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The EAL domain protein YciR acts as a trigger enzyme in a c-di-GMP signalling cascade in *E. coli* biofilm control

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C-di-GMP—which is produced by diguanylate cyclases (DGC) and degraded by specific phosphodiesterases (PDEs)—is a ubiquitous second messenger in bacterial biofilm formation. In *Escherichia coli*, several DGCs (YegE, YdaM) and PDEs (YhjH, YciR) and the MerR-like transcription factor MlrA regulate the transcription of *csgD*, which encodes a biofilm regulator essential for producing amyloid curli fibres of the biofilm matrix. Here, we demonstrate that this system operates as a signalling cascade, in which c-di-GMP controlled by the DGC/PDE pair YegE/YhjH (module I) regulates the activity of the YdaM/YciR pair (module II). Via multiple direct interactions, the two module II proteins form a signalling complex with MlrA. YciR acts as a connector between modules I and II and functions as a trigger enzyme: its direct inhibition of the DGC YdaM is relieved when it binds and degrades c-di-GMP generated by module I. As a consequence, YdaM then generates c-di-GMP and—by direct and specific interaction—activates MlrA to stimulate *csgD* transcription. Trigger enzymes may represent a general principle in local c-di-GMP signalling.
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Introduction

Nucleotide second messenger signalling in bacteria has recently moved back into the focus of attention when bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) was discovered to ubiquitously promote biofilm formation (for recent reviews, see Wolfe and Visick, 2010). C-di-GMP is synthesised by diguanylate cyclases (DGCs) characterised by GGDEF domains, with this amino-acid motif constituting the active site (A-site). Its degradation is catalysed by specific 3′-phosphodiesterases (PDEs) with either EAL or HD-GYP domains (Schirmer and Jenal, 2009). C-di-GMP-binding effector components include proteins of different families as well as RNAs (Hengge, 2010a). Protein effectors operate as transcription factors (Lee *et al*, 2007; Hickman and Harwood, 2008; Leduc and Roberts, 2009) or by directly interacting with enzymes (Amikam and Galperin, 2006) or complex cellular structures (Duerig *et al*, 2009; Boehm *et al*, 2010; Fang and Gomelsky, 2010; Paul *et al*, 2010). RNA-based c-di-GMP effectors are 5′-untranslated regions of mRNA molecules that act as c-di-GMP-controlled riboswitches (Sudarsan *et al*, 2008; Smith *et al*, 2009; Lee *et al*, 2010). c-di-GMP nearly ubiquitously downregulates bacterial motility and stimulates biofilm formation, that is, the synthesis of bacterial adhesins and extracellular matrix components. Also, virulence genes involved in acute infections by some pathogenic bacteria are downregulated by c-di-GMP, while chronic infections are often associated with high c-di-GMP levels and biofilm formation (Tamayo *et al*, 2007).

The minimal 'module' required for c-di-GMP signalling consists of a DGC, a PDE and an effector component that directly controls the output of a specific target (Hengge, 2009). Yet, many bacterial species possess multiple DGCs and PDEs (Galperin, 2005). For instance, *E. coli* K-12 has 29 GGDEF and EAL domain proteins (Hengge, 2010b). This questioned the concept that the activities of dozens of DGCs and PDEs simply converge to control a cellular pool of freely diffusible c-di-GMP and therefore a common spectrum of outputs differentiated only by different affinities of various c-di-GMP-binding effectors. As an alternative concept, sequestration of c-di-GMP control modules has been suggested, which would allow different modules to act on different outputs with no or minimal 'cross-talk' between each other (Jenal and Malone, 2006; Kader *et al*, 2006; Kulasakara *et al*, 2006; Weber *et al*, 2006; Güvener and Harwood, 2007; Hengge, 2009; Ryan *et al*, 2010). Temporal sequestration of c-di-GMP signalling modules can be achieved by differential regulation of expression of specific DGCs, PDEs and effectors. Functional sequestration, that is, operation on different targets in parallel, implies the formation of multiprotein complexes, in which local c-di-GMP concentrations and/or direct protein–protein interactions are of key importance for the regulatory output (Hengge, 2009).

A candidate for such local c-di-GMP signalling is provided by the system that regulates the expression of CsgD (Pesavento *et al*, 2008), a key biofilm regulator that activates the genes for amyloid curli fibres which accumulate in the biofilm matrix of *E. coli* and other enteric bacteria (Römling *et al*, 2000). Activation of the *csgD* promoter requires RNA polymerase (RNAP) containing the stationary phase sigma subunit σ^S (RpoS) and the regulator MlrA, which is a MerR-like transcription factor whose expression is itself σ^S-dependent (Brown *et al*, 2001;

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Weber *et al*, 2006). In addition, two c-di-GMP control modules activate *csgD* transcription (Figure 1A). One consists of the DGCs YegE and YedQ (the latter contributes to a very minor extent only) and the antagonistically acting PDE YhjH. This system downregulates flagellar activity via the c-di-GMP-binding YcgR protein and activates *csgD* transcription in a YcgR-independent manner, that is, via an unknown effector component (Girgis *et al*, 2007; Pesavento *et al*, 2008; Boehm *et al*, 2010). Since c-di-GMP modulated by this system acts by more than one effector component on different targets, it is most likely free to diffuse (Pesavento *et al*, 2008). The second c-di-GMP control module involved consists of the DGC YdaM and its cognate PDE YciR. *ydaM* and *yciR* knockout mutations strongly affect *csgD* transcription and curli fimbriae synthesis, but do not influence motility, which is in contrast to the phenotypes observed with *yegE* and *yhjH* mutants (Weber *et al*, 2006; Pesavento *et al*, 2008). Microarray transcriptome studies (Weber *et al*, 2006) indicated that the YdaM/YciR system as well as the transcription factor MlrA act highly specifically

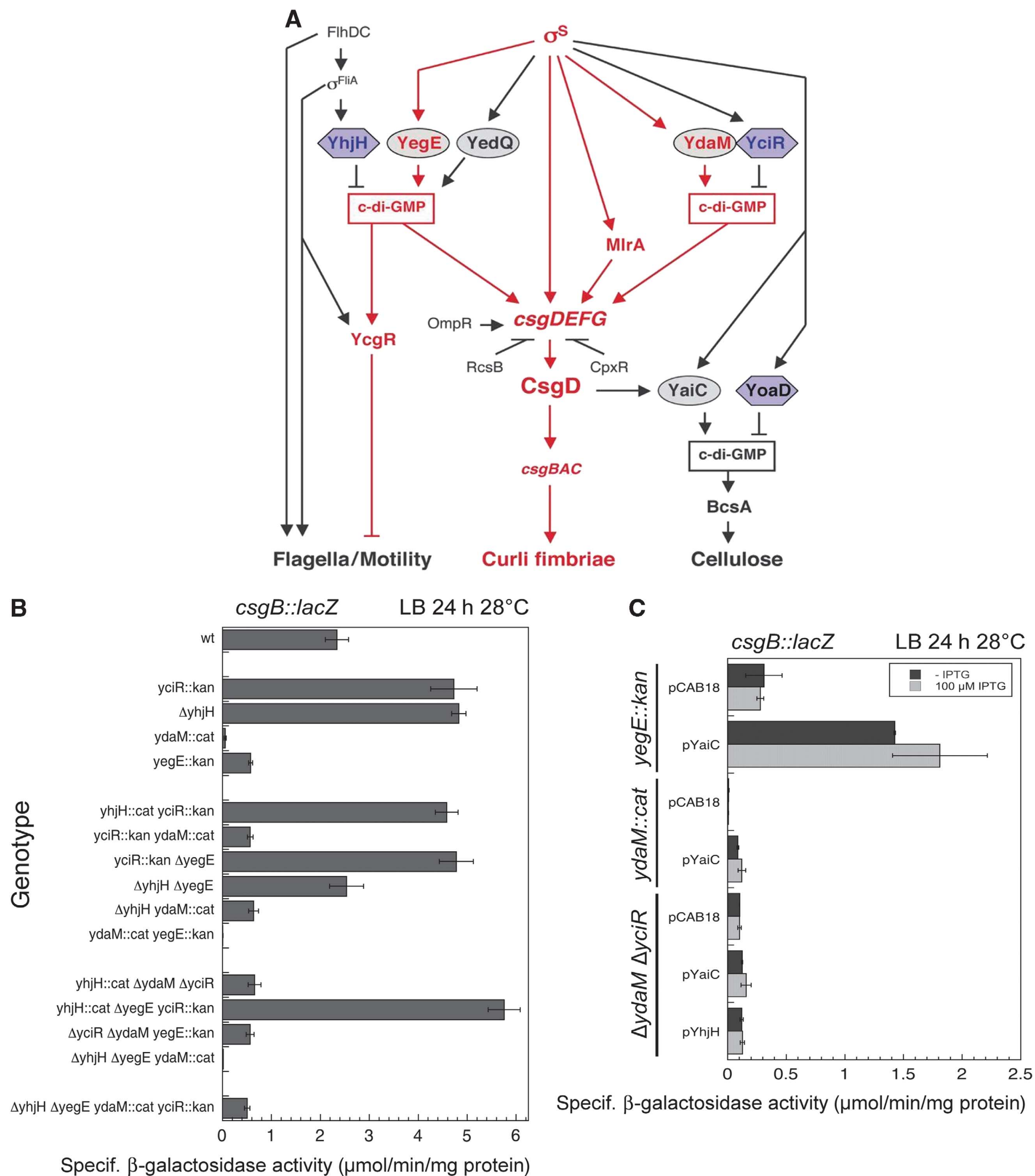

Figure 1 Integration of two c-di-GMP control modules in the regulation of the biofilm regulator CsgD. (**A**) Components involved in the regulation of the biofilm regulator CsgD and curli fibres in *E. coli*. DGCs are indicated by ovals, PDEs by hexagons, DGCs and PDEs relevant for this study are labelled by red and blue letters, respectively. This figure is a revised version of a figure previously published in the supplement of Mika *et al* (2012). (**B**) Curli gene expression in mutants with single or multiple knockout mutations in the YegE/YhjH and YdaM/YciR c-di-GMP control modules. (**C**) Curli gene expression in strains with mutations in YegE, YdaM and YciR that also carry low copy number plasmids (derived from the vector pCAB18) expressing the IPTG-inducible DGC YaiC or PDE YhjH. Derivatives of *E. coli* K-12 W3110 carrying a single copy *csgB*::*lacZ* reporter fusion as well as the indicated mutant alleles and plasmids were grown in LB at 28°C for 24 h and optical densities (OD_{578}) as well as specific β-galactosidase activities were determined.

