Regulation by cyclic di-GMP in *Myxococcus xanthus*

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DOROTA SKOTNICKA

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Erstgutachterin: Prof. Dr. Lotte Søgaard-Andersen

Zweitgutachterin: Prof. Dr. Anke Becker

Weitere Mitglieder der Prüfungskommission:

Prof. Dr. Lars-Oliver Essen

Prof. Dr. Hans-Ulrich Mösch

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c-di-GMP regulates type IV pili-dependent-motility in *Myxococcus xanthus*.
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A minimal threshold of c-di-GMP is essential for fruiting body formation and sporulation in *Myxococcus xanthus.*
Skotnicka D, Smaldone GT, Petters T, Liang J, Kaever V, Singer M & Søgaard-Andersen L. (manuscript submitted)

c-di-GMP regulates multiple cellular functions in the symbiotic α-proteobacterium *Sinorhizobium meliloti.*
Schäper S, Krol E, **Skotnicka D**, Kaever V, Søgaard-Andersen L, Becker A.
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Abstract

The nucleotide-based second messenger bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) is involved in regulating a plethora of processes in bacteria that are typically associated with lifestyle changes. *Myxococcus xanthus* undergoes major lifestyle changes in response to nutrient availability with the formation of spreading colonies in the presence of nutrients and spore-filled fruiting bodies in the absence of nutrients. Here, we investigated the function of c-di-GMP in *M. xanthus.* We show that this bacterium synthesizes c-di-GMP. Manipulation of the cellular c-di-GMP level by expression of either an active, heterologous diguanylate cyclase or an active, heterologous phosphodiesterase in vegetative cells caused defects in type IV pili (T4P)-dependent motility whereas gliding motility was unaffected. An increased level of c-di-GMP caused reduced transcription of the *pilA* gene that encodes the major pilin of T4P, reduced assembly of T4P and altered cell agglutination whereas a decreased level of c-di-GMP caused altered cell agglutination. The systematic inactivation of the 24 genes in *M. xanthus* encoding proteins containing GGDEF, EAL or HD-GYP domains, which are associated with c-di-GMP synthesis, degradation or binding, identified three genes encoding proteins important for T4P-dependent motility. These three proteins named DmxA, TmoK and SgmT all contain a GGDEF domain. Purified DmxA had diguanylate cyclase activity whereas the TmoK and SgmT (both hybrid histidine protein kinases) did not have diguanylate cyclase activity.

During starvation, the c-di-GMP level in *M. xanthus* increases significantly. Manipulation of this level revealed that a low c-di-GMP level negatively affects the developmental program while an increased level does not interfere with development. Moreover, among the 24 genes encoding proteins containing GGDEF, EAL or HD-GYP domains, we identified two which are specifically involved in development: *pmxA* and *dmxB*. *pmxA* codes for an enzymatically active phosphodiesterase with an HD-GYP domain. *dmxB* codes for a developmentally induced, enzymatically active diguanylate cyclase. DmxB is essential for the increased c-di-GMP level and regulates exopolysaccharide accumulation during starvation. Our results show that c-di-GMP acts as an important signaling molecule during *M. xanthus* development, and suggest a model in which a minimal threshold level of c-di-GMP is essential for the successful progression and completion of the developmental program.

Additionally, candidates for c-di-GMP effectors in *M. xanthus* were identified using a capture compound mass spectrometry approach. Some of the candidates were confirmed to bind c-di-GMP *in vitro* and deletion mutants for genes encoding those proteins were characterized in terms of T4P-dependent motility and development.

Zusammenfassung

Der nukleotid-basierte, sekundäre Botenstoff bis-(3‘-5‘)-cyclic GMP (c-di-GMP) ist an einer Vielzahl von regulatorischen Prozessen im Zusammenhang mit Veränderungen des Lebenszyklusses in Bakterien beteiligt. *Myxococcus xanthus* reagiert entsprechend der Nährstoffverfügbarkeit in seiner Umgebung. Bei ausreichenden Nährstoffen bildet *M. xanthus* sich ausbreitende Kolonien. Unter nahrungslimitierenden Bedingungen hingegen werden mit Sporen gefüllte Fruchtkörper geformt. In dieser Arbeit wurde die Funktion von c-di-GMP in *M. xanthus* untersucht. *M. xanthus* kann c-di-GMP produzieren. Die Manipulation der zellulären c-di-GMP Konzentration durch Expression einer heterologen, aktiven Diguanylatzyklase oder Phosphodiesterase in lebenden Zellen führte zu einem Defekt der „type-IV-pili“ (T4P) abhängigen Beweglichkeit. Die Gleitbewegung von *M. xanthus* hingegen blieb dadurch unberührt. Eine erhöhte Konzentration von c-di-GMP reduzierte die Transkription des *pilA* Genes, welches für das wichtigste Pilin des T4P codiert, reduzierte das Vorkommen von T4P generell und veränderte die Zellagglutination. Ein niedriges Niveau von c-di-GMP führte lediglich zu veränderter Zellagglutination.

Die systematische Inaktivierung von 24 Genen in *M. xanthus*, welche für Proteine mit GGDEF, EAL oder HD-GYP Domänen kodieren, die im Zusammenhang mit der Synthetisierung, dem Abbau oder dem Binden von c-di-GMP stehen, identifizierte drei Gene, die wichtig für die T4P abhängige Bewegung sind. Die dazugehörigen Proteine DmxA, TmoK und SgmT enthalten alle eine GGDEF Domäne. DmxA besitzt Diguanylatzyklaseaktivität, TmoK und SgmT (beide Hybrid Histidinkinasen) zeigen keine Diguanylatzyklaseaktivität *in vitro*.

Die Konzentration von c-di-GMP steigt während nahrungslimitierenden Bedingungen signifikant an. Artifiziell herbeigeführtes niedriges c-di-GMP Niveau beeinflusst das Entwicklungsprogramm, hohes jedoch nicht. Zudem konnten wir aus den 24 Genen, die für Proteine mit GGDEF, EAL und HD-GYP Domänen kodieren, zwei Gene identifizieren, welche spezifisch im Entwicklungsprogramm von *M. xanthus* involviert sind: *pmxA* und *dmxB. pmxA* kodiert für eine enzymatisch aktive Phosphodiesterase mit einer HD-GYP-Domäne. *dmxB* kodiert für eine im Entwicklungsprogramm induzierte, enzymatisch aktive Diguanylatzyklase. DmxB ist essentiell um ein erhöhtes c-di-GMP Niveau in den Zellen aufrechtzuerhalten und reguliert außerdem Exopolysaccharide während des Nährstoffmangels.

Unsere Resultate zeigen, dass c-di-GMP ein wichtiges Signalmolekül im Entwicklungsprogramm von *M. xanthus* ist und weist auf ein Model hin, in dem ein

minimaler Schwellenwert an c-di-GMP Konzentration erreicht sein muss, um ein erfolgreiches Fortschreiten des Entwicklungsprogrammes zu gewährleisten.

Zusätzlich konnten wir c-di-GMP spezifische Effektormoleküle mit Hilfe von Massenspektrometrie identifizieren und teilweise charakterisieren. Für einige dieser Kandidaten konnte bestätigt werden, dass sie *in vitro* c-di-GMP binden und die Deletionsmutanten der korrespodierenden Gene wurden hinsichtlich ihrer Fähigkeit des T4P abhängigen Beweglichkeit und ihres Entwicklungsprogrammes charakterisiert.

Abbreviations

ATP/ADP	adenosin tri-/diphosphate
bp	base pairs
cAMP	3’,5′-cyclic monophosphate
CCMS	capture compound mass spectrometry
cdG-CC	c-di-GMP specific capture compound
c-di-AMP	cyclic di-3′,5′-adenosine monophosphate
c-di-GMP	bis-(3'-5')-cyclic dimeric guanosine monophosphate
cDNA	single-stranded complementary DNA
cGMP	guanosine 3‘,5‘-monophosphate
CR	congo red
C-terminus	carboxyl-terminus
CTT	casitone Tris medium
DGC	diguanylate cyclase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRaCALA	differential radial capillary action of ligand assay
DTT	dithiothreitol
ECM	extracellular matrix
EM	electron microscopy
EPS	exopolysaccharides
GTP/GDP/GMP	guanosine tri-/di-/monophosphate
h	hours
HPK	histidine protein kinase
HTH	helix-turn-helix
IM	inner membrane
IPTG	isopropyl β-D-1-thiogalaktopyranoside
kDa	kilodalton
LPS	lipopolysaccharides
min	minutes
MOPS	3-(N-morpholino) propanesulfonic acid
OD	optical density
OM	outer membrane
PDE	phosphodiesterase
pGpG	5'- phosphoguanylyl- (3' −> 5')- guanosine
(p)ppGpp	guanosine 3'-diphosphate 5'-triphosphate
RNA	ribonucleic acid
RR	response regulator
s	seconds
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrilamide gel electrophoresis
T4P	type IV pili
TCSS	two-component systems
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLC	thin layer chromatography
WT	wild type

1. Introduction

1.1. Nucleotide based second messengers

In order to survive, all organisms need to sense and respond to changes in the surrounding environment. In the past years, significant effort has been dedicated to understand the mechanisms that bacteria use to adapt to their environments. One of the strategies that microorganisms use is based on so-called second messenger systems in which the sensing of an environmental signal, i.e. the first messenger, results in the production of a small cytoplasmic molecule, i.e. the second messenger, which regulates cellular processes involved in adaptation.

The list of second messengers used by bacteria include guanosine 3‘,5‘-monophosphate (cGMP), cyclic di-3′,5′-adenosine monophosphate (c-di-AMP), adenosine 3’,5′-cyclic monophosphate (cAMP), guanosine-3',5'-bis-pyrophosphate ((p)ppGpp) as well as cyclic di-3’,5’-guanosine monophosphate (c-di-GMP), which is the focus of this study (Figure 1) (Gomelsky, 2011). The two most studied second messengers are cAMP and (p)ppGpp.

