduced bdm::lacZ expression, and also in the ymgB-negative background, the ycgE mutation did not enhance bdm::lacZ expression [Fig. 6D]; in fact, bdm expression was even slightly reduced, consistent with higher expression of YcgZ in the ycgE ymgB double mutant than in the ymgB mutant. We conclude that also with wild-type expression levels of all components involved, the YcgF/YcgE system acts via YmgB and the Rcs phosphorelay system on downstream targets.

Discussion

The EAL protein YcgF is not involved in c-di-GMP metabolism but acts by direct protein–protein interaction with the MerR-like repressor YcgE

As GGDEF and EAL domains have been associated with DGC and c-di-GMP phosphodiesterase activities, respectively, proteins carrying these domains are often assumed to be such enzymes without further testing. However, many bacteria have proteins in which the GGDEF and EAL motifs are degenerate, and as these amino acid motifs crucially contribute to the active centers of the enzymes [Malone et al. 2007; Wassmann et al. 2007; Rao et al. 2008], the degenerate proteins cannot have enzymatic activity. Nevertheless, degenerate GGDEF/EAL domain proteins can control various output functions, as shown for YhdA in E. coli [Suzuki et al. 2006; Sommerfeldt et al. 2009] and GdpS in Staphylococci [Holland et al. 2008]. However, the direct molecular mechanisms of action of these proteins have remained unknown.

In the present study, we analyzed the function of a degenerate EAL protein in E. coli, YcgF, which also carries a blue-light-sensing BLUF domain at its N terminus. We found that YcgF acts by protein–protein interaction independently of c-di-GMP biochemistry. Purified YcgF did not degrade or bind c-di-GMP [Supplemental Fig. S1], nor did overproduction of an active DGC [YaiC] or an active PDE [YjhF] affect the expression of the YcgF-controlled target gene ycgZ [Supplemental Fig. S2]. Instead, YcgF directly interacts with the MerR-like repressor YcgE [Fig. 2] and, in a blue-light-dependent manner, releases it from the DNA, where YcgE specifically binds to the ycgZ promoter region [Fig. 3; Supplemental Fig. S3]. Thus, YcgF is a blue-light-controlled direct anti-repressor for YcgE [for a summarizing model, see Fig. 7].

As both YcgF and YcgE are two-domain proteins, we studied the role of the specific domains in this interaction. The N-terminal BLUF domain of YcgF alone can be purified as a soluble protein, but it is unable to interact with YcgE. We conclude that the C-terminal EAL domain of YcgF is involved in this interaction, either on its own or in conjunction with the BLUF domain. Consistently, not only the BLUF domain but the entire YcgF protein changes conformation upon blue-light irradiation [Hasegawa et al. 2006; Nakasone et al. 2007]. On the YcgE side, it is the MerR-like DNA-binding N-terminal domain that binds to YcgF [Fig. 2]. The molecular role of the YcgE-CTD is currently unknown, but it seems to contribute to repression as (1) expression of the DNA-binding YcgE-NTD alone is not sufficient to achieve complementation of a ycgE mutation (assayed as repression of ycgZ::lacZ), and (2) expression of YcgE from a leaky overexpression vector resulted in repeatedly picking up inactivating point mutations not only in the DNA-binding helix of YcgE-NTD, but also at a specific position in the C-terminal domain [our unpublished results].

Here, we show that blue light is required for YcgF to release YcgE from its operator in the ycgZ promoter region in vitro [Fig. 3]. In vivo, blue light further increased YcgF-mediated activation of ycgZ expression at low temperature [Fig. 5]. Interestingly, also nonirradiated YcgF can bind to free YcgE [no DNA present] [Fig. 2]. However, only irradiated YcgF can efficiently release YcgE bound to its operator DNA [Fig. 3]. In principle, two different mechanisms could account for this regulation by blue light: Either YcgF binds equally well to free and DNA-bound YcgE, and the blue-light-induced conformational change in YcgF induces a conformational change in the MerR-like DNA-binding N-terminal domain that binds to YcgF [Fig. 2]. The molecular role of the YcgE-CTD is currently unknown, but it seems to contribute to repression as (1) expression of the DNA-binding YcgE-NTD alone is not sufficient to achieve complementation of a ycgE mutation (assayed as repression of ycgZ::lacZ), and (2) expression of YcgE from a leaky overexpression vector resulted in repeatedly picking up inactivating point mutations not only in the DNA-binding helix of YcgE-NTD, but also at a specific position in the C-terminal domain [our unpublished results].