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on *csgD* transcription only. This suggested that the YdaM/YciR system could be a candidate for local signalling and may act via MlrA. In addition, the question arose how this system is integrated with the more globally acting YegE/YhjH module.

In this study, we demonstrate that these two c-di-GMP control modules function in a cascade, with YciR acting as a bi-functional trigger enzyme (Commichau and Stülke, 2008) that connects modules I and II. Thus, the enzymatic activity of YciR, that is, the binding and degradation of c-di-GMP generated by module I (YegE/YhjH), interferes with its second activity, that is, an inhibition of YdaM by direct interaction. As a consequence, high cellular c-di-GMP levels relieve this inhibition and thus allow YdaM to also generate c-di-GMP and—again by direct interaction—to activate the transcription factor MlrA, which then drives *csgD* transcription. Such a c-di-GMP control cascade, which implies local signalling, as well as a c-di-GMP-related enzyme acting as a trigger enzyme represent novel concepts in c-di-GMP signalling.

Results

Genetic analysis of the interplay of two c-di-GMP control modules in the regulation of the biofilm regulator CsgD

Knockout mutations in the two DGC genes *yegE* and *ydaM* strongly reduce CsgD and therefore curli expression, whereas mutations in the PDE genes *yhjH* or *yciR* generate the opposite phenotype (Weber *et al*, 2006; Pesavento *et al*, 2008). These effects can easily be assayed with a *lacZ* reporter fusion to the CsgD target gene *csgB* (Figure 1B).

In an epistasis analysis designed to get first hints on how the two c-di-GMP modules, that is, YegE/YhjH and YdaM/YciR, are integrated in the control of CsgD and curli, we combined *yegE*, *yhjH*, *ydaM* and *yciR* knockout alleles in all possible double, triple and quadruple mutants and tested the effects on the expression of *csgB*::*lacZ* (Figure 1B). Overall, we observed four distinct levels of expression of *csgB* with different combinations of mutations (Figure 1B): (i) wild-type level of expression, (ii) hyperactivated expression (approximately twice as high as wild-type expression), (iii) c-di-GMP-independent 'basal' expression (in the quadruple mutant) and (iv) hyperrepressed, that is, essentially no expression (e.g., in the *ydaM* mutant).

Directly comparing *csgB*::*lacZ* expression levels in distinct mutants (Figure 1B) allowed first insights into signalling relationships. First, the effects of knocking out the two PDEs YhjH and YciR were not additive. Second, in a *yciR* mutant background, also knocking out the DGC YdaM strongly reduced *csgB* expression (to the 'basal' level), whereas knocking out the other DGC, that is, YegE, did not produce any effect. In other words, a YciR-deficient strain is 'blind' to what happens to the components of the YegE/YhjH system, but still clearly reacts to a knockout of the DGC YdaM. This epistatic asymmetry suggested that the YegE/YhjH module acts upstream of and via the YdaM/YciR module on CsgD and curli expression.

Furthermore, the quadruple mutant exhibited a 'basal' expression which by definition is unregulated by c-di-GMP, since those DGCs and PDEs that affect curli expression (Pesavento *et al*, 2008) are all eliminated. However, the triple mutant, in which YciR is still present, showed no expression at all, that is, YciR *alone* still has the potential to further downregulate 'basal' expression. This YciR-dependent inhibition could also be seen when the *ydaM* mutant was compared to the *ydaM yciR* double mutant. By contrast, the other PDE, YhjH, does not have this regulatory power, since *csgB* expression was similar in the quadruple mutant and the triple mutant with an intact *yhjH*$^{+}$ allele. Yet, YhjH *is* able to downregulate *csgB* expression in the absence of YdaM, provided its cognate DGC YegE as well as YciR are present (compare the *ydaM* and *ydaM yhjH* mutants). Conversely, when the DGC YegE was present but not its antagonistic PDE YhjH (again in the absence of YdaM), YciR did *not* exert its inhibitory effect on the basal level of *csgB::lacZ* expression (the *yhjH ydaM* and the *yhjH ydaM yciR* strains showed similar *csgB* expression). Again, all these data indicate that the YegE/YhjH module acts upstream and actually can exert its effect even via YciR alone (in the absence of YdaM). In the absence of YdaM *and* YciR, however, system output becomes entirely unresponsive to variations in c-di-GMP—neither mutations in the genes for any DGC or PDE (Supplementary Figure 1) nor artificial overproduction of a DGC (YaiC) or a PDE (YhjH) (Figure 1C) generated any effect on *csgB::lacZ* expression. All these data point to YciR as the component that responds to the c-di-GMP generated by YegE and thereby connects the two signalling modules.

Another interesting observation was an 'assymmetric' behaviour of the two DGCs YegE (module I) and YdaM (module II) (Figure 1C). Whereas the *yegE* mutation could be fully complemented by the heterologous DGC YaiC (expressed from a low copy number plasmid; compare to wild-type levels of *csgB::lacZ* expression shown in Figure 1B), this was not the case for the *ydaM* mutation. In the latter mutant, the presence of the YaiC-encoding plasmid increased expression of *csgB::lacZ* only slightly to the same point as by introducing a secondary mutation in *yciR* (Figure 1C), indicating that c-di-GMP generated by YaiC just relieves inhibition by YciR, but does not compensate for the lack of YdaM. Overall, these 'assymmetric' behaviour indicates that YegE is a c-di-GMP releasing DGC, whereas YdaM not only has DGC activity (Weber *et al*, 2006) (and see below), but also plays a more complex and specific role in the control of *csgD* and curli expression.

The other essential and highly specific player in the control of *csgD* and therefore *csgB::lacZ* is the transcription factor MlrA (Brown *et al*, 2001; Weber *et al*, 2006). With respect to *csgB::lacZ* expression, knocking out *mlrA* was epistatic to mutations in *ydaM*, *yciR*, *yegE* and *yhjH* in various combinations, indicating that the two c-di-GMP control modules act upstream of MlrA (Supplementary Figure 2A). The finding that expression of MlrA itself was not affected by mutations in *ydaM* and *yciR* (Supplementary Figure 2B) indicates that YdaM/YciR controls the *activity* of MlrA. In the absence of all relevant c-di-GMP signalling (i.e., in the *yegE yhjH ydaM yciR* quadruple mutant), the low 'basal' *csgB::lacZ* expression was fully dependent on a basal activity of MlrA (Supplementary Figure 2A).

Overall, the results of this genetic analysis suggested the following working hypothesis: (i) the YegE/YhjH c-di-GMP control module acts upstream of the YdaM/YciR module; (ii) YciR acts as a connector or mediator between the two

modules, with c-di-GMP modulated by YegE/YhjH somehow counteracting the inhibitory role of YciR in the control of *csgD*; (iii) the YdaM/YciR module affects the activity of the transcription factor MlrA; and (iv) the DGC YdaM is not only essential for *csgD* expression, but also plays a highly specific role that goes beyond just producing c-di-GMP. All this would be consistent with the hypothesis already-mentioned above that YdaM/YciR may be a locally operating module that directly cooperates with MlrA to activate *csgD* transcription. In the following, we focus on more mechanistic aspects of this complex c-di-GMP signalling pathway by mainly addressing two questions: Do YdaM, YciR and MlrA exhibit the protein–protein interactions that may be expected for such a locally operating system? And, how does YciR exert its putative function as a mediator between the YegE/YhjH module and the downstream components of the pathway, that is, YdaM and MlrA?

Complex formation between the DGC YdaM, the PDE YciR and the MerR-like transcription factor MlrA in vitro

Putative protein–protein interactions between YdaM, YciR and MlrA were assayed by affinity chromatography ('pull-down') experiments. YdaM and MlrA were expressed with S tags that bind to S-protein agarose (YciR was found to strongly aggregate when expressed with an S tag). All three proteins were also expressed with His6 tags that served for detection of potentially interacting and therefore co-eluting proteins by immunoblot analysis. For the pull-down experiments we used cellular extracts instead of purified proteins, because in the presence of the other cellular proteins only highly specific interactions should be detected. Each of the extracts used contained only one of the tagged proteins.