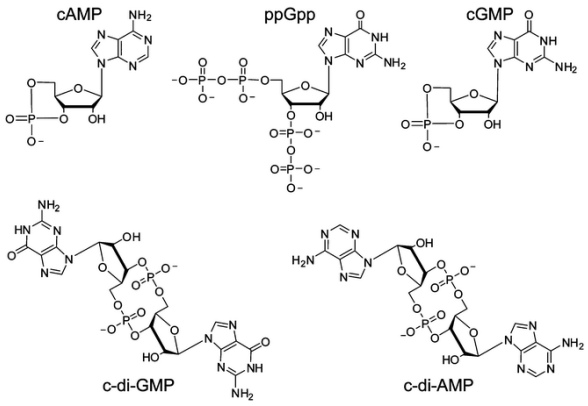


Figure 1. Chemical structures of known nucleotide based second messenger signaling molecules used by bacteria

Figure was reproduced from Shanahan & Strobel, 2012.

cAMP is a universal second messenger that functions in both prokaryotic and eukaryotic cells. It is synthesized from ATP by adenylate cyclases and degraded into AMP by phosphodiesterases. cAMP is produced by bacterial cells in response to carbon starvation and allosterically activates its receptor, the transcription factor called catabolite activator protein (CAP), in order to transcriptionally regulate different catabolic operons for the use of alternative carbon sources or, in the absence of other substrates, switches the central carbon metabolism to slow growth (Harman, 2001, Lee *et al.*, 2012b). The number of adenylate cyclase per genome varies between bacterial species. *Mycobacterium tuberculosis* genome contains 17 genes encoding for proteins

with adenylate cyclase domains. In contrast, *Pseudomonas aeruginosa* and *Myxococcus xanthus* have two adenylate cyclases (He & Bauer, 2014, Kimura *et al.*, 2002, Kimura *et al.*, 2005). *Escherichia coli*, *Corynebacterium glutamicum* and *Streptomyces coelicolor* only have one adenylate cyclase (Agarwal *et al.*, 2009).

(p)ppGpp is produced only by bacteria and chloroplasts. It accumulates upon starvation with the initiation of the stringent response, and is produced from GTP or GDP by the ribosome-associated protein RelA in response to uncharged tRNA molecules in the acceptor site of ribosomes. (p)ppGpp is hydrolysed to GTP/GDP and pyrophosphate by SpoT. It was suggested that (p)ppGpp causes a redirection of transcription, so that genes important for starvation survival are activated at the cost of genes required for growth and proliferation (Magnusson *et al.*, 2005). All bacteria contain RelA-SpoT homologues (Tozawa & Nomura, 2011). *E. coli and M. xanthus* possess both of those enzymes, while in *Streptococcus equisimilis, C. glutamicum*, *Bacillus subtilis* and others, there seems to be only one RelA/SpoT homologue, which encodes both synthetic and degradative functions (Sun *et al.*, 2001, Harris *et al.*, 1998).

1.2. c-di-GMP in bacteria

c-di-GMP is a ubiquitous nucleotide-based second messenger and a global regulator of a variety of processes that are typically associated with lifestyle changes in response to environmental cues in bacteria. An overall function of c-di-GMP signaling is to regulate the transition between motile and sessile bacterial lifestyles. Generally, elevated c-di-GMP levels are associated with inhibition of motility, increased adhesion and biofilm formation while low levels of c-di-GMP are associated with motile, free-living cells (Hengge, 2009, Krasteva *et al.*, 2012, Jenal & Malone, 2006, Boyd & O'Toole, 2012, Römling *et al.*, 2013). c-di-GMP was first identified in the 1980s as an allosteric activator of cellulose synthase, used by *Gluconacetobacter xylinus* to produce an extracellular cellulose matrix (Ross *et al.*, 1987).

It is now well known that c-di-GMP is produced by enzymes called diguanylate cyclases (DGCs) that contain GGDEF domains, and it is degraded by c-di-GMP-specific phosphodiesterases (PDEs) that contain EAL or HD-GYP domains (Figure 2). EAL domain phosphodiesterases degrade the second messenger to its linear form, pGpG (Ross *et al.*, 1986) while HD-GYP domain phosphodiesterases fully hydrolyse c-di-GMP to GMP (Ryan *et al.*, 2006). Recently, two studies described the oligoribonuclease Orn as the primary enzyme responsible for pGpG degradation in *P. aeruginosa* (Cohen *et al.*, 2015, Orr *et al.*, 2015). High level of pGpG reduces c-di-GMP

degradation in cell lysates and inhibits the activity of EAL type PDEs (Cohen *et al.*, 2015). This result supports the already existing idea that pGpG can function as a signalling molecule itself.

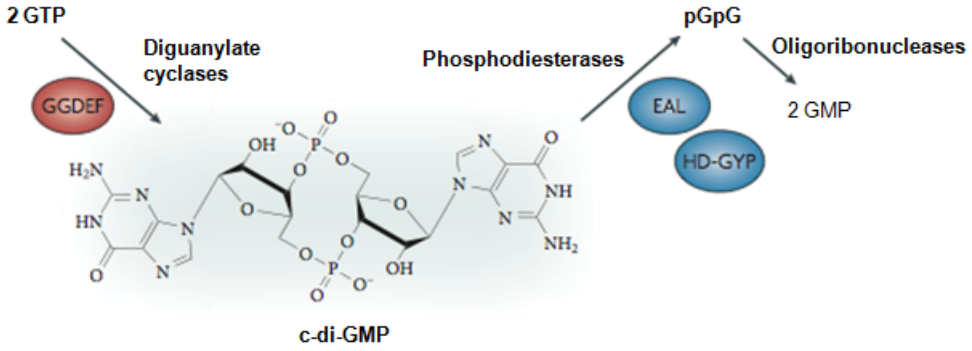


Figure 2. c-di-GMP structure and metabolism

c-di-GMP is a nucleotide-based second messenger. Its level in the cells is antagonistically controlled by diguanylate cyclases (DGC) that carry GGDEF domains and phosphodiesterases (PDE) that carry EAL or HD-GYP domains. Figure was modified from Hengge, 2009.

In recent years, genome sequencing allowed to reveal many proteins involved in c-di-GMP signaling in the genomes of diverse bacterial species. The number of GGDEF and EAL/HD-GYP domain proteins is highly variable and differs from organism to organism (Jenal & Malone, 2006). For example, *E. coli* encodes 29 of them (Weber *et al.*, 2006), *Clostridium difficile* encodes 37 (Bordeleau *et al.*, 2011) and *P. aeruginosa* PA01 encodes 38 (Kulasakara *et al.*, 2006). Some proteins contain both GGDEF and EAL domains, but in many of them just one is enzymatically active, with the other domain having a regulatory function (Cotter & Stibitz, 2007, Tamayo *et al.*, 2007).

Such an abundance of c-di-GMP-metabolizing enzymes, combined with the fact that many of them also contain different sensory and regulatory domains, suggests that the c-di-GMP signaling network in the cell is precisely regulated in response to environmental conditions.

1.2.1. c-di-GMP metabolism

Diguanylate cyclases

c-di-GMP is produced from two molecules of GTP by diguanylate cyclases (DGCs), enzymes that contain a GGD(E)EF (Gly-Gly-Asp(Glu)-Glu-Phe) motif in the

active site (A-site) and substitutions that alter this motif usually abolish the diguanylate cyclase activity (Ferreira *et al.*, 2008). The two glycine residues of the GGDEF motif are involved in GTP binding. The third amino acid (aspartate/glutamate) is required for catalysis and metal ion coordination, while the fourth residue (glutamate) is involved in metal ion coordination (Chan *et al.*, 2004). The fifth residue, phenylalanine, is highly conserved and also essential for catalysis, but its exact role remains unknown.

Structural studies of the full-length PleD response regulator from *Caulobacter crescentus* in complex with c-di-GMP allowed proposing a catalytic mechanism for the condensation of two GTP molecules into c-di-GMP (Figure 3AB). Structurally, the DGC enzyme is a homodimer and consists of two monomers that are in close physical contact to form the active site at the interface between the two monomers, and create the catallycally active DGC enzyme (Römling *et al.*, 2013, Hengge, 2009) (Figure 3AB). The diguanylate cyclase catalyzes the formation of phosphodiester bonds between two GTP molecules with the presence of two Mg^{2+} or two Mn^{2+} leading to the production of c-di-GMP (Römling *et al.*, 2013).

Most GGDEF domains also contain an allosteric inhibitory site (I-site), defined by an RxxD motif (where x indicates any amino acid) and typically located five amino acids N-terminal of the GGDEF motif. The I-site allows product inhibition and by this limits the concentration of c-di-GMP (Christen *et al.*, 2006).

A. **B.**

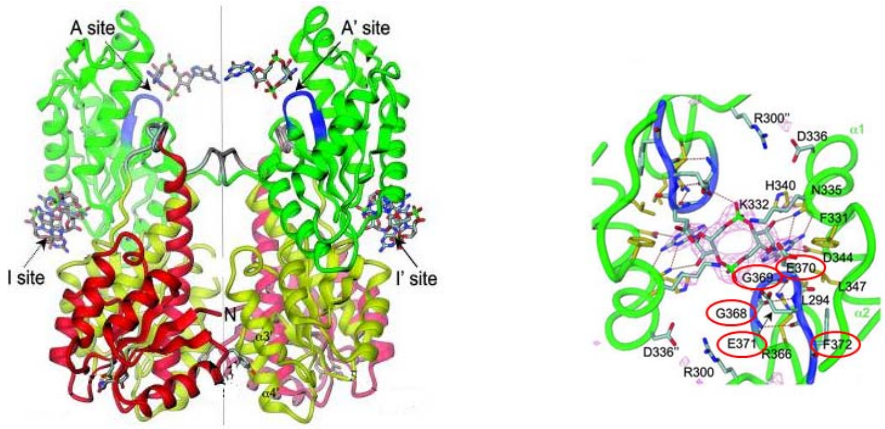


Figure 3. Crystal structure of PleD form *C. crescentus*

(A) DGC dimer. The monomer consists of three domains. Domain D1 (residues 2–140) is shown in red. Domain D2 (residues 141–285) is shown in yellow. The catalytic DGC domain (residues 286–454) is shown in green. The GGEEF motif is located on the β-hairpin (blue) and constitutes part of the active site (A-site) to which a c-di-GMP molecule is bound. Two c-di-GMP molecules are found at the D2/DGC interface (I-site).
(B) Close-up view of the active site. Conserved residues important for protein activity and stability are indicated. The GGEEF motif in the active site is marked in red.
Figure was modified from Chan *et al.*, 2004.

