Supplementary Material for:

Molecular function and potential evolution of the biofilm-modulating blue light-signaling pathway of *Escherichia coli*

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Figure S1. EAL-domain sequence alignment of various BluF homologs in comparison to the EAL-domain of the active phosphodiesterase (PDE) YahA. The alignment was generated using CLUSTAL W (Larkin et al., 2007) and further adjusted manually to highlight key amino acid positions, which have been assigned to conserved amino acids in active PDEs required for c-di-GMP binding (red), Mg\(^{2+}\)-binding (blue) and catalysis (green) using RocR from Pseudomonas aeruginosa as a model system (Rao et al., 2008). YahA from E. coli was experimentally demonstrated to be active as a c-di-GMP specific phosphodiesterase by Schmidt et al. (2005). The EAL domain sequences of BluF homologs from Bordetella avium 197N (BAV1542), Klebsiella pneumoniae 342 (KPK_2789 and KPK_3794), Enterobacter sp. 638 (Ent638_2032 and Ent638_1757) and for E. coli K-12 (BluF) are depicted. KPK_3794 and Ent638_1757 contain a partially degenerate EAL-domain and are encoded next to the genes for BluR homologs (see Fig. 1C). KPK_2789 (BlrP1), which is encoded independently of BluR, contains a conserved EAL-domain and was shown to act as a blue-light regulated PDE (Barends et al., 2009). Due to amino acid sequence, Ent638_2032 and BAV1542 very likely represent active PDEs and are not associated with BluR in terms of their genetic location. BluF from E.coli, which is encoded next the gene for BluR lacks all four amino acids involved in c-di-GMP binding, one of eight amino acids involved in Mg\(^{2+}\)-binding and an essential catalytic glutamate residue. It represents the most degenerated BluF variant and was shown to be unable to bind or degrade c-di-GMP, but to act as anti-repressor protein (Tschowri et al., 2009). Key amino acids essential for PDE activity that were re-introduced into BluF (resulting in BluF M2-M8 variants; see Figs. 2, 3, 4) are indicated by asterisks.
**Figure S2. Sequence comparison of BluR with other MerR-like proteins from E. coli.**

Alignments were performed using CLUSTAL W (Larkin et al., 2007). Residues identical to BluR were manually coloured in red. The protein sequences for BluR (Tschowri et al., 2009), MlrA (Brown et al., 2001), SoxR (Nunoshiba et al., 1992), CueR (Outten et al., 2000), and ZntR (Brocklehurst et al., 1999) are shown. The conserved DNA binding helix-turn-helix region is indicated above the alignment according to (Brown et al., 2001). The N-terminal domains were determined using SMART (Letunic et al., 2012) and are highlighted in gray. Based on the analysis using CLUSTAL W, BluR shows 49% identity to MlrA, 12% identity to SoxR, 17% to CueR and 14% to ZntR.
Figure S3. Restoration of consensus amino acids in the EAL domain of BluF does not reconstitute PDE activity. (A) Phosphodiesterase assay with radiolabeled c-di-GMP. Wildtype BluF (WT), the mutant variants BluF-M4 (I193L+Q195R+M362E+A365E), BluF-M5 (M4+T247N), BluF-M6 (M5+H177Q), BluF-M7 (M6+H306D), BluF-M8 (M7+S328D) and the known c-di-GMP phosphodiesterase YhjH were purified and tested for phosphodiesterase activity performed under blue-light conditions according to (Tschowri et al., 2009). (B) Detection of c-di-GMP binding by UV-crosslinking. The same purified BluF proteins as used in (A) as well as the mutationally activated diguanylate cyclase PleD* were incubated with radiolabeled c-di-GMP and UV-crosslinked. (C) Motility of a W3110 yhjH::kan mutant carrying derivatives of pQE30Xa containing wild-type or mutated bluF variants as in (A) including the M2 variant (BluF<sup>I193L,Q195R</sup>). YhjH was expressed from the low copy number p(tac) vector pCAB18. Cells were incubated on motility plates containing 100µg/ml ampicillin at 28°C for 5 hours.
Figure S4. Two-hybrid analysis to detect *in-vivo* interaction between BluF-WT and BluF-M8 and MrA as well as the single domains of MrA and BluR. Using the Bacterio-Match two-hybrid system, reporter cells were co-transformed with a vector control and/or derivatives of the pBT and pTRG plasmids. Wildtype (WT) BluF and the M8 mutant version (I193L+Q195R+M362E+A365E+T247N+H177Q+H306D+S328D) were expressed as hybrid proteins fused to cI-NTD on pBT. MrA as well as the N-terminal (NTD) and C-terminal (CTD) domains of MrA and BluR were synthetised from pTRG as fusions to RNAP alpha-NTD. Interaction was detected by growth of cotransformants in the presence of 5 mM His3 inhibitor 3-AT (selective medium) at 37°C for 24 hours following incubation at 28°C for 48 hours. Numbers of cotransformants able to grow on selective screening medium normalized to numbers on non-selective medium are shown.
**Supplementary Table**