By affinity chromatography on S-protein agarose, YdaM-S in one extract could pull-down YciR-His6 from the second extract. This interaction was specific, since the similarly expressed EAL domain protein and PDE, YhjH-His6, was not bound (Figure 2). A potential interaction of YdaM-S with MlrA-His6 could not be tested since the latter alone bound to S-protein agarose. However, MlrA-S as a bait could pull down YdaM-His6 as well as YciR-His6, no matter whether only two or all three extracts were combined

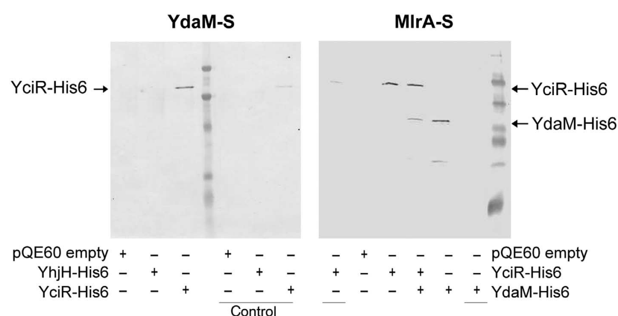

Figure 2 *In vitro* interactions between the diguanylate cyclase YdaM, the phosphodiesterase YciR and the transcription factor MlrA. By affinity chromatography of extracts of total soluble cellular proteins, S-tagged YdaM (left panel) and S-tagged MlrA (right panel) were bound to S-protein agarose and secondary extracts containing His-tagged YciR, YdaM or YhjH as indicated or no His-tagged protein ('pQE60 empty') were added. His-tagged proteins co-eluted with the S-tagged bait proteins were detected by immunoblotting with anti-His antibodies. 'Control' samples did not include the S-tagged proteins. Unlabelled lanes contain coloured size markers.

(Figure 2). The same interactions of MlrA with YdaM and YciR were also observed when the unspecific but strong interaction of MlrA-His6 with S-protein agarose was exploited for the pull-down experiments (data not shown). Moreover, in protein purification experiments performed in parallel, we always observed co-purification with YciR of proteins that by mass spectrometry were identified as MlrA and YdaM, which also indicated that these proteins do interact (data not shown). Taken together, these data indicated that the DGC YdaM, the PDE YciR and the transcription factor MlrA can all bind to each other.

In vivo interaction of YdaM, YciR and MlrA and identification of interacting domains

YdaM, YciR and MlrA have complex domain structures (see Figure 3B). Both YdaM and YciR harbour N-terminal extensions containing PAS domains of unknown function, the GGDEF domain in YdaM provides the DGC activity, whereas YciR has a GGDEF domain of unknown function and a C-terminal EAL domain with PDE activity (Weber *et al*, 2006). As a MerR-like transcription factor, MlrA has an N-terminal DNA-binding domain featuring a helix-turn-helix motif, followed by a coiled-coil linker and a C-terminal putative ligand-binding domain (Brown *et al*, 2003).

In order to confirm the interaction of YdaM, YciR and MlrA *in vivo* and to assign their interactions to specific domains, we used a bacterial two-hybrid system, in which the two potentially interacting proteins or domains are fused to the N-terminal domain of the cI repressor of phage lambda (cI-NTD; expressed from pBT) and to the N-terminal domain of the alpha-subunit of *E. coli* RNAP (alpha-NTD; expressed from pTRG) (Dove and Hochschild, 2004). Interaction of the two fusion proteins in co-transformants drives the expression of the yeast *His3* gene, which is required for growth of the histidine-auxotrophic *E. coli* host on selective plates (see Materials and methods for details). Full-size proteins as well as isolated domains of YciR and MlrA were fused to cI-NTD and alpha-NTD, respectively, YdaM as well as its domains were fused to both components in order to allow for all combinations. As a positive control, we used corresponding constructs with σ^S (RpoS) and RssB, a tightly σ^S-binding proteolytic targeting factor (Becker *et al*, 1999). In all the binary combinations tested (YdaM/YciR, YciR/MlrA, etc), we combined the two full-size proteins, one full-size protein with the domains of the other and vice versa, as well as domains with domains (Figure 3A).

The two-hybrid system clearly reproduced the interaction between YdaM and YciR (see Figure 3B for a graphical summary). Using YdaM domains together with full-size YciR showed the YdaM-NTD to be involved in interaction with YciR. In YciR, the EAL domain was the most strongly interacting domain, but also the YciR-NTD and YciR-GGDEF exhibited some interaction with the YdaM-NTD, consistent with YciR$^{\Delta EAL}$ still reducing *csgB*::*lacZ* expression to some extent (Supplementary Figure 3). As isolated domains, YdaM-GGDEF could interact with YciR-GGDEF. This interaction was specific since it did not occur with the GGDEF domain of another DGC, YeaP (Figure 3A, lower right panel). In the presence of *all* domains in the full-size YciR, however, the GGDEF domains of YdaM and YciR did not interact, that is, this interaction seems cryptic or conditional.

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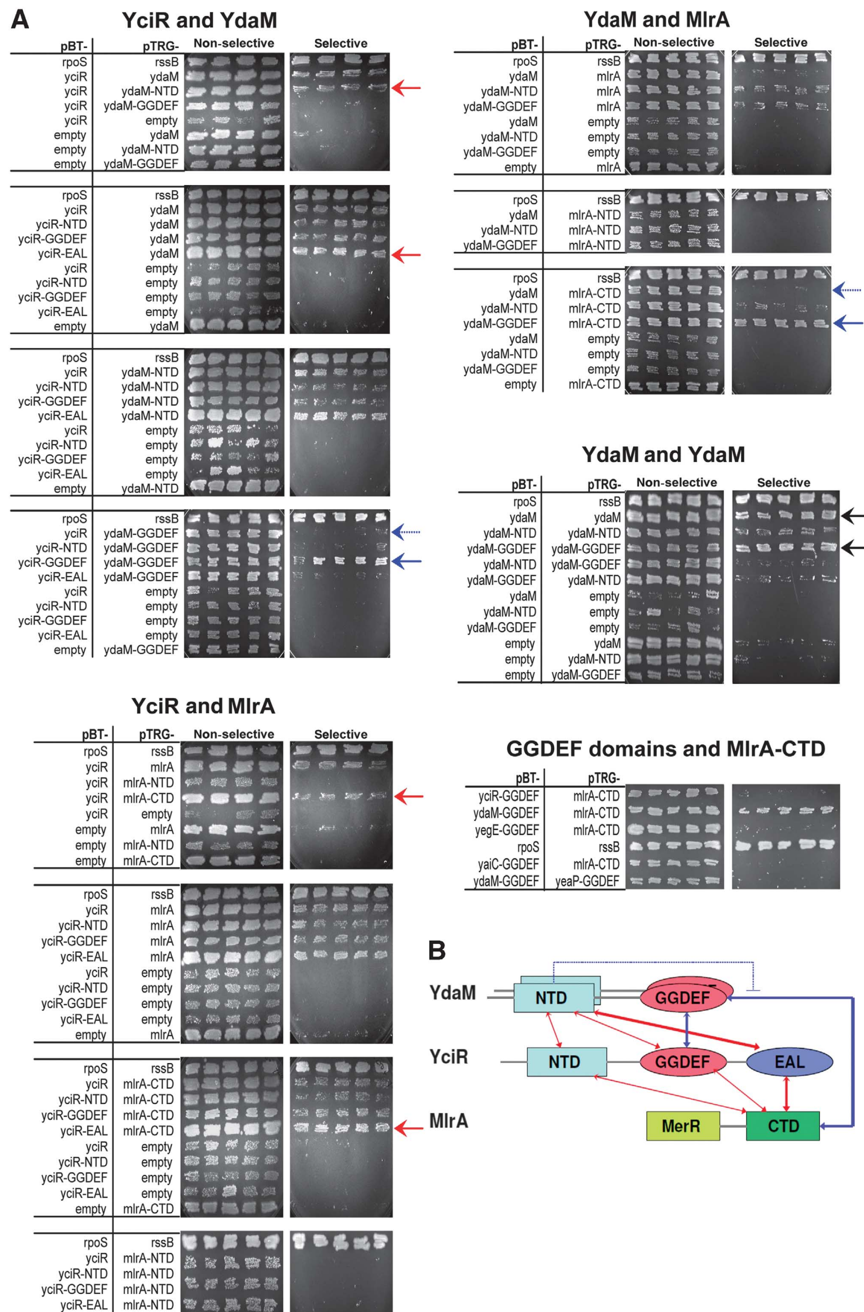

Figure 3 Detection of *in vivo* interactions between YdaM, YciR and MlrA and the identification of interacting domains. (**A**) Using the BacterioMatch®-II two-hybrid system, the indicated proteins or protein domains were co-expressed as hybrids to lambda cI-NTD (on pBT) and RNAP alpha-NTD (on pTRG). Interaction in the combinations indicated is detected by growth on selective plates (note that in the last panel, conditions were more stringent; see Supplementary data for details). The previously known strong interaction between σ^S (RpoS) and its proteolytic targeting factor RssB (Becker *et al*, 1999) serves as a positive control. (**B**) Interactions between different domains are schematically summarised. Strong and weak interactions are indicated by the thickness of the lines, blue lines indicate conditional interactions, which are seen only with the isolated domains, but not in the presence of additional and therefore inhibitory domains (for YdaM-NTD, this inhibition is indicated as a dotted line). YdaM is drawn as a dimer, since GGDEF domains have to dimerise to be active as a DGC. Arrows in (**A**) point to key results, with arrow colours referring to the colouring of the strong interactions schematically summarised in (**B**).