PleD is activated upon phosphorylation of the N-terminal receiver domain (Chan *et al*., 2004) (Figure 4). Phosphorylation is an important and widespread mechanism of GGDEF domain activation. For example Rrp1 from *Borrelia burgdorferi* consists of REC-GGDEF domains and is not functional *in vitro* without the REC domain being phosphorylated (Ryjenkov *et al*., 2005).

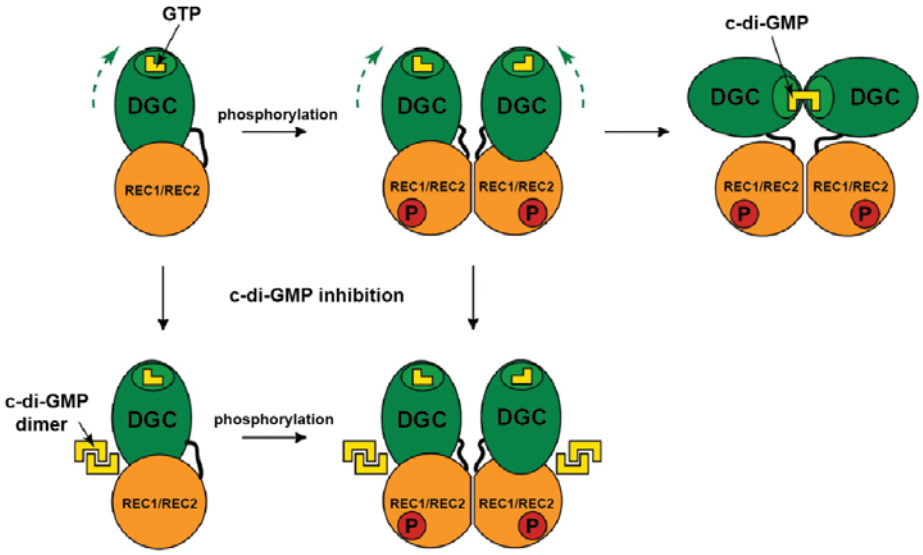


Figure 4. Model of PleD regulation

Activation and product-inhibition of DGC PleD. Phosphorylation of REC1-REC2 triggers PleD to dimerise and activates DGC activity. Binding of dimeric c-di-GMP at the I-site abolishes DGC activity. The catalytic DGC domain is indicated in green, REC1 and REC2 domains are indicated in orange, GTP and c-di-GMP are indicated in yellow, phosphoryl group is indicated in red. Figure was modified from Chan *et al*., 2004.

Phosphodiesterases

The phosphodiester bond in c-di-GMP is hydrolyzed by specific phosphodiesterases (PDEs). c-di-GMP-specific PDE activity is associated with EAL and HD-GYP domains. The first step of the reaction (hydrolysis of c-di-GMP into pGpG) is performed by EAL type phopshodiesterases, while the second step is performed by different enzymes with affinity for pGpG (Römling *et al*., 2013). The HD-GYP domain PDEs catalyse the complete hydrolysis of c-di-GMP to 2 GMP (Tamayo *et al*., 2007, Hengge, 2009). PDE reaction depends on the presence of Mg^{2+} or Mn^{2+} ions, and is inhibited by Ca^{2+} ions (Christen *et al*., 2005).

1.2.2. Regulation of diguanylate cyclase and phophodiesterase activity

The activities of DGCs and PDEs are regulated on different levels. In *E. coli,* the majority of the genes encoding GGDEF/EAL domain proteins are transcriptionally regulated under the control of the general stress sigma factor σ^S (Sommerfeldt *et al.*, 2009). However, the most common mechanism for regulation of enzymatic activity of DGCs and PDEs seems to be posttranslational control. Some of these proteins contain just GGDEF or EAL domains, but most are present in combination with other domains such as PAS, GAF, HAMP, receiver (REC), and helix-turn-helix (HTH) (Tamayo *et al.*, 2007). Those domains have been described to participate in phosphorylation and DNA binding, sense small molecules, light, redox potential, voltage, oxygen, nutrients, osmolarity and other signals.

Among those processes, phosphorylation is a common mechanism regulating protein activity. The signaling proteins composed of GGDEF and/or EAL or HD-GYP domains with the REC domain belong to the group of response regulators (RR) of two-component signal transduction systems (TCSS). Domains involved in c-di-GMP metabolism are found in around 5.4% of all bacterial response regulators (Römling *et al*., 2013). This makes them an important component of the two-component signal transduction machinery.

Proteins of two-component systems have important functions in sensing signals in bacteria, in the generation of responses, in adaptation and differentiation (Beier and Gross, 2006). They regulate many bacterial processes such as virulence, motility, secondary metabolite production and cell division (Stock *et al*., 2000). The number of two-component system proteins differs greatly between different bacteria (Stock *et al*., 2000). Response regulators are either single-domain proteins consisting only of the receiver domain with the conserved aspartate residue, or multi-domain proteins containing a receiver domain and an output domain (Stock *et al*., 2000).

Typically, TCS consists of a histidine protein kinase (HPK) and a response regulator (RR) that are usually encoded in the same operon. HPKs sense a signal and autophosphorylate on the conserved histidine residue using ATP. Then, the phosphoryl group is transferred to the conserved aspartate residue in the receiver domain of the response regulator, resulting in activation of the response regulator and the generation of a response (Figure 5).

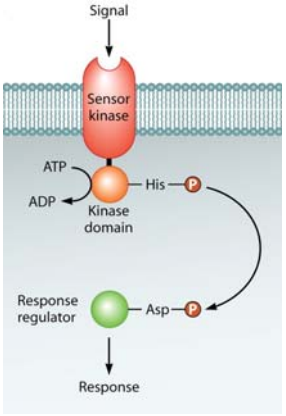


Figure 5. Typical organization of the two component systems (TCSS) in bacteria

The typical TCSS is comprised of a single sensor kinase and a single response regulator. Signal sensing by the input domain causes activation of the autokinase domain, which results in phosphorylation of a specific histidine residue in the phosphotransfer subdomain of the kinase. The phosphoryl group is then transferred to a conserved aspartate residue in the receiver domain of the cognate response regulator protein. Phosphorylation results in modulation of the function of the linked output domain. Figure was modified from Bretl *et al.*, 2011.

The presence of multiple GGDEF/EAL/HD-GYP domain enzymes encoded in the genomes of many bacterial species and the complex regulation of their activity raises a problem of specificity. That is why it is generally believed that c-di-GMP signaling may involve local, rather than global, pools of c-di-GMP, which would overcome the problem of unwanted cross-talk among individual signaling systems. These local pools are thought to be a result of the temporal and spatial separation of the c-di-GMP metabolizing enzymes as well as their receptors. Temporal separation would be accomplished by activation of gene expression and protein function only under certain conditions. Local separation would mean that c-di-GMP control modules would operate in physically separated cellular compartments (Römling *et al.*, 2013, Hengge, 2009).

1.2.3. c-di-GMP specific receptors

While c-di-GMP metabolizing proteins are easy to identify due to the characteristic conserved domains, identifying the c-di-GMP specific effectors is much more challenging. It is now known that cyclic di-GMP has a variety of cellular receptors. They include degenerate GGDEF, HD-GYP and EAL domain proteins that do not have catalytic activity (Duerig *et al.*, 2009, Petters *et al.*, 2012, Qi *et al.*, 2011, Newell *et al.*, 2009, Hengge, 2009, Navarro *et al.*, 2009, Boyd & O'Toole, 2012), transcription factors of the TetR, CRP/FNR, NtrC, FixJ/LuxR/CsgD and BldD families (Li & He, 2012, Chin *et al.*, 2010, Fazli *et al.*, 2011, Hickman & Harwood, 2008, Srivastava *et al.*, 2011, Krasteva *et al.*, 2010, Tschowri *et al.*, 2014), stand-alone PilZ domains as well as PilZ domains that are part of multidomain proteins (Amikam & Galperin, 2006, Ryjenkov *et al.*, 2006, Christen *et al.*, 2007, Pratt *et al.*, 2007, Ramelot *et al.*, 2007, Merighi *et al.*, 2007, Wilksch *et al.*, 2011, Tschowri *et al.*, 2014), riboswitches (Sudarsan *et al.*, 2008) as well as proteins which do not belong to any of these groups like the PgaCD complex

involved in poly-β-1,6-*N*-acetylglucosamine synthesis in *E. coli* (Steiner *et al.*, 2013). Based on this, c-di-GMP can serve as a regulatory molecule at the transcriptional, post-transcriptional and post-translational level affecting many different processes in the cell (Ryan *et al.*, 2012b, Sondermann *et al.*, 2012).

Proteins with a PilZ domain

PilZ domain represents the best studied type of c-di-GMP specific receptors. PilZ domain-containing proteins were reported to function either as single domains or to be linked to other regulatory domains (Römling *et al*., 2013). The most highly conserved residues include the motif RxxxR in the N-terminus of the protein, and a second motif, D/NxSxxG, which participate in di-nucleotide binding (Shin *et al.*, 2011) (Figure 6). The two described mechanisms of regulation by PilZ domain include direct protein-protein interactions and DNA binding (Ryan *et al*., 2012b).

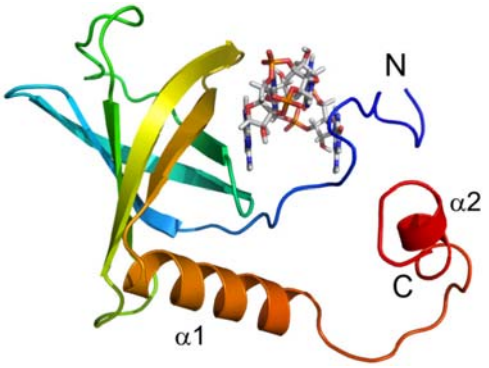


Figure 6. Structure of PilZ domain protein PA4608 with bound c-di-GMP

Cyclic di-GMP (in stick representation) binds as an intercalated, symmetric dimer to one side of the β-barrel. N-terminus containing the RxxxR motif wraps around the ligand. Secondary structure elements are color-coded from N-terminus (blue) to C-terminus (red). Figure was reproduced from Habazettl *et al.*, 2011.