Figure 7. Model for the YcgF/YcgE-controlled small regulatory protein network. At the top of the network, YcgF acts as a direct antagonist for the MerR-like regulator YcgE. The roles of the N- and C-terminal domains of YcgF and YcgE are indicated [for details, see the Discussion]. Inactivation of YcgE results in the induction of eight small proteins [YcgZ, YmgA, YmgB, YmgC, Yil, YnaK, Bdm, and YbgS; all between 71 and 126 amino acids long]. YcgE directly represses the ycgZ-ymgABC operon and down-regulates also yilIL and ynaK by yet uncharacterized mechanisms. Among the small proteins induced, YmgA and YmgB activate the Rcs phosphorelay pathway, and YcgZ has a weak opposite effect. Expression of the small protein genes bdm and ybgS [but not yilIL and ynaK] is activated by YmbB. The bdm gene is a known member of the RcsB regulon; how YmbB affects the ybgS gene has yet to be clarified [therefore, ybgS is not included in the figure]. Several signals are integrated by the YcgF/YcgE pathway: Blue light affects the activity of YcgF protein, low temperature induces the expression of YcgF, YcgE, and σ52; and starvation [and other stresses] results in σ54 accumulation due to post-transcriptional control mechanisms. For further details and references, see the Discussion.
YcgE followed by release from the DNA, or, alternatively, YcgF may bind better to free YcgE as YcgF has to compete with operator DNA for overlapping binding sites in the MerR-like domain of YcgE, and blue light would increase the affinity of YcgF for YcgE such that it competes better with operator DNA. Several observations seem to support the second model. First, increased YcgF:YcgE stoichiometry can clearly induce target gene expression in vivo (overexpression of YcgF from a plasmid as in Fig. 1C, or stronger induction of YcgF by low temperature as in Fig. 5; see also below); this is a key prediction of the competition model, but does not seem consistent with the first model. Also, the first model, in which YcgF binding does not discriminate between free and DNA-bound YcgE, seems at odds with the finding that the order of addition of components and blue-light irradiation clearly affects the efficiency of release of YcgE from the DNA [Fig. 3]. In conclusion, the current data indicate that blue-light irradiation increases YcgF affinity for YcgE, thereby allowing it to release YcgE from its operator site, but this will have to be confirmed by further biochemical and structural analyses.

A similar situation as in the YcgF/YcgE system has been described for CarS, which is a direct antagonist for the MerR-like regulator CarA that controls carotenoid biosynthesis in Myxococcus xanthus. The acidic CarS protein (theoretical pI of 4.8) structurally mimics operator DNA and thereby occludes the DNA-binding site in CarA (Navarro-Aviles et al. 2007). The theoretical pI of the YcgF-EAL domain is also acidic (4.82), which is in contrast to the more consensus-like YcgF-EAL domains of two bacterial species that do not have YcgE (5.29 and 5.28 in Bordetella avium and Alteromonas macleodii, respectively) [see Supplemental Fig. S1] and to the EAL domain of known PDE (e.g., YciR, pI of 6.03).

It should also be noted that the only paralog of YcgE in E. coli, which contains both a MerR-like NTD as well as a YcgE-like CTD, is MlrA. In contrast to the repressor YcgE, this protein acts as an activator that is essential and highly specific for the transcription of the curli activator gene csgD (Brown et al. 2001). The c-di-GMP phosphodiesterase YciR equally selectively inhibits csgD transcription [Weber et al. 2006; Pesavento et al. 2008]. Preliminary data suggest that YciR (a GGDEF+EAL protein) directly interacts with MlrA and that this interaction is important for inhibiting csgD expression (S. Lindenberg, C. Pesavento, and R. Hengge, unpubl.). Thus, control of the activity of regulators by physical interaction with an EAL domain protein may be a more general principle and may be one way to achieve a more local action of GGDEF/EAL proteins, as suggested by several investigators [Jenal and Malone 2006; Kader et al. 2006; Ryan et al. 2006; Weber et al. 2006; Pesavento et al. 2008]. C-di-GMP production and degradation may still play a role in such a complex [as it does for curli regulation; Weber et al. 2006]. Alternatively, as in the case of YcgF/YcgE, it may have been “given up” during the course of evolution [see Supplemental Fig. S1], with the system now relying completely on protein–protein interaction.