Following the same logics, the interaction between YciR and MlrA was analysed (Figure 3A, lower left panel). Again, the interaction of the two full-size proteins seen in the pull-down assays was confirmed *in vivo*. Here, the C-terminal domain of MlrA was found to be involved in the interaction. The YciR-EAL domain contributed most to this interaction, but also the other two YciR domains seemed involved to some extent.

A complex pattern of interaction was observed for YdaM and MlrA (Figure 3A, upper right panel). Full-size YdaM did hardly show any indication of interacting with MlrA, nor with the two isolated MlrA domains. However, the isolated YdaM domains showed some interaction with full-size MlrA. Strikingly, the YdaM-GGDEF domain alone exhibited strong interaction with MlrA-CTD, that is, the c-di-GMP producing domain of YdaM can make a direct contact with the putative

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ligand-binding domain of MlrA. This interaction of MlrA-CTD was clearly not observed with the full-size YdaM protein, which means that the presence of the YdaM-NTD can inhibit a therefore conditional interaction between the YdaM-GGDEF domain and MlrA-CTD. Moreover, this interaction was specific, because MlrA-CTD did not interact with the GGDEF domains of YciR, YegE or YaiC (Figure 3A, lower right panel).

Finally, since DGCs are active upon dimerisation (Chan *et al*, 2004; Schirmer and Jenal, 2009), we assayed potential interactions of the two YdaM domains to each other (Figure 3A, lower right panel). Testing full-size YdaM against itself clearly produced interaction. This is in line with *in vitro* crosslinking experiments showing purified YdaM to form dimers and even predominantly tetramers (Supplementary Figure 4). In addition, we observed strong interaction of the YdaM-GGDEF domain tested against itself. YdaM-NTD showed a weak dimerisation potential and also the YdaM-NTD and YdaM-GGDEF domains could weakly interact. *In vitro*, YdaM-NTD was required for tetramerisation (Supplementary Figure 4).

Taken together, all these data suggest that YdaM, YciR and MlrA undergo complex formation with numerous domain contacts, consistent with local signalling. Some of these domain contacts depend on the status of other domains and thus are likely to be regulated.

Biochemical activities of YciR and its physiological function as a mediator between c-di-GMP signalling modules I and II

YciR, which has a GGDEF as well as an EAL domain, has PDE activity (Weber *et al*, 2006) and acts as an inhibitory factor in *csgD* expression (Pesavento *et al*, 2008) (Figure 1; Supplementary Figure 3). In addition, our genetic data (Figure 1) suggested that this inhibitory role of YciR is antagonised by c-di-GMP controlled by the YegE/YhjH module. The c-di-GMP sensor function of YciR could be provided either by its EAL domain, which not only binds but also degrades c-di-GMP, or by its GGDEF domain.

To clarify the molecular basis of these complex functions of YciR, we reexamined the enzymatic activity of purified YciR in closer detail. As previously shown, YciR hydrolysed highly purified c-di-GMP to pGpG and GMP, with this activity requiring an intact EAL motif (Figure 4A). In addition, we observed that it further processed GMP to radiolabelled phosphate, with this last step being inhibited by GTP. By contrast, YhjH, a simple PDE consisting of an EAL domain only, generated GMP as an end product from c-di-GMP (Figure 4A). In fact, YciR could also generate a radiolabelled fast running product from GTP directly (Figure 4A). Due to the radioactive labelling of GTP in the αP position, this product has to be tri-phosphate that further hydrolyses to pyrophosphate and phosphate (collectively designated as $(PP)P_i$). In contrast to the 3′-PDE activity that generates pGpG and GMP from c-di-GMP, this 5′-PDE reaction was not inhibited by adding excess c-di-GMP (data not shown).

In UV-crosslinking assays, YciR was found to bind radiolabelled c-di-GMP as well as GTP (Figure 4B). This binding was specific as it could be chased by non-radiolabelled c-di-GMP and GTP, respectively (in concentrations as low as 1 μM; Supplementary Figure 5). In order to assign these binding activities to distinct domains and motifs of YciR, several YciR variants with mutations in crucial amino-

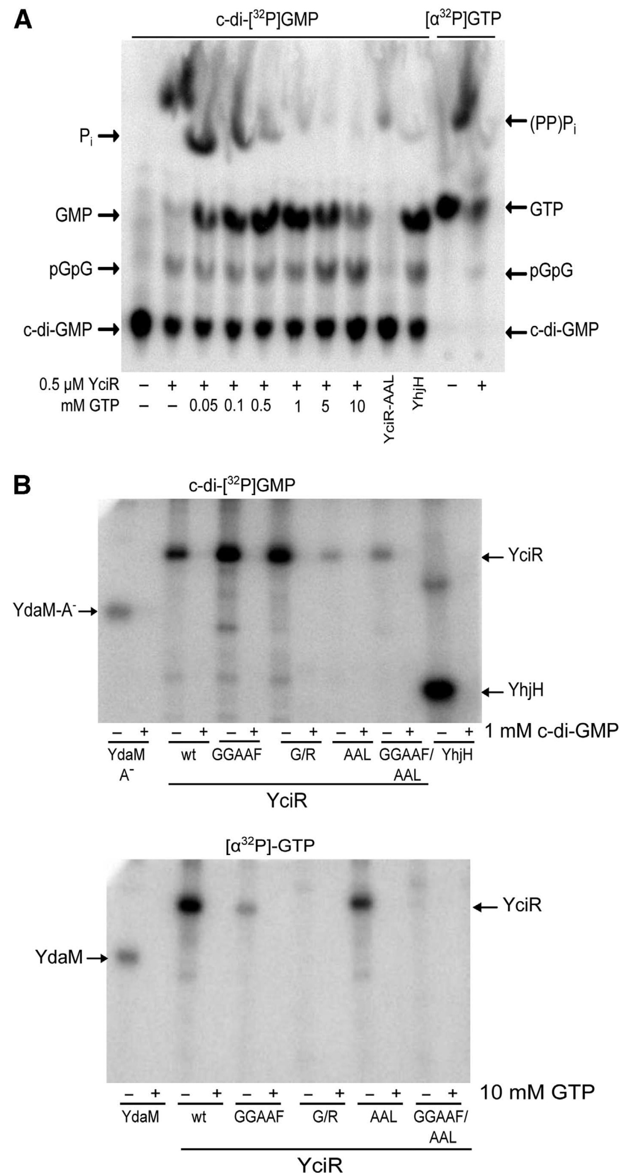

Figure 4 Enzymatic activities and binding of c-di-GMP and GTP by YciR. In (**A**), purified YciR, $YciR^{AAL}$ and YhjH (all at 0.5 μM) were assayed for PDE activity with [^{32}P]-c-di-GMP. Where indicated, samples also contained unlabelled GTP or [α-^{32}P]-GTP alone (last two lanes). (**B**) UV crosslinking with [^{32}P]-c-di-GMP and [α-^{32}P]-GTP (according to Christen *et al*, 2005) was tested for purified YciR and the indicated YciR mutant versions (0.5 or 1 μM), using YdaM (1.6 μM) and YhjH (2.5 μM) as controls. Mutations in the GGDEF and EAL motifs of YciR are as indicated, 'G/R' refers to a mutant YciR in which alterations in the GGDEF motif (AAAAF) and R248A were combined.

acid positions were tested: (i) the GGDEF motif replaced by GGAAF, (ii) AAAAF (instead of the GGDEF motif) combined to R248A ('G/R'; in GGDEF domains this arginine residue contributes to GTP binding; Schirmer and Jenal, 2009), (iii) the EAL motif replaced by AAL and (iv) GGAAF and AAL combined. While c-di-GMP binding required an intact EAL domain but was independent of the GGDEF domain (Figure 4B; note that this GGDEF domain does not feature an I-site motif), GTP binding was clearly a function of the GGDEF domain (Figure 4C). The latter finding raised the question whether the YciR GGDEF domain may have some residual DGC activity. As previously observed