Proteins with degenerated GGDEF, EAL or HD-GYP domains

Some proteins with GGDEF, EAL or HD-GYP domains carry degenerated motifs, meaning that they have lost catalytic activity but maintain the ability to bind c-di-GMP and function as effectors. Several examples of such receptors were described in *P. aeruginosa*. In the case of PelD, c-di-GMP binds to the conserved I-site of the GGDEF domain (Lee *et al.*, 2007), in the case of LapD, c-di-GMP binds to the EAL domain (Newell *et al*., 2009). According to the Römling *et al*., no enzymatically inactive HD-GYP domains have been identified so far to act as c-di-GMP receptors molecules although they are expected to exist (Römling *et al*., 2013).

Transcription factors

In a number of bacterial species like *P. aeruginosa* (FleQ, BrlR)*, Vibrio cholerae* (VpsT), *Xanthomonas campestris* (Clp), *Klebsiella pneumonia* (MrkH), *Streptomyces venezuelae* (BldD), *Mycobacterium smegmatis* (LtmA), *Burkholderia cenocepacia* (Bcam1349) and others – c-di-GMP has been shown to modulate the activity of transcription factor (Römling *et al*., 2013).

Riboswitches

The group of Breaker discovered that two classes of riboswitches (class I and II) can bind c-di-GMP (Sudarsan *et al*., 2008). Binding of c-di-GMP to these riboswitches was shown to exert a regulatory role towards the target mRNA, affecting transcription termination or translation (Hengge, 2010).

1.2.4. Processes regulated by c-di-GMP

The most general function of c-di-GMP is being a key regulator in the transition process from a motile and planktonic to a sessile lifestyle of bacteria. This is accomplished by regulation a plethora of cellular processes including biofilm formation, motility, differentiation, virulence, cell cycle and others. Due to its main function, c-di-GMP is best studied in terms of its effect on motility and biofilm formation.

Regulation of motility

There are numerous ways by which cyclic c-di-GMP influences motility. Its targets include flagellar motility, T4P motility as well as gliding motility (Römling *et al*., 2013).

c-di-GMP inhibits flagellar motility in a variety of ways, either at the level of gene expression, flagellar assembly or function. In *E. coli* and *Salmonella enterica* c-di-GMP binds to the receptor protein YcgR, which in turn interacts with the FliG and FliM subunits of the flagellum switch complex FliGMN and interferes with flagellar motor function (Paul *et al.*, 2010, Boehm *et al*., 2010). The same is true also for *B. subtilis*, where the homolog of YcgR, YpfA, was found to interfere with flagellar rotation through its interaction with the flagellar motor protein MotA (Chen *et al.*, 2012).

c-di-GMP was found to be also involved in transcriptional regulation of flagellar genes *via* VpsT in *V. cholerae* (Krasteva *et al*., 2010).

c-di-GMP signaling also controls T4P biogenesis and twitching motility in *P. aeruginosa.* T4P biogenesis requires the GGDEF-EAL domain protein FimX localized at one cell pole (Huang *et al.*, 2003). FimX binds c-di-GMP via its degenerate EAL domain (Navarro *et al.*, 2009). Studies of the FimX homolog in *X. campestris* allowed proposing molecular mechanisms by which FimX affects type IV pilus biogenesis and twitching motility. Upon binding of c-di-GMP, FimX interacts with a degenerated PilZ domain protein. PilZ domain protein subsequently interacts with an ATPase PilB, to control T4P pilus polymerization (Guzzo *et al.*, 2009) (Figure 7). Additionally, in *X. campestris* the complex of RpfG (HD-GYP domain response regulator) and GGDEF domain proteins (XC_0420 and XC_0249) recruits a specific PilZ domain protein that interacts with the pilus motor proteins PilU and PilT controlling motility (Ryan *et al.*, 2012a, Ryan *et al.*, 2010).

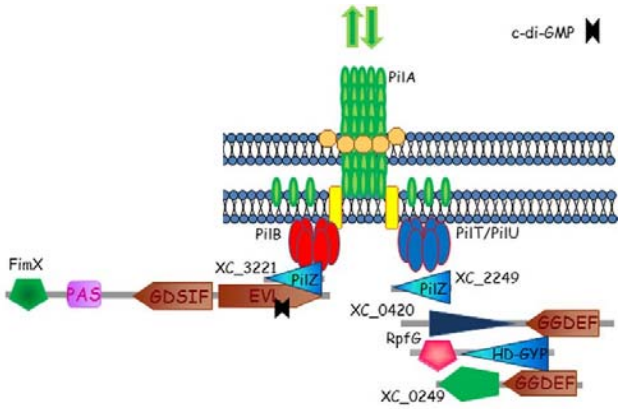


Figure 7. c-di-GMP dependent regulation of T4P motility in *Xanthomonas*

PilZ domain protein XC_3221 mediates interactions between the c-di-GMP binding protein FimX and the pilus polymerization ATPase PilB. RpfG in a complex with the diguanylate cyclases XC_0249 and XC_0420 recruits the XC_2249 adaptor and interacts with the PilT/PilU ATPases required for pilus retraction. Figure was modified from Römling *et al*., 2013.

Regulation of gliding motility by c-di-GMP has been discovered in *Bdellovibrio bacteriovorus*, where one of three active DGCs, DgcA, is important for gliding. This type of motility is required for the *B. bacteriovorus* to exit the exhausted prey debris and to move off to regions where new prey can be found (Hobley *et al.*, 2012).

Regulation of biofilm formation

c-di-GMP regulates many extracellular matrix components contributing to biofilm formation, like diverse exopolysaccharides, adhesive pili, adhesins, as well as extracellular DNA. Biofilm formation can be controlled by c-di-GMP on the level of transcription, posttranscription and posttranslation.

Synthesis of cellulose in *G. xylinus*, *E. coli* and *S. typhimurium* is regulated by c-di-GMP. Bacterial cellulose is produced and translocated across the inner membrane by a cellulose synthase BcsA. c-di-GMP directly activates cellulose biosynthesis in these bacteria by binding to the PilZ domain of BcsA (Figure 8) (Ross *et al.*, 1987, Morgan *et al.*, 2014, Whitney *et al.*, 2012).

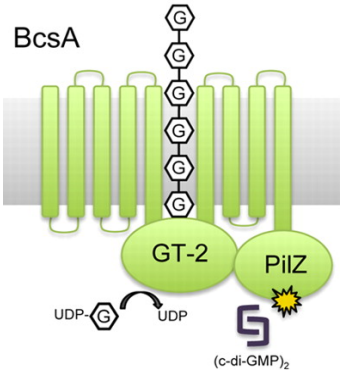


Figure 8. Schematic representation of the *E. coli* cellulose biosynthesis systems

The *E. coli* cellulose synthase protein BcsA is colored *green*. GT-2 indicates the family of glycosyl transferases. The chains of hexagons containing G represent 1,4-linked β-d-glucose (cellulose). Figure was modified from Whitney *et al.*, 2012.

Activation of biosynthesis of Pel and Psl polysaccharides by c-di-GMP has been studied mostly in *P. aeruginosa*. Pel and Psl production is increased in the presence of high levels of c-di-GMP, for example upon constitutive activation of the REC-GGDEF diguanylate cyclase WspR (Hickman *et al.*, 2005). The NtrC-like transcriptional regulator FleQ is a c-di-GMP receptor that, upon binding c-di-GMP, promotes *pel* and *psl* transcription (Hickman & Harwood, 2008). Biosynthesis of Pel is also regulated by c-di-GMP at the posttranslational level by an I-site c-di-GMP receptor encoded in the *pel* operon, PelD, possibly through activation of the associated glycosyl transferases (Lee *et al.*, 2007, Whitney *et al.*, 2012).

1.3. *Myxococcus xanthus* as a model organism

In this study, we focused on the c-di-GMP signaling pathways in the soil bacterium *Myxococcus xanthus*. *M. xanthus* is a Gram-negative, rod-shaped deltaproteobacterium. If present on a solid surface at a high cell density, *M. xanthus*

cells can self-organize into three morphologically distinct patterns: spreading colonies, ripples or fruiting bodies (Konovalova *et al*., 2010) (Figure 9). Due to its complex lifecycle, *M. xanthus* serves as a model for investigating motility, cell polarity, social behaviour and starvation induced development.

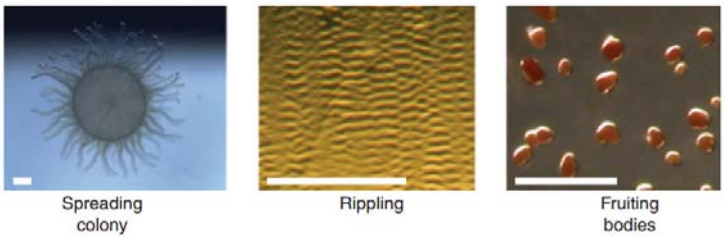


Figure 9. Three cellular patterns formed by *M. xanthus* cells

Scale bars=1 mm. Figure was reproduced from Konovalova *et al*., 2010.

1.3.1. Developmental program

M. xanthus undergoes major lifestyle changes in response to nutrient availability (Konovalova *et al*., 2010). If nutrients become limited, cells initiate a developmental program that results in the formation of multicellular fruiting bodies inside which the rod-shaped motile cells differentiate to spherical spores. Spores germinate in the presence of nutrients.