Integration of blue light, low temperature, and general stress signals in the YcgF/YcgE signaling pathway

The expression of both ycgF and ycgE as well as their target operon ycgZ-ymgABC is clearly cold-induced [Figs. 4, 5; Polissi et al. 2003, White-Ziegler et al. 2008]. While the ycgZ-ymgABC operon is under σ5 control [Table 1; Weber et al. 2005], and therefore cold induction of σ5 itself [Sledjeski et al. 1996] may contribute to up-regulation of these genes, ycgF and ycgE expression is independent of σ5, and cold induction occurs by a currently unknown mechanism. Our data indicate that a shift to low temperature (16°C) alone can already trigger a YcgF-mediated relief of repression by YcgE, which is further enhanced by blue-light irradiation [Fig. 5]. We noted that low-temperature induction of ycgF expression is clearly stronger than that of ycgE (150-fold and sevenfold, respectively, when cells were shifted from 37°C to 16°C). Blue light promotes a conformational change of YcgF [Hasegawa et al. 2006; Nakasone et al. 2007, Schroeder et al. 2008] that probably increases YcgF affinity for YcgE and allows it to release YcgE from its operator site [as discussed above]. Yet, by simple mass action, the cold-induced increase in the YcgF:YcgE stoichiometry also could have similar consequences, i.e., generate enough active YcgF to sequester YcgE and therefore induce the YcgE regulon. Interestingly, the recovery of the dark ground state of YcgF also is strongly delayed at lower temperature [Schroeder et al. 2008]. Therefore, blue light and low temperature can act in an additive manner on target gene expression.

In addition, the general stress response feeds into this complex regulatory circuit. Five of the eight target genes of the YcgF/YcgE system require σ5 for expression [Table 1]. σ5 accumulates under a variety of stress conditions [nutrient limitation, hyperosmotic, acidic, and other conditions] [Hengge-Aronis 2002]. Given the combination of nutrient scarcity, low temperature, and intensive blue-light irradiation as inducing conditions, it is tempting to speculate that the YcgF/YcgE pathway is important for E. coli growth and/or survival in a marine or freshwater environment.

The YcgF/YcgE pathway controls the expression of small regulatory proteins that modulate biofilm functions via the Rcs two-component pathway

Is blue light just a signal indicative of an aquatic environment, or can blue light also be harmful on its own; i.e., cause cellular damage? We observed that blue-light irradiation indeed reduces growth of E. coli specifically at low temperature (<23°C), but does not lead to increased cell death [Supplemental Figs. S5, S6]. It could be that some particularly sensitive target for blue-light-induced photo-oxidation [Rajagopal et al. 2004] is formed at low temperature only [e.g., cold-adapted phospholipids with unsaturated fatty acid side chains and other modifications; Cronan 2003]. However, mutants defective for YcgF, RcsB, or even σ5 do not show further reduced growth or even cell death upon blue-light irradiation [Supplemental Fig. S6], indicating that the YcgF/YcgE pathway does not
cope with blue-light/cold-induced cellular stress or damage, but uses blue light and low temperature as distinct environmental indicator signals.

What are the output functions of the YcgF/YcgE pathway, and how are these of adaptive value in a nutrient-limited, cold, and radiation-rich environment? The primary output of this pathway is the expression of six small proteins (Fig. 7). Four are encoded in an operon, *ycgZ-ymgABC*, which is under direct control of the YcgE repressor. It is not yet clear whether the target genes *yIL* and *ynaK* are under similar direct control, but they are at least not activated by the primary target Ymgb, as are two additional genes, *bdm* and *ybgS*, which therefore are indirect downstream targets of the YcgF/YcgE pathway (Fig. 6D, our unpublished microarray data). This distinct group of eight coregulated small proteins (78–126 amino acids) is so far unique.