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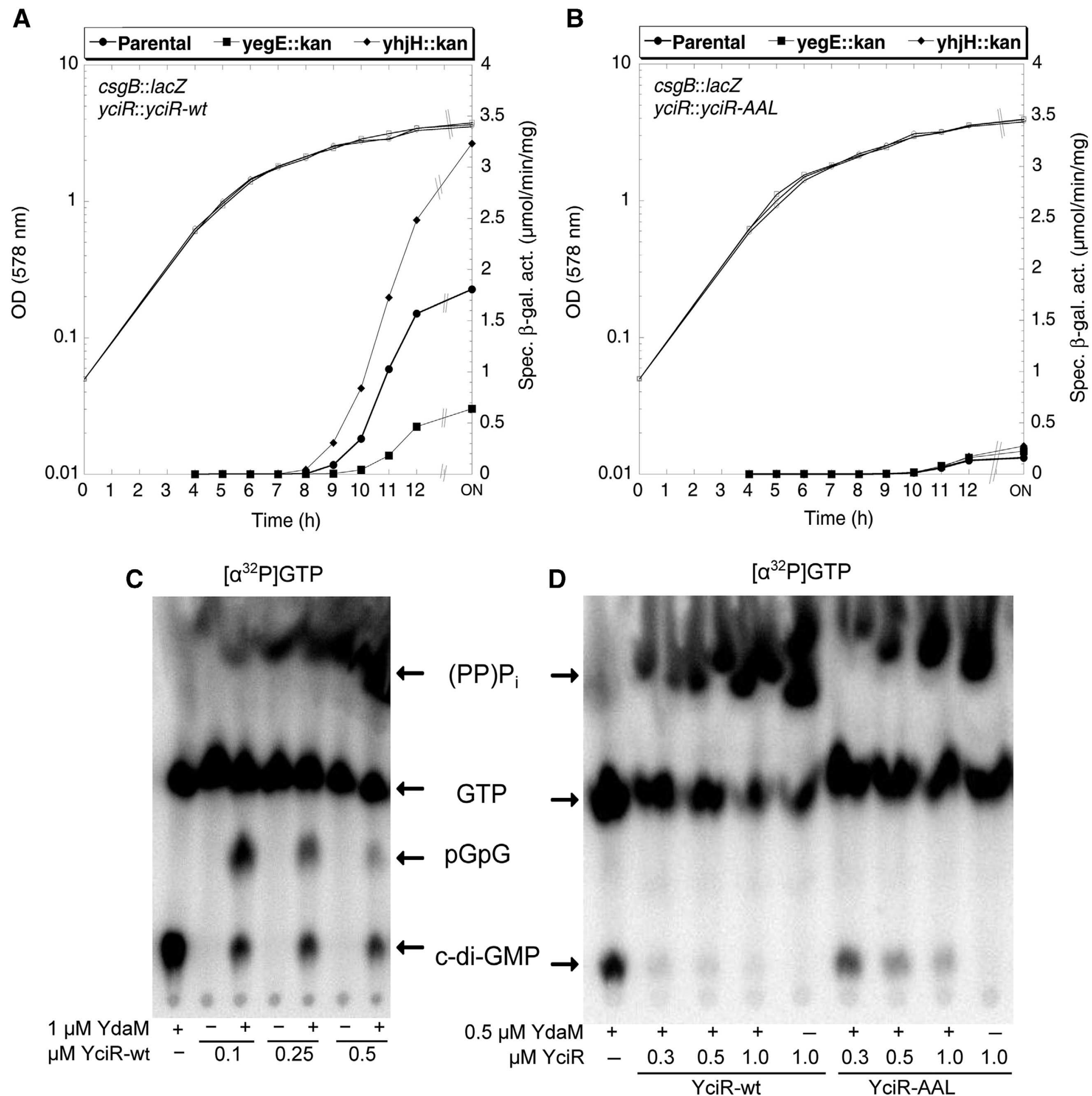

Figure 5 YciR inhibits the YdaM/MlrA-generated output and is antagonised by YegE-generated c-di-GMP acting via the EAL domain of YciR. W3110 derivatives carrying the single copy *csgB*::*lacZ* reporter fusion as well as either wild-type *yciR* (**A**) or the point-mutated chromosomal *yciR*^{AAL} allele (**B**) and *yegE* or *yhjH* knockout mutations as indicated were grown in LB at 28°C. OD_{578} as well as specific β-galactosidase activities were determined. The wild-type *yciR* strain carries the *yciR*$^{+}$ allele 'back-crossed' into the chromosome by the same procedure that was used to integrate *yciR*^{AAL}. In (**C**) and (**D**), DGC assays were performed with [α-^{32}P]-GTP, YdaM and substoichiometric concentrations of YciR (**C**), or YciR and YciR^{AAL} in further increasing, equal and superstoichiometric concentrations (**D**).

(Weber *et al*, 2006), no c-di-GMP could be detected when YciR was incubated with radiolabelled GTP. However, occasionally (with very fresh preparations of YciR only; see Materials and methods) we observed that YciR could produce traces of pGpG from GTP (Figure 4A). This suggests that the GGDEF domain of YciR has a very weak DGC activity, which usually remains cryptic as the c-di-GMP generated by it is immediately hydrolysed to pGpG by the PDE activity of the EAL domain.

In order to find out which YciR domains and biochemical activities are crucial for its *in vivo* function as a mediator between c-di-GMP control module I (YegE/YhjH) and the module II-generated output, that is, CsgD/curli expression, we crossed the *yciR*^{AAL} and *yciR*^{GGAAF} alleles back into the chromosome such that expression levels of these YciR variants and stoichiometries to YdaM and MlrA are as in the wild type (Supplementary Figure 6). If one of these sites represents an effector site for YegE/YhjH-controlled c-di-GMP, then its mutation should render the output of the system insensitive to knockout mutations in *yegE* and *yhjH*. While the *yciR*^{GGAAF} strain (Supplementary Figure 7) showed similar *csgB*::*lacZ* expression and regulation by YegE/YhjH as the original or the 'back-crossed' *yciR*$^{+}$ strains, the *yciR*^{AAL} allele produced a striking phenotype (Figure 5A and B). First, it strongly reduced *csgB*::*lacZ* expression (in contrast to the hyperactivating *yciR* null mutation; compare to Figure 1B); second, this low level expression was indeed insensitive to the *yegE* and *yhjH* mutations suggesting constitutive inhibition by YciR^{AAL}. These data indicate that the EAL domain of YciR not only acts as a PDE, but also serves as a

sensor or effector domain that transmits the information about YegE/YhjH-controlled c-di-GMP to the downstream part of pathway, that is, YdaM/MlrA-driven *csgD* transcription.

Our protein–protein interaction data (Figures 2 and 3) suggested that inhibition of YdaM/MlrA by YciR relies on direct interaction. We therefore tested direct inhibition of YdaM by YciR with purified proteins *in vitro*. When YciR was present in substoichiometric concentrations and therefore excess YdaM (not in a complex with YciR) could generate and release c-di-GMP, pGpG, that is, the degradation product of the PDE reaction of YciR, could be detected (Figure 5C). However, the amount of pGpG produced became *lower* with *increasing* YciR concentrations, that is, inhibition of the DGC reaction of YdaM replaced c-di-GMP hydrolysis when YdaM:YciR ratios reached appropriate stoichiometry (Figure 5D). Since also $\mathrm{YciR^{AAL}}$—despite its strongly reduced PDE activity (Figure 4A)—produced an inhibitory effect of almost similar strength (Figure 5D), this reduced c-di-GMP amount indicated an inhibition of the DGC activity of YdaM by YciR rather than degradation of YdaM-generated c-di-GMP. Thus, YciR can inhibit the DGC activity of YdaM by direct stoichiometric interaction.

In addition, YciR interacts with MlrA (Figures 2 and 3) and our genetic data (Figure 1; Supplementary Figure 1) showed that YciR can prevent MlrA from driving the low basal expression of *csgD*/curli in the absence of the other signalling components (in the *yegE yhjH ydaM* triple mutant). Also for the inhibition of basal activity by MlrA alone, $\mathrm{YciR^{AAL}}$ was as efficient as wild-type YciR (Supplementary Figure 8). Overall, we conclude that YciR is a direct antagonist not only for YdaM but also for MlrA.

Role of the DGC YdaM in the control of csgD transcription

Finally, we examined the role of YdaM, its DGC activity and its I-site motif in generating the pathway output. Its c-di-GMP binding as detected by UV crosslinking (Figure 6A) is mainly dependent on an intact I-site, and is in fact improved when the enzyme is inactivated and thereby intrinsically immobilised by mutating the active centre or A-site (to GGAAF). The I-site as well as the N-terminal PAS domain are dispensible for DGC activity, although I-site mutated YdaM ($\mathrm{YdaM^{I\text{-}site}}$) and the GGDEF domain alone show somewhat reduced DGC activity (Figure 6B).

Despite the presence of the c-di-GMP-binding I-site, the addition of up to 100 μM non-radiolabelled c-di-GMP did not inhibit the DGC activity of YdaM (Figure 6C). This is in pronounced contrast to other DGCs such as PleD* (Figure 6C) (Christen *et al*, 2005, 2006), but is consistent or actually a prerequisite for YdaM being *activated* by module I-generated c-di-GMP sensed by YciR. Alternatively, a putative positive function of c-di-GMP binding to the I-site in YdaM in this signalling pathway could be excluded, since mutation of the I-site (in the chromosomal *ydaM* allele) did not disturb the responsiveness to mutations in module I components, that is, *yegE* and *yhjH* (Supplementary Figure 9).

Most strikingly, when we introduced the A-site mutation (GGAAF)—which eliminates DGC activity (Figure 6B)—into the chromosomal copy of *ydaM*, *csgB::lacZ* expression was not eliminated but was reduced only by $\sim$50% (Figure 6D). This means that production of c-di-GMP by YdaM somewhat contributes to but is *non-essential* for activating MlrA to drive *csgD* transcription, that is, YdaM may activate MlrA by direct interaction. Moreover, activation by the A-site-deficient $\mathrm{YdaM^{GGAAF}}$ still responded to mutations in *yegE* and *yhjH* (Figure 6E).

Nevertheless, examples of c-di-GMP binding transcription factors have been found in other systems (Hickman and Harwood, 2008; Chin *et al*, 2009). Also, the c-di-GMP-generating GGDEF domain of YdaM could strongly bind to the C-terminal domain of MlrA (see Figure 3), which in MerR-like regulators serves as the ligand-binding domain. Therefore, we tested whether purified MlrA is able to bind c-di-GMP either free in solution or when produced from GTP by YdaM in the same sample. In our standard crosslinking assay, c-di-GMP could indeed bind to MlrA (Figure 7A)—however, this binding was unspecific as it could not be chased by up to 1 mM non-radiolabelled c-di-GMP (data not shown). When MlrA was co-incubated with YdaM-generating c-di-GMP from GTP, no transfer of c-di-GMP to MlrA was observed (Figure 7B). Moreover, no c-di-GMP binding to MlrA was detected, when a *csgD* promoter fragment containing the MlrA binding site (Ogasawara *et al*, 2010b) was added (Figure 7B) or when we used the His6-tagged YdaM-GGDEF domain alone (data not shown; this construct does not contain the PAS domain that inhibits the interaction of YdaM-GGDEF with MlrA-CTD; see Figure 3). Yet, YdaM did produce c-di-GMP in these assays as can be seen from c-di-GMP binding to YciR when the latter was added (Figure 7C). Finally, we noticed that the MlrA sequence features two I-site-like putative c-di-GMP-binding RxxD motifs (R55/D58 and R107/D110). However, replacing these R and D residues by alanines did neither affect the unspecific c-di-GMP binding of the purified proteins *in vitro*, nor *csgB::lacZ* expression *in vivo* (data not shown).