Fruiting body formation proceeds in distinct morphological stages that are separated in time and space. After 4-6 h of starvation, cells change motility behaviour and start to aggregate to form translucent mounds (Jelsbak & Søgaard-Andersen, 1999, Jelsbak & Søgaard-Andersen, 2002). By 24 h the aggregation process is complete and cells that have accumulated inside fruiting bodies differentiate to spores with spore maturation complete by 72 h. Only 1 to 3% of cells differentiate into spores while up to 30% of cells remain outside of the fruiting bodies as so called peripheral rods (O'Connor & Zusman, 1991b, O'Connor & Zusman, 1991a). The remaining cells undergo cell lysis (Wireman & Dworkin, 1977) (Figure 10).

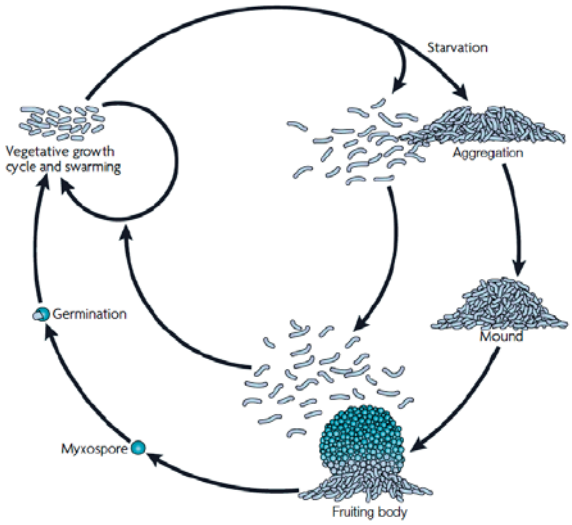


Figure 10. Schematic representation of the *M. xanthus* life cycle

Various stages are described in the main text. Figure was reproduced from Zusman *et al.*, 2007.

M. xanthus multicellular development is mediated by series of signaling events. The first response of starving cells is the stringent response, in which the cell produce the second messenger (p)ppGpp in response to elevated levels of uncharged tRNAs. The stringent response in *M. xanthus,* as in other bacteria, is based on the RelA (p)ppGpp synthase. The intracellular level of (p)ppGpp is important for the expression of many genes involved in development (Singer & Kaiser, 1995, Harris *et al*., 1998). After starvation is sensed and early development genes are upregulated, the cells start to form fruiting bodies.

Fruiting body development depends extensively on intercellular signaling between *M. xanthus* cells. Five intercellular signals (A-, B-, C-, D-, and E-signals) have been suggested to be involved in development of *M. xanthus*. However, only two of them, the A- and C-signals, have been characterized in details.

The A-signal becomes important for development after 2 h. A-signal is mostly composed of six amino acids (Trp, Pro, Phe, Tyr, Leu, and Ile) and peptides. The A-signaling system functions to ensure that a sufficiently large population of starved cells is present to make a fruiting body (Shimkets, 1999, Konovalova *et al*., 2010). C-signal becomes important after 6 h of starvation, acts in a threshold dependent manner and functions to ensure the correct temporal order of rippling, aggregation and sporulation (Konovalova *et al*., 2010). The intercellular C-signal is a 17 kDa protein (p17), which is generated by proteolytic cleavage of the full-length 25 kDa CsgA protein (p25) (Lobedanz & Sogaard-Andersen, 2003).

Sporulation of *M. xanthus* cells can also be chemically induced by compounds such as glycerol, DMSO or ampicillin in vegetatively growing cultures (O'Connor & Zusman, 1997). These artificially induced spores have a thinner spore coat and lack

some proteins that are found in starvation induced spores (Kottel *et al.*, 1975, Mccleary *et al.*, 1991) but as starvation induced spores they will germinate once plated on a rich media.

1.3.2. Two motility systems

The social lifestyle of *M. xanthus* depends on the ability of cells to display active movement. When nutrients are available, cells grow, divide and form colonies in which cells at the edge spread outwards in a coordinated fashion using two motility systems: T4P dependent motility (also called S or social) and gliding motility (also called A or adventurous). These two forms of motility are genetically independent. Gliding motility is largely dispensable for fruiting body formation (Hodgkin & Kaiser, 1979) while lack of T4P dependent motility causes a delay or even blocks fruiting body formation (Wu *et al.*, 1998, Hodgkin & Kaiser, 1979).

Gliding motility

A-motility (gliding) is favoured on hard and dry surfaces and enables the movement of single cells. There are different models proposed for this motility system. One model, called the slime gun model, implies that A-motility depends on polyelectrolyte gel (slime) actively secreted at the lagging cell pole (Wolgemuth *et al.*, 2002). In an alternative model, gliding motility is based on motility complexes that are distributed along the cell length (Luciano *et al.*, 2011, Sun *et al.*, 2011, Nan *et al.*, 2010, Mignot *et al.*, 2007, Nan *et al.*, 2011, Jakobczak *et al.*, 2015).

T4P-dependent motility

T4P-dependent motility, favoured on wet and soft surfaces, occurs when cells move in groups. It is comparable to twitching motility of *Pseudomonas* species and depends on T4P (Kaiser, 1979, Wu & Kaiser, 1995). T4P are highly dynamic structures undergoing cycles of extension, attachment to the substratum and retraction. Retractions generate a force that pulls bacterial cell forward (Merz *et al.*, 2000). In *M. xanthus* 5-10 T4P can be found exclusively at the leading cell pole (Sun *et al.*, 2000, Kaiser, 1979).

The *M. xanthus pil* locus is composed of 17 genes, 14 of them share similarity with their *Pseudomonas* orthologs and are designated with the same names (Wall & Kaiser, 1999) (Figure 11). PilA is the major pilin assembled into filaments. PilB and PilT

share homology and have ATPase activity. PilB drives the assembly of PilA subunits into T4P and is referred to as the extension motor. PilT is the retraction motor and cause the depolymerisation of the pilus during retraction (Jakovljevic *et al.*, 2008). Other core members crucial for T4P biogenesis are PilCDMNOPQ, Tgl and TsaP (Figure 11). Mutation of any gene described above leads to loss or, in case of *pilT*, significant impairment of T4P-motility (Bulyha *et al.*, 2009, Friedrich *et al.*, 2014, Siewering *et al.*, 2014).

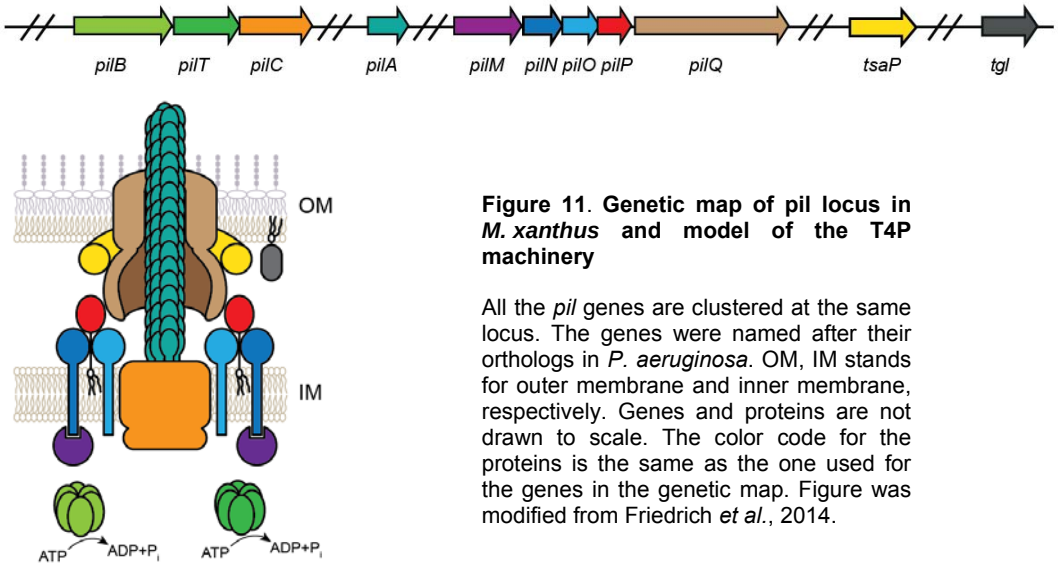


Figure 11. Genetic map of pil locus in *M. xanthus* and model of the T4P machinery

All the *pil* genes are clustered at the same locus. The genes were named after their orthologs in *P. aeruginosa*. OM, IM stands for outer membrane and inner membrane, respectively. Genes and proteins are not drawn to scale. The color code for the proteins is the same as the one used for the genes in the genetic map. Figure was modified from Friedrich *et al.*, 2014.

Moreover, additional genes were described, that encode components of the regulatory systems for T4P. Both *P. aeruginosa* and *M. xanthus* possess the PilR-PilS TCS that is involved in the regulation of the *pilA* gene expression. Similarly to *P. aeruginosa, pilA* transcription in *M. xanthus* depends on the response regulator PilR and is most likely driven by a σ^{54} promoter. The histidine kinase PilS is a negative regulator of *pilA* expression (Hobbs *et al.*, 1993, Wu & Kaiser, 1997, Ishimoto & Lory, 1992).

1.3.3. *M. xanthus* extracellular matrix

The *M. xanthus* extracellular matrix (ECM) is composed of 55% carbohydrate and 45% protein (Behmlander & Dworkin, 1994a). The ECM proteins are tightly associated with the exopolysaccharide (EPS), requiring detergent and boiling to remove them

(Behmlander & Dworkin, 1994b). The function of most of the ECM proteins remains unclear. One of them, FibA, is a zinc metalloprotease and the most abundant protein associated with the ECM (Curtis *et al.*, 2007). FibA is important for developmental progression (Bonner *et al.*, 2006). EPS is comprised of the monosaccharides mannose, galactosamine, galactose, glucosamine, *N*-acetylated-amine sugars, glucose, rhamnose and xylose but its macromolecular structure is unknown (Lu *et al.*, 2005).

In *M. xanthus* T4P-dependent motility is cell-cell contact-dependent because EPS stimulates T4P retraction (Li *et al.*, 2003). Lack of EPS blocks fruiting body formation and sporulation (Shimkets, 1986b, Shimkets, 1986a, Yang *et al.*, 2000, Chang & Dworkin, 1994). Many mutants with altered EPS accumulation have been identified and often these mutants not only have defects in T4P-dependent motility but also in development (Yang *et al*., 2000, Caberoy *et al.*, 2003, Berleman *et al.*, 2011, Overgaard *et al.*, 2006, Lancero *et al.*, 2004, Petters *et al*., 2012, Weimer *et al.*, 1998, Dana & Shimkets, 1993, Lancero *et al.*, 2005, Lu *et al*., 2005, Moak *et al.*, 2015).