**Table S1.** Oligonucleotides used in the present study

I. Primers for generating knockout mutations by one-step inactivation

| Primer Name | Sequence | Description |
|-------------|----------|-------------|
| \( \Delta \text{ymgA::kan} \) | 5’- ATGAAGTCGTTATGAAGACATCTGATAATGAACGTATAA AATATGAAATTGTGGCTGGAGCTGCTTC -3’ 5’- TGTATTTCTGTATTTTCTATTACATTGAACATACTTTTATT GTCAGCTGTATTCCGGGGATCCGTCGACC -3’ |

II. Primers for generating DNA fragments used for electrophoretic mobility assays

| Primer Name | Sequence | Description |
|-------------|----------|-------------|
| \( csgD \) | 5’- CACCGAATATTTTTTATATGC -3’ 5’- CAATCTAGGCATTACAATCTTA -3’ |
| \( ycgZ \) | 5’- ATGCATTAGACACTATTTGCA -3’ 5’- GTGATTGCTCCCGCAGAATC -3’ |

III. Primers for generating a \( ycgZ \) promoter-containing DNA fragment for DNase I footprint assays

| Primer Name | Sequence | Description |
|-------------|----------|-------------|
| \( ycgZp-fw \) | 5’- DIG-GTATAGATTGTCAGTTAAATGATGC -3’ |
| \( ycgZp-rev \) | 5’- GATGCGATGCTACGCTCTG -3’ |

IV. Primers for determining transcriptional start sites by primer extension

| Primer Name | Sequence | Description |
|-------------|----------|-------------|
| \( bluR \) | 5’- GCCCAAAATTCTCCTCCTTGCGTGTC -3’ |
| \( bluF \) | 5’- CGTCGTCACGTATATGGCTACG -3’ |
| \( ycgZ \) | 5’- GTGATTGCTCCCGCAGAATC -3’ |

V. Primers for cloning and mutagenizing the \( ycgZ \) promoter on pJL28

| Primer Name | Sequence | Description |
|-------------|----------|-------------|
| \( ycgZ\)-EcoRI-fw | 5’- CGGAATTCGACAGGTTCTGATCGTCACGTATATGC -3’ 5’- CGTGACAAAGCT7GAGTGATTGCCGCCGAGAATCC -3’ |
| \( ycgZ\)-HindIII-rev | 5’- ATCATTTAGTACACATATTTTCTACATAG -3’ 5’- GAAATATGTGACCTAATGAT -3’ |
| C-39A, T-38G | 5’- ATCATTTAGTACACATATTTTCTACATAG -3’ 5’- GAAATATGTGACCTAATGAT -3’ |
| C-39A, T-38G, G-37A | 5’- ATCATTTAGTACACATATTTTCTACATAG -3’ 5’- GAAATATGTGACCTAATGAT -3’ |
| G-24A, C-21A | 5’- CACATATTTCATACGAGTCTTG -3’ 5’- GCAAACCTTTTATGAAATATGTC -3’ |