Based on all these results, it seems quite unlikely that MlrA is activated by binding of YdaM-generated c-di-GMP. Rather, YdaM activates MlrA by direct interaction. How then does c-di-GMP produced by YdaM contribute to activation as indicated by the twofold reduced *csgB::lacZ* expression in a mutant carrying the $ydaM^{A\text{-}site}$ allele (Figure 6D)? In order to test whether YdaM-generated c-di-GMP contributes to the primarily YegE-generated c-di-GMP pool in a positive feedback loop (see summarising model in Figure 8), we tested the effect of the $ydaM^{A\text{-}site}$ allele in a *yciR* knockout mutant, that is, in the absence of the sensor for this c-di-GMP pool. In this background, the inability of $\mathrm{YdaM^{A\text{-}site}}$ to produce c-di-GMP still reduced *csgB::lacZ* slightly but reproducibly (by $<$20%; Figure 6F). We conclude that the DGC activity of YdaM indeed contributes to the module-I-generated c-di-GMP pool that antagonises the inhibitor YciR, but that to some minor degree its enzymatic activity can also stimulate its activation of MlrA that relies on direct protein–protein interaction.

Discussion

Local signalling in a functional cascade of two c-di-GMP control modules

The genetic and biochemical data presented in this study indicate that activation of the biofilm regulatory gene *csgD* by c-di-GMP occurs in a serial arrangement of two distinct DGC/PDE modules. Module I (YegE/YhjH) increasingly

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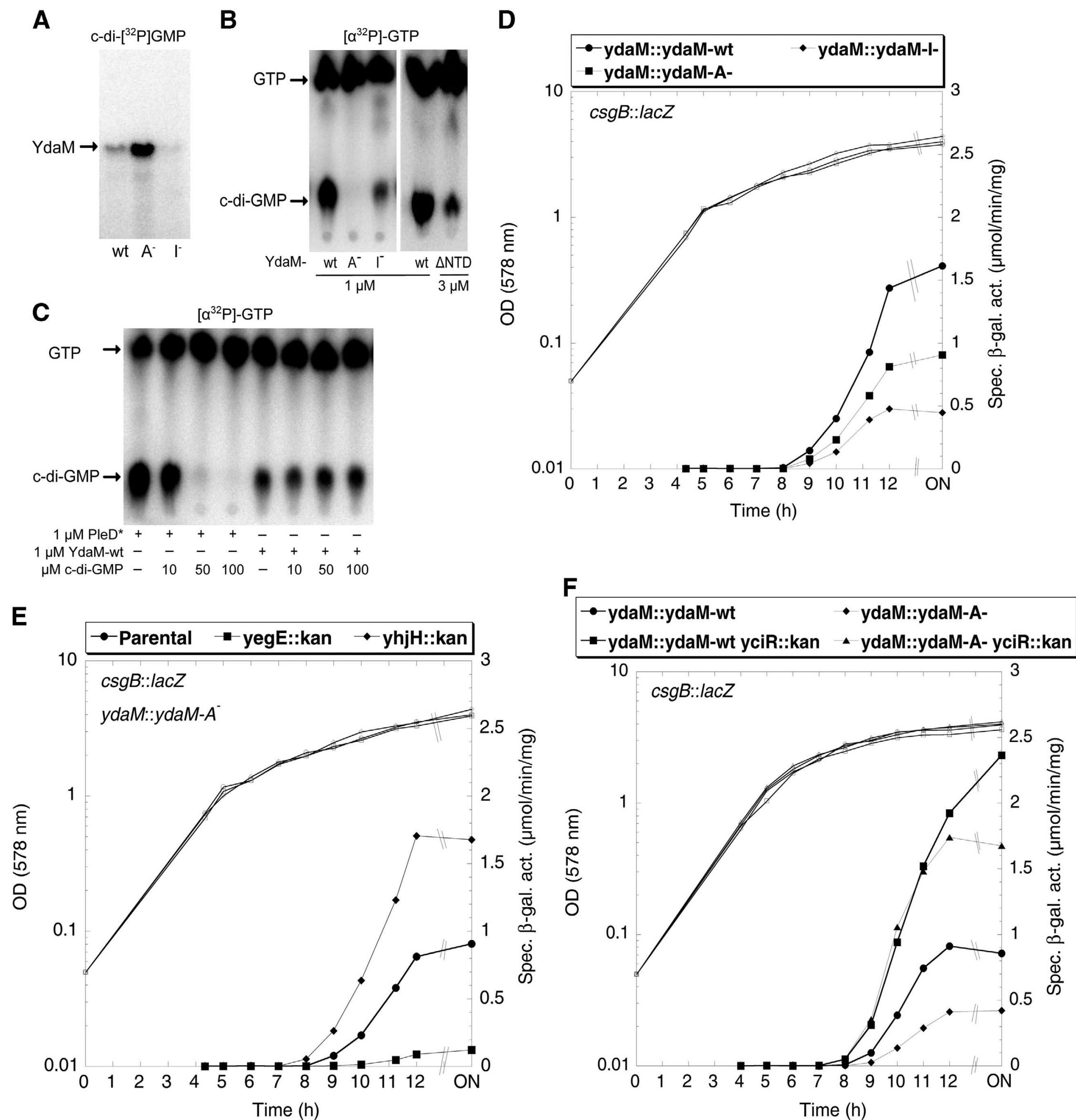

Figure 6 The function of the DGC activity of YdaM in the YdaM/MlrA-generated output. (**A**) Binding of c-di-GMP to the 'I-site' in YdaM. UV crosslinking of [^{32}P]-c-di-GMP to wild-type YdaM as well as to the A-site and I-site-mutated YdaM variants was assayed as in Figure 4. (**B**) DGC assays were performed with purified YdaM (wt), YdaM^{GGAAF} (A$^-$), YdaM$^{I\text{-}site}$ (I$^-$) and the N-terminally truncated YdaM$^{\Delta NTD}$ using [α-^{32}P]-GTP as a substrate. Proteins were used at a concentration of 1 μM, except for YdaM$^{\Delta NTD}$ (3 μM). (**C**) Inhibition of DGC activity by excess non-radiolabelled c-di-GMP (up to 100 μM) was tested for PleD* and YdaM proteins (1 μM). (**D**, **E**) Single copy *csgB*::*lacZ* expression was tested in W3110 derivatives carrying either wild-type *ydaM* or the chromosomal *ydaM$^{A\text{-}site}$* and *ydaM$^{I\text{-}site}$* alleles (**D**), as well as *yegE* or *yhjH* knockout mutations as indicated (**E**). (**F**) The influence of the *ydaM$^{A\text{-}site}$* allele on *csgB*::*lacZ* expression was tested in W3110 derivatives carrying either *yciR*$^+$ or *yciR* knockout alleles.

generates c-di-GMP when cells approach stationary phase, since *yegE* is activated under σ^S control, whereas *yhjH* is co-regulated with flagellar genes that are shut off under these conditions (Pesavento *et al*, 2008). This c-di-GMP slows down flagellar rotation and swimming speed via the effector protein YcgR (Pesavento *et al*, 2008; Boehm *et al*, 2010; Fang and Gomelsky, 2010; Paul *et al*, 2010). Here, we have demonstrated that in parallel it also affects module II (YdaM/YciR) by preventing YciR from inhibiting YdaM, which allows YdaM to generate c-di-GMP and to activate MlrA-driven *csgD* transcription (summarised in Figure 8).