Most mutations causing a defect in EPS accumulation are in the genes encoding regulatory proteins. It was shown that the production of EPS in *M. xanthus* is regulated by different genetic loci, such as the *dif* operon (Yang *et al*., 2000); *pilA*, the gene encoding the pilus structural protein (Black *et al.*, 2006) and *stkA* and *sglK***,** encoding DnaK homologues (Yang *et al.*, 1998a). Transposon mutagenesis studies revealed two genetic regions that are required for *M. xanthus* EPS accumulation: *eps* and *eas* regions that carry genes coding for proteins required for the assembly and export of the EPS polymer (Lu *et al*., 2005). *M. xanthus* genome encodes 70 different glycosyltransferases and 47 glycosyl hydrolases, as described in the Carbohydrate Active Enzymes (CAzy) database (Lombard *et al.*, 2014) .

The Dif chemosensory system is essential for EPS synthesis, however, the regulatory mechanism remains unknown. The Dif system consists of five proteins: MCP (DifA), CheW adaptor protein (DifC), and CheA histidine protein kinase (DifE), CheY response regulator (DifD) and CheC phosphatase (DifG) (Yang *et al.*, 1998b) (Figure 12). DifA, DifC and DifE build the core of the system and stimulate EPS synthesis (Yang *et al*., 1998b). In contrast, DifD and DifG negatively regulate EPS accumulation (Yang *et al*., 1998b, Black & Yang, 2004). It has been proposed that the DifE kinase stimulates EPS accumulation by phosphorylation of a yet to be identified response regulator (Black & Yang, 2004) while DifD and DifG function synergistically to divert phosphates away from DifE-P (Black *et al.*, 2010) (Figure 12).

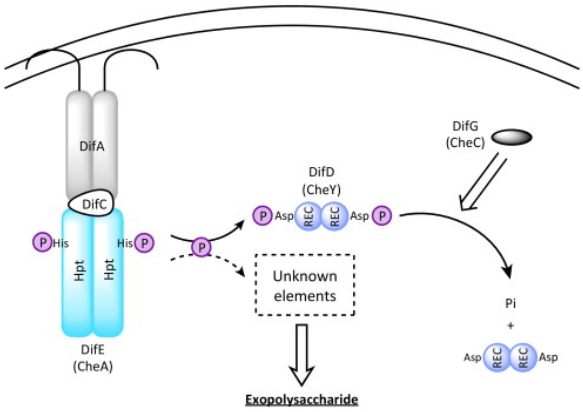


Figure 12. *M. xanthus* Dif system

Schematic diagram of the Dif signalling pathway. Indicated homology of *M. xanthus dif* locus with bacterial chemotaxis proteins. Details are described in the text. Figure was reproduced from He & Bauer, 2014.

Based on the fact that mutants lacking T4P accumulate reduced amounts of EPS, that a Δ*pilT* mutant, which is hyperpiliated, accumulates increased amounts of EPS, and that *dif* mutations are epistatic to mutations affecting T4P function, it has been suggested that T4P serve as a sensor for the Dif pathway (Black *et al*., 2006). Studies by Li and colleagues reported, that the components of EPS such as N-acetylglucosamine trigger pilus retraction and that T4P are likely to directly bind EPS components (Li *et al*., 2003).

1.3.4. Lipopolysaccharide O-antigen

Another component essential for T4P-dependent motility in *M. xanthus* is the lipopolysaccharide (LPS) O-antigen. LPS consist of a lipid moiety called lipid A, a core of approximately 10 monosaccharides, and an O-antigen consisting of repetitive subunits of monosaccharide (Caroff & Karibian, 2003) (Figure 13). LPS is attached to the cells via the lipid A embedded in the outer membrane, while EPS can be bound to the cell surface or released into the environment (free EPS) (Whitfield & Valvano, 1993). The *M. xanthus* LPS O-antigen is generally similar to that in other Gram-negative bacteria. The *M. xanthus* LPS consists of glucose, mannose, rhamnose, arabinose, xylose, galactosamine, 2-keto-3-deoxyoctulosonic acid, 3-O-methylpentose and 6-O-methylgalactosamine (Yang *et al.*, 2007). When O-antigen production in *M. xanthus* is interrupted, several distinct colony behaviours are affected. Mutants show defects in fruiting body formation and T4P-dependent motility although they still produce T4P and EPS (Bowden & Kaplan, 1998).

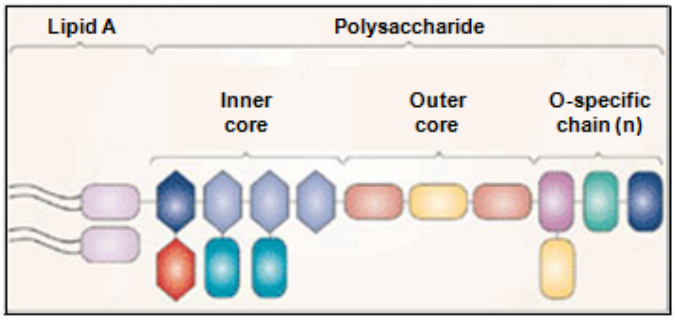


Figure 13. Structure of LPS in Gram-negative bacteria

Hexagon and oblongs represent units of various sugars. Figure was modified from Mahenthiralingam *et al.*, 2005.

1.3.5. c-di-GMP signaling in *M. xanthus*

Prior to this study, there was not much known about c-di-GMP signaling pathways in *M. xanthus*. The only information come from studies on TCS proteins. 272 genes encoding proteins of TCS were identified in *M. xanthus* (Shi *et al*., 2008). They have essential functions in regulation of motility and fruiting body formation. They are unusually organized: only 29% display the standard paired gene organization, 55% are orphan and 16% are in complex gene clusters. The most frequently occurring output domains of response regulators are involved in DNA binding and, importantly for this study, in c-di-GMP metabolism (Shi *et al*., 2008).

The first GGDEF domain protein studied in *M. xanthus* was a response regulator ActA, part of the *act* operon. ActA possesses degenerated A-site but intact I-site and was suggested to regulate production of the C-signal, a cell surface-associated protein required for aggregation of cells into fruiting bodies and sporulation but the connection with c-di-GMP was not studied (Gronewold & Kaiser, 2001).

The first hint that c-di-GMP may be important in the *M. xanthus* lifecycle came from studying the hybrid histidine protein kinase SgmT. SgmT is a partner kinase of the orphan DNA binding response regulator DigR (Petters *et al*., 2012). Together those two proteins are essential for regulating the composition of the extracellular matrix.

SgmT consists of N-terminal GAF domain, kinase and receiver domains, and a C-terminal GGDEF domain. SgmT activity is regulated by ligand binding to the GAF domain resulting in SgmT activation (Petters *et al*., 2012). A DigR binding site was identified in the promoter of the *fibA* gene, which encodes an abundant extracellular matrix metalloprotease. Based on microarray experiment the authors suggested that

SgmT/DigR regulates the expression of genes for secreted proteins and enzymes involved in secondary metabolite synthesis (Petters *et al*., 2012).

The A-site in GGDEF domain of SgmT is degenerated (GGGVF motif) but the I-site is intact and binds c-di-GMP *in vitro* (Figure 14A). c-di-GMP binding to SgmT mediates spatial localization of this cytoplasmic histidine kinase, without any obvious change in functionality (Petters *et al*., 2012) (Figure 14B).

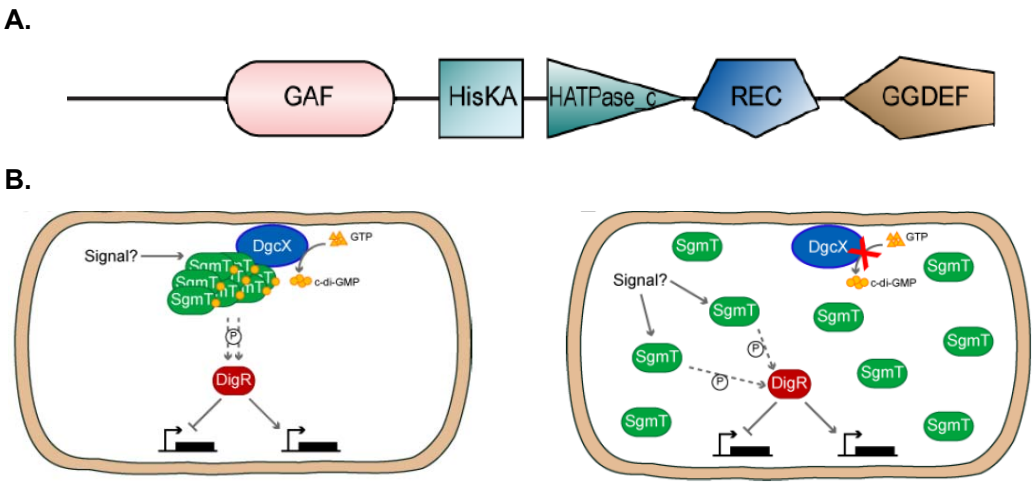


Figure 14. Model of SgmT/DigR action in regulating extracellular matrix composition

(A) Domain structure of DmxB protein. Domain annotation was performed using the SMART web tool, domains are not drawn to scale.
(B) SgmT localizes in the c-di-GMP-bound form in clusters in non-polar regions of the cell, presumably together with a yet unknown c-di-GMP diguanylate cyclase (DgcX). After the sensing of an unknown signal SgmT phosphorylates DigR which activates or represses the transcription of various genes. In the absence of DgcX or when the cellular c-di-GMP concentration is low, SgmT in the c-di-GMP-unbound form can freely diffuse in the cell. Whether and under what circumstances the cellular localization of SgmT is essential, is so far unknown.
Figure was reproduced from Petters, 2012.