We showed here that Ymgb, and to some extent also Ymga [in particular at 16°C], can strongly promote the synthesis of the exopolysaccharide and biofilm matrix component colanic acid and virtually eliminates the expression of the adhesive curli fibritae genes, whereas YcgZ seemed to partially antagonize these effects [Fig. 6]. In addition, Ymgb moderately stimulated the expression of several acid resistance genes including *gadB*, *gadC*, *gadE*, and *hdeA* (data not shown). These findings are consistent with a recent report showing that Ymgb reduces biofilm formation (tested only in the "crystal violet assay," which, however, is known to be sensitive to curli expression) and promotes survival at low pH [Lee et al. 2007]. Moreover, all Ymgb-regulated functions that we describe here—i.e., colanic acid production, curli biosynthesis, and the expression of acid resistance genes, *bdm* and many other genes—are under direct or indirect control of the Rscc/Rscd/Rcsb two-component phosphorelay system [Gottesman et al. 1985; Hagiwara et al. 2003; Francez-Charlot et al. 2005; Vianney et al. 2005; Castanie-Cornet et al. 2007]. As these Ymgb effects were eliminated in an *rcsc* mutant background [Fig. 6; data not shown], we conclude that Ymgb acts upstream of and via Rcsb [Fig. 7]. Moreover, Ymgb stimulates the activity rather than the expression of the Rscc system, as *rcsb*, *rcsc*, and *rscd* were not among the genes activated by Ymgb. Using the known Rcsb-targeted *bdm* gene as a reporter in an epistasis analysis, we show that also a knockout mutation in *ycgE* affects *bdm* expression via Ymgb and Rcsb [Fig. 6D]. That the YcgF/YcgE/Ymgb pathway can activate the Rcs system is consistent with the *ycgZ-ymgABC* genes belonging to a cohort of genes that exhibit intermediate timing of expression during biofilm development [Domka et al. 2007], which fits the concept that the Rcs system is involved in biofilm maturation rather than initiation [Majdalani and Gottesman 2005; Beloin et al. 2008]. Thus, via Ymgb/Ymga and Rcsb, the YcgF/YcgE system can stimulate biofilm maturation and possibly differential gene expression within a biofilm. In a natural aquatic environment, a thick biofilm matrix may not only afford protection against abiotic stresses, but, e.g., also against grazing microbial predators.

What is the molecular mechanism of action of Ymgb, Ymga, and the other small proteins under YcgF/YcgE control? As Ymgb is a small three-helix protein that exhibits similarity to Hha, a protein that interacts with the DNA-binding protein H-NS, it was speculated that Ymgb might be a novel type of transcriptional (co)regulator [Lee et al. 2007]. As Ymgb clearly acts upstream of and via Rcsb (see above), we propose the hypothesis that Ymgb (and perhaps also Ymga) could be a two-component system "connector" that specifically activates the Rcs phosphorelay system. Connectors are a currently emerging group of typically small proteins (~60–150 amino acids) that influence the activity of specific two-component systems. They provide additional signal input not perceived by the sensory input domains of the sensor kinases, and thereby connect two-component systems to other regulatory pathways. Connectors can affect phosphorylation, phosphotransfer, and dephosphorylation reactions within simple two-component or more complex phosphorelay systems or they may sequester a component of such a pathway [Kato and Groisman 2004; Bougdour et al. 2006, 2008; Eguchi et al. 2007; Kato et al. 2007]. How Ymgb and probably also Ymga and YcgZ affect the activity of the Rcs phosphorelay will have to be investigated in future genetic and biochemical studies. Furthermore, it is conceivable that other small proteins under the control of the YcgF/YcgE system might also act as connectors. In this case, the YcgF/YcgE system would not only modulate biofilm formation, but could reorganize the two-component network, when *E. coli* has to survive in a marine or freshwater environment characterized by starvation, cold, and sun radiation.

Materials and methods

**Bacterial strains and growth conditions**

All strains used in this study are derivatives of the *E. coli* K-12 strain MC4100 [Casadaban 1976]. Mutations were introduced by P1 transduction [Miller 1972]. The *rpoS359::Tn10* mutation was described previously [Lange and Henge-Aronis 1991]. The newly constructed mutant alleles *ycgF::kan*, *ycgE::cat*, *Δ[ycgZ-ymgABC]::cat*, *ymgb::kan*, and *rcsb::kan* are all deletion-insertion mutations generated by one-step inactivation according to Datsenko and Wanner [2000] using the primers listed in Supplemental Table S2. In order to avoid polarity on downstream genes in an operon, insertions were also flipped out according to Datsenko and Wanner [2000]. The construction of plasmids and single-copy *lacZ* reporter fusions is described in detail in the Supplemental Material [including relevant references]. Note that *ycgF* and *ycgE* do not constitute an operon (for experimental evidence, see Supplemental Material). Thus, the *ycgF* insertion is also not polar on *ycgE*.