*Nucleotides in **bold** indicate mutations introduced, nucleotides in **bold italics** indicate restriction sites.*
### VI. Primers used for cloning of *bluR* into pCAB18

| Primer          | Sequence                                                |
|-----------------|---------------------------------------------------------|
| *bluR*-EcoRI-fw | 5’- CGGAATTCAGGAGGTACTGAGGTGGCTTATTACAGCATTGGTG-3’      |
| *bluR*-HindIII-rev | 5’- CGTGACAAGCTTCGCAACGTTGACGAAACATCACC-3’           |

### VII. Primers used for cloning into pBT

| Primer                | Sequence                                                                 |
|-----------------------|--------------------------------------------------------------------------|
| *bluF* (WT) and       | 5’- CGGAATTCCTTACCACCCTTTATTTATC-3’                                      |
| *bluF* (M8)           | 5’- CCGCTCAGAGATTTTTTCTGTGCGTGTTGCAAGGAG-3’                               |
| *bluF*-NTD            | 5’- CGGAATTCCTTACCACCCTTTATTTATC-3’                                      |
| *bluF*-CTD (WT) and   | 5’- CGGAATTCCTTACCACCCTTTATTTATC-3’                                      |
| *bluF*-CTD (M8)       | 5’- CGCTCAGAGATTTTTTCTGTGCGTGTTGCAAGGAG-3’                               |
| *rcsD*                | 5’- CGGAATTCCTTACCACCCTTTATTTATC-3’                                      |

### VIII. Primers used for cloning into pTRG

| Primer          | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| *mlrA*          | 5’- GTACGAATTCAGATGGCGCTTTACACAAATTTGGTG-3’                               |
| *mlrA*-NTD      | 5’- GTACGAATTCAGATGGCGCTTTACACAAATTTGGTG-3’                               |
| *mlrA*-CTD      | 5’- GTACGAATTCAGATGGCGCTTTACACAAATTTGGTG-3’                               |
| *bluR*          | 5’- CGGAATTCAGGAGGTACTGAGGTGGCTTATTACAGCATTGGTG-3’                        |
| *bluR*-NTD      | 5’- CGGAATTCAGGAGGTACTGAGGTGGCTTATTACAGCATTGGTG-3’                        |
| *bluR*-CTD      | 5’- CGGAATTCAGGAGGTACTGAGGTGGCTTATTACAGCATTGGTG-3’                        |
| *rcsC*          | 5’- CGGAATTCAGGAGGTACTGAGGTGGCTTATTACAGCATTGGTG-3’                        |
IX. Primers used for cloning and mutagenizing *bluF* on pQE30Xa-LacIq

| Primer                  | Sequence                                      |
|-------------------------|-----------------------------------------------|
| bluF-Phos-fw            | 5’- Phos-ATGCTTACCCACTATTATATC-3’              |
| bluF-HindIII-rev        | 5’- GACAAGCCTTTATTTTTTCTCTGGCCACGCTATG-3’     |
| H177Q                   | 5’- TTTGCCCTTCAGCCTATTGTGC-3’                 |
|                         | 5’- GACAATAGGCTGAAGGAGCAA-3’                  |
| H306D                   | 5’- GCAATTTGATGCATTGCGCA-3’                   |
|                         | 5’- TGGGCCAAAGTCATCAATTGC-3’                  |
| M362E, A365E            | 5’- GTCAGTGCTGAAGCGTGGGAAACACCAGAA-3’         |
|                         | 5’- TTCTGGTGTTCACGCCTTCACGACTGAC-3’           |
| S328D                   | 5’- ATTAAATCGACCAGAAATTG-3’                   |
|                         | 5’- CAATTCCTGCGTATTTTAAT-3’                   |
| T247N                   | 5’- TTACCTATGAAACCTGGTTAAC-3’                 |
|                         | 5’- GTTACCCAGGTTCACTAGGTAA-3’                 |

X. Primers used for cloning of *mlrA* into pETDuet

| Primer                  | Sequence                                      |
|-------------------------|-----------------------------------------------|
| mlrA-NdeI               | 5’- GTCCATATGGCCGCTTTACACAAATTGGT-3’           |
| mlrA-XhoI               | 5’- GCTCTCGAGGATGCGCAGGTTGGAAATATCATGGG-3’    |

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