1.4. Scope of the study

M. xanthus is a model organism to study social behaviour, cell-cell communication and development in bacteria. c-di-GMP is a second messenger that antagonistically control cellular motility and biofilm formation in many bacteria. Although many studies have revealed a general role of c-di-GMP in bacterial physiology, its metabolism and function in *M. xanthus* has never been described so far. Based on previous data, we hypothesized *M. xanthus* cells produce c-di-GMP and that c-di-GMP may be important for the cellular functions in this bacterium.

In this study, we investigated the role of c-di-GMP in *M. xanthus*. We manipulated the c-di-GMP level in the cells by heterologous overproduction of DGC and PDE enzymes during vegetative growth as well as during starvation and determined the effect of high as well as of low c-di-GMP levels on motility and development. Additionally, we bioinformatically predicted proteins encoded in *M. xanthus* genome putatively involved in c-di-GMP metabolism and we used molecular biology and biochemical methods to investigate their function. Finally, we aimed to experimentally identify and characterize c-di-GMP specific effectors in *M. xanthus*.

2. Results

2.1. *M. xanthus* accumulates constant level of c-di-GMP during vegetative growth and increasing level during starvation

To determine if *M. xanthus* cells synthesize c-di-GMP and at which level, we quantified c-di-GMP in wild type (WT) DK1622 cells during vegetative growth and during starvation, by using a liquid chromatography coupled tandem mass spectrometry method (Spangler *et al.*, 2010). For vegetative growth, cells in exponential growth phase and in stationary phase were used. For starvation, exponentially growing cells were removed from rich medium, transferred to MC7 buffer and starved in suspension for 48 h. For both experiments, three independent cultures were grown in parallel and then analyzed. Next, nucleotides were extracted and c-di-GMP level was quantified at indicated time-points. This experiment was performed by Dr. Tobias Petters.

c-di-GMP was detected at the same level throughout the exponential growth phase and in stationary phase cells (Figure 15A). As shown in Figure 15A, exponentially growing cells had a level of c-di-GMP of 4.4 ± 1.7 pmol/mg protein and stationary phase cells a level of 4.2 ± 0.7 pmol/mg protein. These data suggest that the level of c-di-GMP is not growth phase regulated in *M. xanthus*. In contrast, in *E. coli* the level of c-di-GMP increases at the entry into stationary phase and then decreases again during stationary phase (Spangler *et al*., 2010).

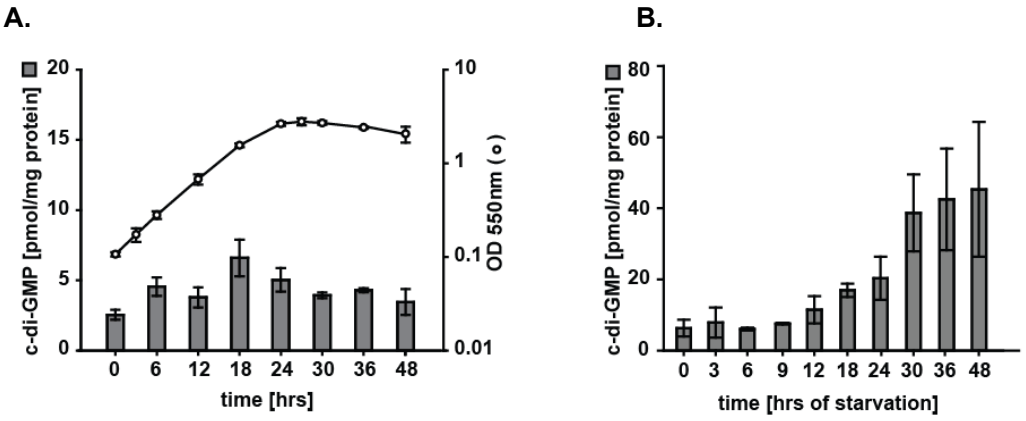


Figure 15. c-di-GMP accumulates in vegetative *M. xanthus* cells and during starvation

(A) c-di-GMP levels during vegetative growth of DK1622 WT cells in rich medium in suspension culture. Levels of c-di-GMP are shown as mean ± standard deviation (SD) calculated from three biological replicates. Circles represent growth measured as OD_{550}.
(B) c-di-GMP levels during starvation in MC7 buffer in suspension of DK1622 WT cells. Levels of c-di-GMP are shown as mean ± standard deviation (SD) calculated from three biological replicates.

During starvation c-di-GMP was detected at all time-points and the level did not change significantly from 0 h (6.3 ± 2.36 pmol/mg protein) to 9 h (7.6 ± 0.23 pmol/mg protein) of starvation but then increased approximately 4-fold to 45.3 ± 19.0 pmol/mg protein at 48 h (Figure 15B). It should be noted that *M. xanthus* cells neither form fruiting bodies nor sporulate when starved in suspension. However, this method was used because c-di-GMP levels in cells starved on a surface are highly variable even between technical replicates. Since the level of c-di-GMP does not increase significantly in stationary phase cells (Figure 15A), these data suggests that the increase in the c-di-GMP level in starving cells is a specific response to starvation.

2.2. c-di-GMP level is important for T4P-dependent motility

To determine if c-di-GMP level is important for growth or motility in *M. xanthus*, we manipulated the cellular level of c-di-GMP in vegetative cells by overproduction of a heterologous DGC or a heterologous PDE in WT cells as previously described for other bacteria (Thormann *et al.*, 2006, Duerig *et al*., 2009, Tischler & Camilli, 2004, Levi *et al.*, 2011). As a DGC we used $DgcA^{WT}$ of *C. crescentus* (Christen *et al*., 2006)) and as a PDE we used $PA5295^{WT}$ of *P. aeruginosa* (Kulasakara *et al*., 2006). In parallel, we also expressed their active site variants in WT cells: $DgcA^{D164A}$ (active site in WT protein: G^{162}GDEF), $PA5295^{E328A}$ (active site in WT protein: E^{328}AL). All four proteins were C-terminally fused to the StrepII-tag to enable their detection by immunoblotting. This experiment was performed by Dr. Tobias Petters.

We observed that in exponentially growing cells, $DgcA^{D164A}$ accumulated at a significantly higher level than $DgcA^{WT}$ whereas $PA5295^{WT}$ and $PA5295^{E328A}$ accumulated at similar levels (Figure 16A). The c-di-GMP level in exponential $DgcA^{WT}$ expressing cells was ~7-fold higher than in WT cells (60.4 ± 29.1 pmol/mg protein and 8.7 ± 2.1 pmol/mg protein, respectively; $p < 0.001$ Student's T-test) and the c-di-GMP level in $PA5295^{WT}$ expressing cells was ~2-fold lower than in WT cells (4.9 ± 1.6 pmol/mg protein and 8.7 ± 2.1 pmol/mg protein, respectively; $p < 0.05$ Student's T-test) (Figure 16B). Importantly, the c-di-GMP level in $DgcA^{D164A}$ or $PA5295^{E328A}$ expressing cells (8.5 ± 2.4 and 7.7 ± 0.3 pmol/mg protein, respectively) was not significantly different from that in WT cells ($p > 0.2$ Student's T-test). Cells expressing DgcA, $DgcA^{D164A}$, PA5295 or $PA5295^{E328A}$ had the same growth rate as WT in suspension culture (data not shown).

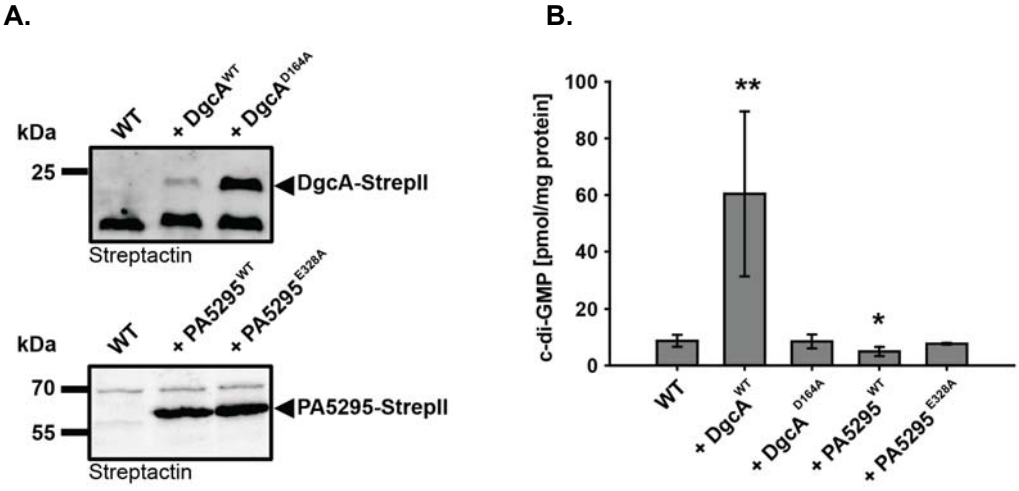


Figure 16. c-di-GMP level in *M. xanthus* vegetative cells can be manipulated

(A) Immunoblot detection of StrepII-tagged DgcA and PA5295 and their active site variants. Total protein was isolated from exponentially growing cells expressing the indicated proteins. Total protein from the same number of cells was loaded per lane and blots probed with streptactin. DgcA^{WT} and PA5295^{WT} have a calculated molecular mass of 26.8 kDa and 63.6 kDa, respectively.
(B) c-di-GMP level in exponentially growing cells expressing the indicated proteins. The levels of c-di-GMP are shown as mean ± SD from six (WT as well as DgcA^{WT} and PA5295^{WT} expressing cells) or three (DgcA^{D164A} and PA5295^{E328A} expressing cells) biological replicates. * $p < 0.05$ in a Students' T-test, ** $p < 0.001$ in a Students' T-test.

On 1.5% agar, which favours gliding motility, WT strain displayed single cells and slime trails characteristic of gliding motility at the edge of the colony, whereas the gliding deficient control strain DK1217 did not (Figure 17). All four strains expressing DgcA or PA5295 variants displayed single cells and slime trails at the colony edges as WT and had the same increase in colony diameter on 1.5% agar as WT, suggesting that level of c-di-GMP is not important for gliding motility. This experiment was performed by Dr. Tobias Petters.