Cells were grown in LB medium [Miller 1972] under aeration at the temperatures indicated in the figure legends. Antibiotics and IPTG were added as recommended [Miller 1972]. Growth was monitored by measuring the optical density at 578 nm ([OD]~578). Blue-light (470 nm) irradiation of growing bacterial cultures or protein samples was performed with an intensity of −100 μM/m2/sec1 using 112 5-mm LEDs (HLMP-HB57-LP0xx, Avago Technologies) arranged as a 16 × 7 array on a 16 × 10-cm plate. Survival of blue-light exposure during growth was monitored.
by determining colony forming units (on LB plates) in samples taken from the irradiated cultures.

**Northern blot analysis**

RNA was prepared and Northern blot analysis was performed as described before (Weber et al. 2006) using a DIG-labeled probe corresponding to the ycgZ coding region (obtained with primers listed in Supplemental Table S2).

**Microarray analysis**

Cell lysis, RNA isolation, DNasel treatment, and phenol/chloroform extraction were previously described (Weber et al. 2006). *E. coli* K-12 microarrays containing 4288 gene-specific 50mer oligonucleotide probes representing the whole *E. coli* genome (MGW) were used for hybridization with Cs3/dCTP-labeled cDNA as described in the supplemental material of Pesavento et al. (2008). Fluorescence detection and image analysis was carried out as described before (Weber et al. 2005) using a GenePix 4100A (Axon, Inc.) laser scanner. Each microarray experiment was repeated two times [biological replicates, with a dye swap in cDNA labeling]. Genes were considered differentially regulated when (1) signal-to-noise ratios exceeded a factor of three, (2) the sum of median intensity counts was >200, and (3) relative RNA level differences [ratios] were at least twofold in both of the two independent experiments. The original data sets can be found in the Array Express database (accession no. E-MEXP-1872 for the YcgE+/− data, E-MEXP-1877 for the YcgE+/− data).

**In vitro protein–protein interaction assay and SDS-PAGE**

Purification of YcgF and YcgE and their respective domains with TRX-His6-S and/or His6 tags is described in detail in the Supplemental Material. In vitro interaction assays were performed by affinity chromatography (‘pull-down’ assays) on S-protein agarose using 0.65 nmol of YcgE or the YcgE domains and 1.3 nmol of YcgF or YcgF-NTD. The procedure was as described by Mika and Hengge (2005), with the exception that the samples were washed five times instead of twice only, and elution was left in the dark in parallel). Samples were then run on a 12% SDS-PAGE and proteins were detected by Coomassie brilliant blue staining.

**Protein–DNA interaction assays**

Electrophoretic mobility shift assays (EMSA) were essentially performed as described (Mika and Hengge 2005). DNA fragments used carried either the ycgZ promoter or the ycgZ-coding regions and were obtained with the primers listed in Supplemental Table S2. Twenty-microliter reaction mixtures contained purified YcgE protein [0.47 μM] and 18 ng of the DNA fragments in 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 5 mM DTT, and 5% glycerol. YcgE, YcgF, and DNA fragments were added in the order and concentrations stated in the text and the figure legends (at room temperature). Preincubation of two components was for 20 min before the third component was added. Blue-light irradiation was also for 20 min (with control samples left in the dark in parallel). Samples were then run on a 1% agarose gel in TAE buffer and stained with ethidium bromide.

**Determination of c-di-GMP binding and phosphodiesterase activity**

In vitro synthesis of radiolabeled c-di-GMP by the purified DGC PleD*, and purification of c-di-GMP was done as described (Paul et al. 2004; Weber et al. 2006). Binding of radiolabeled c-di-GMP to purified proteins in vitro was detected by UV cross-linking according to Christen et al. (2007). Phosphodiesterase activity was tested with purified YcgF and YcgF^{H193L/Q195R} [see the Supplemental Material for details] and C-terminally His6-tagged YhJH (Pesavento et al. 2008) using radiolabeled c-di-GMP as a substrate following the procedure described in detail by Weber et al. (2006).

**Determination of β-galactosidase activity**

β-galactosidase activity was assayed by use of o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate and is reported as micromoles of o-nitrophenol per minute per milligram of cellular protein (Miller 1972). Experiments showing the expression of lacZ fusions along the entire growth cycle were done at least twice, and a representative experiment is shown. Single-value data are the average of at least two independent measurements.

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The BLUF-EAL protein YcgF acts as a direct anti-repressor in a blue-light response of *Escherichia coli*

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