The logics of such a two-module cascade are not compatible with an additive function of two simple c-di-GMP-releasing systems that are present and active in parallel, but rather requires some local activity of at least one of the modules. Local signalling may involve transiently increased local c-di-GMP concentration or even separate pools of freely diffusible and local c-di-GMP as discussed previously (Jenal and Malone, 2006; Kader *et al*, 2006; Kulasakara *et al*, 2006; Ryan *et al*, 2006; Weber *et al*, 2006; Hengge, 2009; Christen *et al*, 2010). As shown in this study, local signalling can also involve secondary activities of the relevant DGCs or PDEs that are based on macromolecular interactions and controlled by their primary, that is, enzymatic activities. Such bifunctional regulatory enzymes are known as 'trigger enzymes' (Commichau and Stülke,

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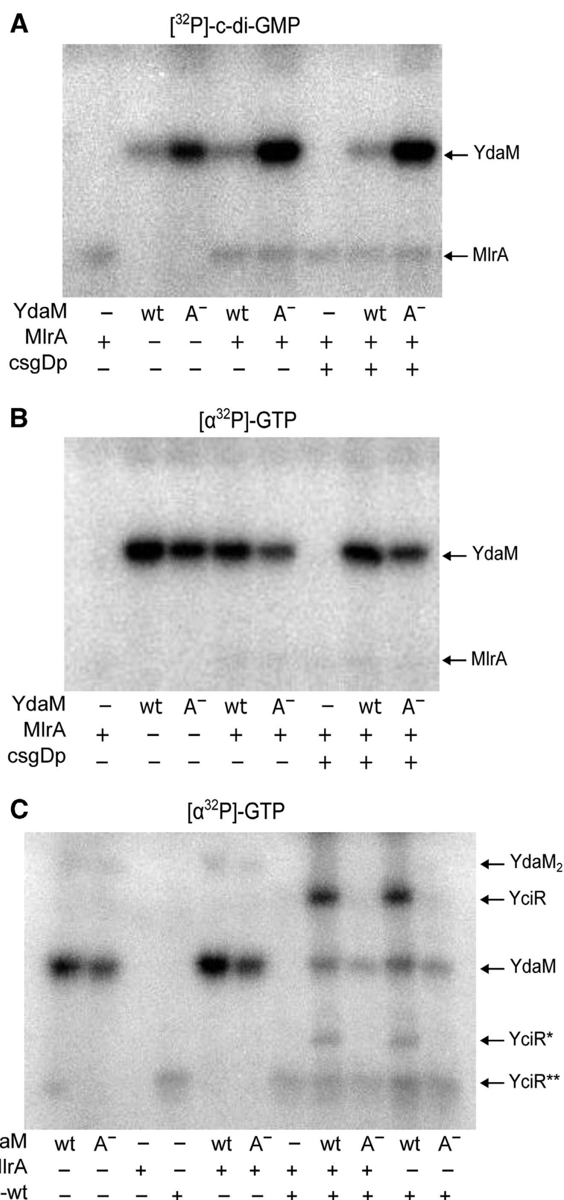

Figure 7 c-di-GMP binding to YdaM, MlrA and YciR. UV cross-linking with [^{32}P]-c-di-GMP (**A**) or [α-^{32}P]-GTP (**B**, **C**) tested for YdaM (1.6 μM), MlrA (2.6 μM) and YciR (0.5 μM) was assayed in the indicated combinations. Where indicated, MlrA was preincubated with a *csgD* promoter-carrying DNA fragment for 60 min. In (**C**) degradation fragments of YciR, which correspond to the EAL (YciR*) and GGDEF (YciR**) domain, were also labelled by [α-^{32}P]-GTP (note that labelling of YciR* requires the presence of YdaM, that is, DGC activity, which—besides it size—indicates that YciR* corresponds to the EAL domain alone). In addition, YdaM dimers were weekly detectable.

2008), with our study being the first that introduces this concept into second messenger signalling.

A common feature of these otherwise rather different modes of local c-di-GMP signalling is their dependence on direct protein–protein interactions. Interactions between distinct DGCs and PDEs (Andrade *et al*, 2006; Bobrov *et al*, 2008; Ryan *et al*, 2010; Abel *et al*, 2011) or between DGC, PDE and/or effector domains—sometimes even in single polypeptides—seem to be relatively common and in some cases were observed at specific subcellular locations (Amikam and Galperin, 2006; Jenal and Malone, 2006; Tuckerman *et al*, 2011). The multiple interactions between YdaM, YciR and MlrA observed in this study (Figures 2 and 3) in fact led us the way to elucidate the local mode of operation of module II.

Molecular interactions between YciR, YdaM and MlrA and their functional implications

The interactions between the various domains of the DGC YdaM, the transcription factor MlrA and the multifunctional GGDEF + EAL domain protein YciR seem to fall into three classes, that is, strong, weak and conditional interactions (Figure 3). The strong interactions of the YciR-EAL domain to YdaM-NTD as well as to MlrA-CTD, which are independent of the presence or absence of other domains, probably contribute to YciR acting as an inhibitor of YdaM/MlrA. Strong interaction also underlies the dimerisation of the YdaM-GGDEF domain, which is required for DGC activity. The multiple weaker interactions observed here may in part be false positives; however, interactions between isolated domains, which seem weak in a two-hybrid system, could be cooperative and therefore more efficient when these domains are linked in a single polypeptide. Thus, the apparently weak interaction between the inhibitory NTD and the GGDEF domain of YdaM may be biological meaningful.

Conditional interactions are strong interactions that became apparent only in the absence of other and therefore inhibitory domains; these were (i) the tight and specific contact between the YdaM-GGDEF domain and MlrA-CTD, which was cryptic with full-size YdaM, suggesting an intramolecular inhibitory role of YdaM-NTD and (ii) the specific interaction between the isolated GGDEF domains of YdaM and YciR, which was not observed with the full-size YciR protein. These conditional interactions bear an obvious potential for regulation, for example, other proteins that interact with the inhibitory domains may regulate these interactions. Moreover, these conditional interactions are the ones that seem crucial for the output of module II and therefore of the entire signalling cascade: (i) DGC activity, which requires homo-dimerisation of the YdaM-GGDEF domain rather than hetero-dimerisation with the YciR-GGDEF domain; and (ii) binding of YdaM-GGDEF to the C-terminal domain of MlrA, which is likely to be the basis for YdaM stimulating MlrA-mediated transcription of *csgD*.

Role of the c-di-GMP PDE YciR as a trigger enzyme that connects c-di-GMP control modules I and II

YciR is a multifunctional three-domain protein, which in principle has two kinds of activities, that is, enzymatic activities and direct inhibitory protein–protein interactions. As an enzyme, it shows (i) the classical 3′-PDE activity of its EAL domain, that is, it binds and degrades c-di-GMP to pGpG and GMP (Weber *et al*, 2006) (Figure 4A and B), (ii) a GTP binding and weak DGC activity of its GGDEF domain that is obliterated by the stronger 3′-PDE activity that eliminates the generated c-di-GMP immediately (Figure 4A and C) and (iii) a novel 5′-PDE activity, which allows it to hydrolyse GTP as well as GMP (Figure 4A). This 5′-PDE activity is unaffected by c-di-GMP but inhibited by excess GTP, but its structural basis and potential physiological function remain to be analysed.

The second class of activities of YciR are direct inhibitory interactions with YdaM and MlrA. Interactions with YdaM

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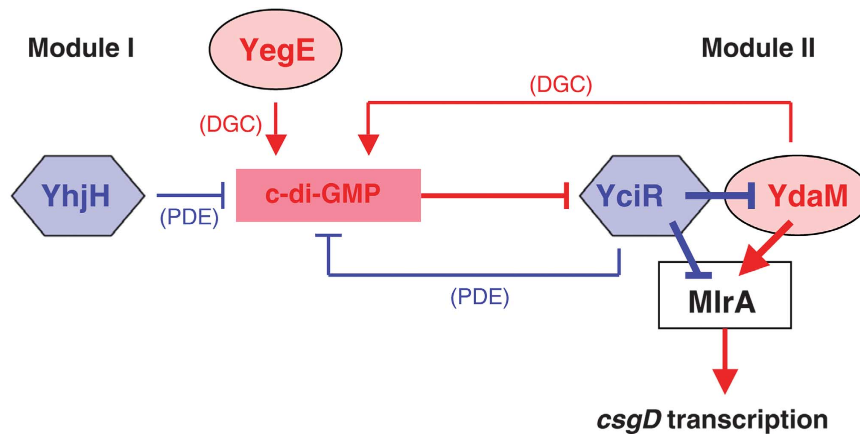

Figure 8 Model of the *csgD*-controlling c-di-GMP signalling cascade and its inherent feedback cycles. Module I (consisting of the DGC YegE and the PDE YhjH) controls c-di-GMP that is sensed and degraded by the PDE YciR, which together with the DGC YdaM constitutes module II. YciR is a also trigger enzyme whose second activity—the direct inhibition of YdaM and MlrA—is relieved when it is active as a PDE. YdaM is equally bifunctional. While its DGC activity contributes to the c-di-GMP pool generated by module I in a positive feedback loop, it also stimulates the transcription factor MlrA by direct interaction. DGCs are indicated by ovals and PDEs by hexagons. DGCs and high c-di-GMP-driven processes are shown in red, and PDEs and processes occurring at low c-di-GMP levels are shown in blue.

are complex and involve contacts between YciR-EAL and YdaM-NTD as well as between the GGDEF domains of both proteins (Figure 3). It is tempting to speculate that the formation of an inactive GGDEF domain heterodimer could allow YciR to inhibit both functions of YdaM, that is, DGC activity (Figure 5C and D) as well as direct activation of MlrA (Figure 3, and genetic data shown in Figure 6), which both rely on the GGDEF domain of YdaM. Furthermore, YciR-EAL directly contacts MlrA-CTD (Figure 3). This contact is likely to interfere with productive interaction between YdaM-GGDEF and MlrA-CTD. In the absence of all other relevant c-di-GMP signalling components (Figure 1B and C; Supplementary Figure 8), YciR also inhibits the basal activity of MlrA, that is, its ability to communicate with RNAP.

The key to understanding the complex role of YciR lies in its 3′-PDE enzymatic activity *controlling* these direct inhibitory interactions. Thus, the enzymatically inactive YciR^{AAL} variant provides strong inhibition but is no longer regulated by the components of c-di-GMP signalling module I (YegE/YhjH) (Figure 5B). We conclude that YciR represents a ‘trigger enzyme’. These are bifunctional enzymes, which control gene expression via direct protein–protein or protein–DNA/RNA interactions in response to the availability of the substrates for their enzymatic activities (Commichau and Stülke, 2008). Other examples of trigger enzymes include the prolin-degrading enzyme PutA, which directly binds to the promoter regions of target genes (Ostrovsky de Spicer and Maloy, 1993); the iron-sulphur cluster enzyme aconitase which in its apo form binds to iron-responsive elements in the mRNAs for other TCA cycle enzymes (Alén and Sonenshein, 1999); the esterase Aes and the βC-S lyase MalY, which by direct interaction control the transcription factor MalT (Zdych *et al*, 1995; Schreiber *et al*, 2000; Joly *et al*, 2002); or the phosphotransferase system involved in glucose uptake that also binds and sequesters the transcription factor Mlc (Tanaka *et al*, 2000).

YciR seems the prototype of a novel class of trigger enzymes, as it enzymatically degrades a second messenger, that is, a small molecule that already has a central regulatory function, rather than a ‘simple’ metabolite. In addition, as a trigger enzyme YciR also serves as a novel type of effector in second messenger signalling, because its interaction with its targets (YdaM, MlrA) not only depends on ligand binding, but also on the conformational alterations associated with ligand degradation. In short, not only ‘degenerate’ EAL domain proteins, which are enzymatically inactive but still bind c-di-GMP, can serve as effectors in c-di-GMP signalling (Jenal and Malone, 2006; Hengge, 2009; Newell *et al*, 2009), but actually *fully active* PDEs can fulfill this function as well. In its trigger enzyme function, YciR thus also emerges as a c-di-GMP-sensing effector for the YegE/YhjH module and thereby serves as a connector between this ‘global’ c-di-GMP control module I and the locally acting YdaM-dependent module II in the cascade that controls *csgD* transcription.

Finally, the enzymatic activity of YciR eliminates, that is, negatively affects c-di-GMP, which on the other hand represents the small molecule that inhibits YciR’s second activity, that is, its direct control of YdaM and MlrA (Figure 8). This sets up a double-negative feedback loop, which is a classical motif able to generate hypersensitive switching and bistable gene expression in a population of cells (Gardner *et al*, 2000; Dubnau and Losick, 2006). CsgD and curli production is heterogeneous in biofilm-derived cells (Grantcharova *et al*, 2010). In fact, CsgD producing and non-producing cells can be observed side by side in a macrocolony biofilm *in situ* (Serra *et al*, 2013). Therefore, this YciR-based double-negative feedback loop may also play a role in generating bistable CsgD expression.

Role of the DGC YdaM in triggering the regulatory output of c-di-GMP signalling module II

Also YdaM has at least two functions, that is, (i) DGC activity and (ii) the activation of MlrA to promote *csgD* transcription. Like for YciR, these two activities can be genetically separated: the A-site mutation in YdaM eliminates its DGC activity (Figure 6B), but YdaM$^{A\text{-}site}$ (expressed from the natural single chromosomal copy context) produces a regulatory output

that still corresponds to about 50% of the activity in the wild type and to even >80% of the activity in the *yciR* knockout mutant background (Figure 6).

These results allow a number of conclusions. First, activation of MlrA is most likely due to the direct interaction of YdaM-GGDEF with MlrA-CTD (Figure 3), which is consistent with the failure of our many attempts to detect specific binding of YdaM-generated c-di-GMP by MlrA (Figure 7) and with basal MlrA activity in the absence of YciR and YdaM being entirely c-di-GMP insensitive (Figure 1C; Supplementary Figure 1). Nevertheless, YdaM-generated c-di-GMP *does* significantly contribute to the output of the system (Figure 6E and F), and even does so much more when YdaM is provided *in trans* from a plasmid (Weber *et al*, 2006). As this contribution is mostly dependent on the presence of YciR, it is largely due to a positive feedback loop with YdaM-generated c-di-GMP adding up to YegE-generated c-di-GMP (Figure 8). Also in the absence of YciR, however, *csgB::lacZ* expression was still reproducibly slightly higher when YdaM had DGC activity (Figure 6F), suggesting that to some extent YdaM also behaves like a trigger enzyme whose direct interaction-based activity (on MlrA) is slightly stimulated by its enzymatic activity.

In addition, YdaM shows two intrinsic features with a potential to inhibit its activity: its c-di-GMP-binding I-site (Figure 6A) and its N-terminal PAS domain, which can interfere with the interaction of the YdaM-GGDEF domain with MlrA-CTD (Figure 3). However, c-di-GMP binding to the I-site is much weaker for active wild-type YdaM than for inactive $YdaM^{A\text{-}site}$, that is, conformational changes associated with DGC activity seem to allow the protein to dispose of c-di-GMP at the I-site. YdaM-NTD, that is, the PAS domain, is contacted by YciR-EAL (Figure 3), which may stabilise the inhibitory interaction of YciR with YdaM. In the *yciR* mutant, however, YdaM/MlrA generate maximal system output (Figures 1 and 6F), which means that a putative inhibitory influence of the YdaM-PAS domain may be somehow neutralised, possibly by interaction with one of the other regulatory proteins that are known to bind in the promoter region of *csgD* (Ogasawara *et al*, 2010a). Work to identify such an additional factor is in progress.

Conclusions and perspectives

With the EAL-domain PDE YciR as a prototype, our study introduces the concept of trigger enzymes into c-di-GMP signalling. Since the secondary activities of trigger enzymes always rely on direct interaction with other proteins or DNA/RNA, trigger enzyme action has a local component by definition. Evidence for local c-di-GMP signalling by DGCs and PDEs that operate on separate targets in parallel with little or no cross-talk has been accumulating for years. This evidence includes clearcut phenotypes for single knockouts in GGDEF/EAL/HD-GYP domain-encoding genes despite large numbers of these genes in most species and/or the absence of measurable changes in cellular c-di-GMP content. In addition, GGDEF/EAL/HD-GYP domain proteins are frequently found in larger protein complexes. So far, local gradients or even microcompartimentation of cellular c-di-GMP pool(s) have been discussed as potential mechanisms (Jenal and Malone, 2006; Kader *et al*, 2006; Kulasakara *et al*, 2006; Ryan *et al*, 2006; Weber *et al*, 2006; Hengge, 2009; Christen *et al*, 2010). However, DGCs and PDEs acting as trigger enzymes seems such a simple, yet powerful principle to confer locality to c-di-GMP signalling that we expect it to be rather widespread. If so, then also cascades of global and local c-di-GMP control modules linked by trigger enzymes may be more common.

Materials and methods

Bacterial strains and growth conditions

All strains used are derivatives of the *E. coli* K-12 strain W3110 (Hayashi *et al*, 2006). The *ydaM::cat, yciR::kan, yegE::kan, yhjH::cat, yhjH::kan* and *mlrA::kan* mutations are full *orf* deletion/resistance cassette insertions previously described (Weber *et al*, 2006; Pesavento *et al*, 2008). When required, cassettes were flipped out (Datsenko and Wanner, 2000). Mutations were transferred by P1 transduction (Miller, 1972). For introducing point mutations in *yciR* and *ydaM* into the otherwise wild-type chromosomal background (W3110), a two-step method related to the one-step inactivation protocol (Datsenko and Wanner, 2000) was applied (see Supplementary data). The single copy *csgB::lacZ* reporter fusion was described before (Weber *et al*, 2006). The construction of plasmids and two-hybrid analysis is detailed in Supplementary data. Cells were grown in LB medium (Miller, 1972) under aeration at 28 or 37°C. Antibiotics were added as recommended (Miller, 1972).

In vitro protein–protein interaction assay

In vitro interaction assays were performed by affinity chromatography ('pull-down' assays) on S-protein agarose (Merck) upon mixing of cellular extracts prepared from strains that express YdaM, YciR or MlrA from a plasmid (S tagged or His6 tagged). In order to minimise interference of the corresponding non-tagged wild-type proteins (expressed from their chromosomal genes), strains used for extract preparation moderately overproduced the tagged protein and were depleted for the respective other two proteins (see Supplementary data). Eluates were analysed by 12% SDS–PAGE and proteins were detected by immunoblotting using a His-tag antibody (Bethyl Laboratories, Inc.).

Two-hybrid analysis for testing protein–protein interactions in vivo

The BacterioMatch®-II two-hybrid system (Agilent Technologies) uses fusions to the NTD of lambda cI (on pBT) and to the bacterial RNAP alpha-NTD (on pTRG) (Dove and Hochschild, 2004). When fusion proteins interact, expression of the *HIS3* gene (originally from *S. cerevisiae*) is activated in the *E. coli* reporter strain (a derivative of XL1-Blue MRF'), which allows growth on selective medium (containing no histidine, but a fine-tunable concentration of the His3 inhibitor 3-Amino-1,2,4-triazole, 3-AT; for details, see Supplementary data.

Determination of c-di-GMP binding to proteins by UV crosslinking

Binding of [α-^{32}P]-GTP (83 nM, 3000 Ci/mmol) and [α-^{32}P]-c-di-GMP (42 nM, 6000 Ci/mmol) to purified proteins *in vitro* was detected by UV crosslinking according to Christen *et al* (2007)). Radiolabelled nucleotides were obtained from Hartmann Analytic GmbH.

Determination of DGC and c-di-GMP PDE activity in vitro

DGC and PDE reactions were performed with purified His6-tagged proteins (for plasmids and protein purification, see Supplementary data). DGC and PDE assays were performed with [α-^{32}P]-GTP and [α-^{32}P]-c-di-GMP (see above; Hartmann Analytic GmbH) with standard incubation times of 60 min, and the products were analysed by thin layer chromatography according to 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 μmol of *o*-nitrophenol/min/mg 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

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two measurements from independent cultures. Error bars represent standard deviation.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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