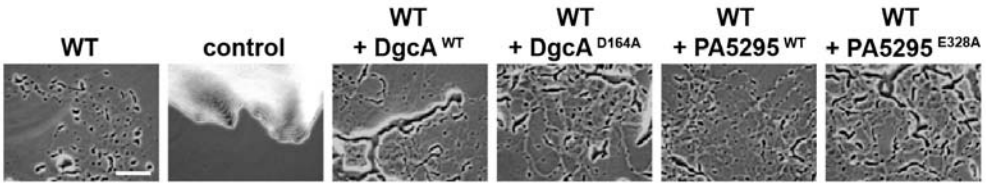


Figure 17. c-di-GMP level is not important for gliding motility

Gliding motility was analyzed on 1.5% agar. DK1217 is deficient in gliding motility and was used as negative control. Scale bar 50 µm.

On 0.5% agar, which favours T4P-dependent motility, WT strain formed the long flares characteristic of T4P-dependent motility whereas the control strain DK1300, which is deficient in T4P-dependent motility, did not (Figure 18). The strains expressing DgcA^{WT} or PA5295^{WT} had reduced T4P-dependent motility as quantified by the increase in colony diameter and the length of the flares at the colony edges, whereas the two strains containing the active site variants had WT T4P-dependent motility. This experiment was performed by Dr. Tobias Petters. We conclude that increased as well as a decreased level of c-di-GMP interferes with T4P-dependent motility.

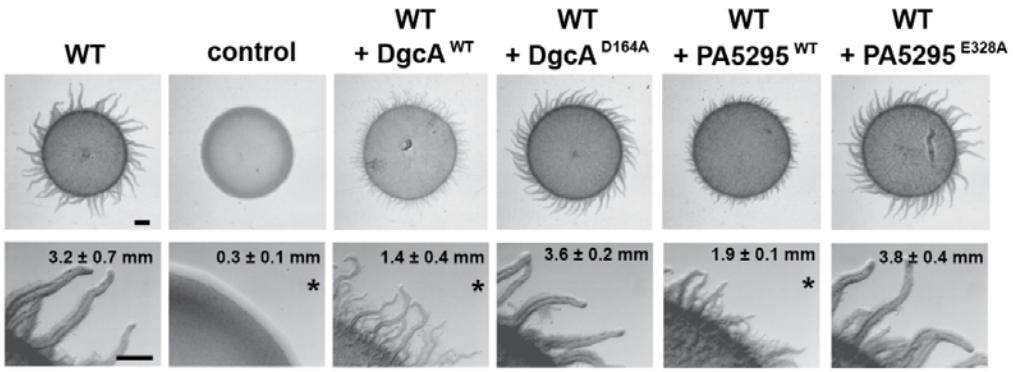


Figure 18. c-di-GMP level is important for T4P-dependent motility

T4P-dependent motility was analyzed on 0.5% agar. DK1300 is deficient in T4P-dependent motility and was used as negative control. T4P-dependent motility was quantified by the increase in colony diameter; numbers indicate the increase in colony diameter in mm ± SD from three biological replicates after 24 h; * $p < 0.05$ in a Students' T-test. Scale bars 1 mm (upper row) and 500 µm (lower row).

2.3. c-di-GMP regulates T4P formation and *pilA* transcription

T4P-dependent motility in *M. xanthus* depends on T4P formation. We used transmission electron microscopy to determine whether the reduced T4P-dependent motility in strains expressing DgcA^{WT} and PA5295^{WT} was due to the lack or reduced functionality of assembled T4P. WT cells assembled a mean of 2.1 T4P per cell in a unipolar pattern whereas the Δ*pilA* control strain, which lacks the pilin subunit of T4P, did not assemble T4P (Figure 19). PA5295^{WT} expressing cells assembled T4P at WT levels (mean of 1.7 T4P per cell) in a unipolar pattern whereas DgcA^{WT} expressing cells were strongly reduced in assembled T4P with less than one T4P per cell (mean of 0.1 T4P per cell) (Figure 19).

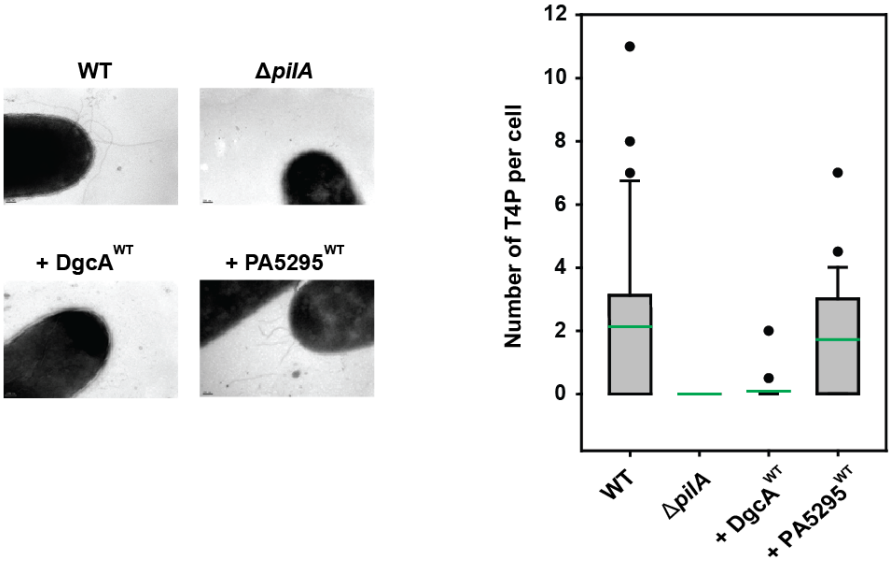


Figure 19. c-di-GMP level is important for T4P formation

Transmission electron microscopy of exponentially growing cells expressing the indicated proteins. Cells were transferred to a grid, stained with 2% (w/v) uranyl acetate and visualized by transmission electron microscopy. Scale bars, 100nm. The box plots show the number of T4P per cell for at least 20 cells. Boxes indicate the 25th and 75th percentile, the green line the mean, whiskers the 10th and 90th percentile, and dots outliers.

To corroborate these observations, the total amount of cellular PilA as well as PilA assembled into T4P were quantified. For this purpose, T4P were sheared-off form the cell surface and analysed using immunoblot. In total cell extracts, the amount of PilA in WT, PA5295^{WT}, DgcA^{D164A} and PA5295^{E328A} expressing cells was similar, whereas DgcA^{WT} expressing cells accumulated PilA at a significantly reduced level (Figure 20A). As expected, PilA was not detected in the Δ*pilA* mutant. In the sheared T4P fraction, WT, PA5295^{WT}, DgcA^{D164A} or PA5295^{E328A} expressing cells contained the same amount of PilA whereas PilA was not detectable in the sheared T4P fraction from Δ*pilA* and DgcA^{WT} expressing cells (Figure 20A). This confirmed the data obtained from electron microscopy experiment and allowed us to conclude that DgcA^{WT} expressing cells accumulate PilA and assemble T4P at a significantly reduced level.

To further understand the mechanism underlying the reduced accumulation of PilA in DgcA^{WT} expressing cells, we used qRT-PCR to determine the level of *pilA* transcription in WT, DgcA^{WT} and in PA5295^{WT} expressing cells. As shown in Figure 20B, the level of *pilA* mRNA in DgcA^{WT} expressing cells was approximately 2.5-fold lower than in WT and slightly reduced in PA5295^{WT} expressing cells suggesting that increased c-di-GMP levels inhibit *pilA* transcription.

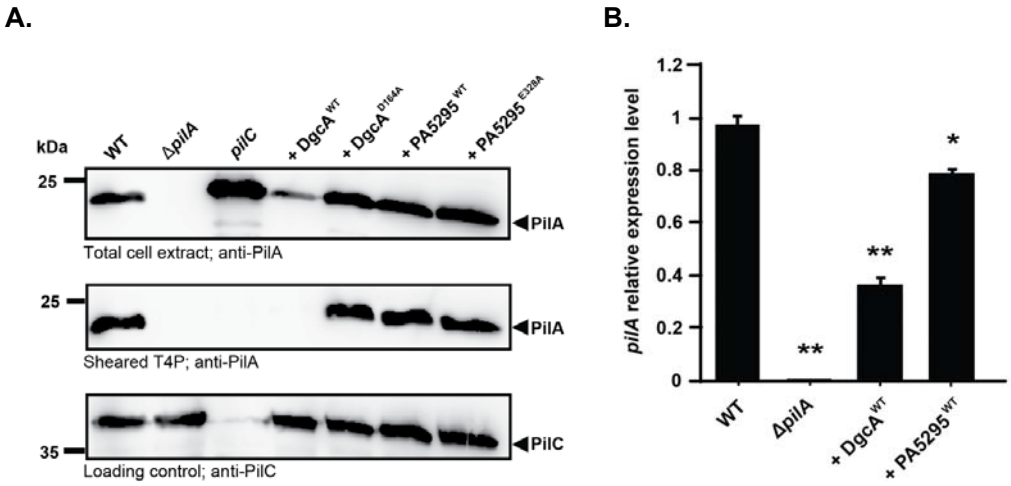


Figure 20. c-di-GMP level is important for T4P formation and *pilA* transcription

(A) Immunoblot detection of PilA in total cell extract and in sheared T4P. In the upper and lower blots, total protein was isolated from the indicated strains grown on 1% CTT/1.5% agar plates. In the middle blot, T4P were sheared off from the cells and concentrated by $MgCl_2$ precipitation. In all three blots, protein from the same number of cells was loaded per lane. The upper and middle blots were probed with anti-PilA antibodies. The lower blot was probed against PilC, which is important for T4P assembly and was used as a loading control. PilA and PilC have a calculated molecular mass of 23.4 kDa and 45.2 kDa respectively.
(B) qRT-PCR analysis of *pilA* expression. RNA was isolated from in the indicated strains grown on 1% CTT/1.5% agar plates. *pilA* transcript level is shown as mean ± SD from two biological replicates each of them with three technical replicates, relative to WT. * $p < 0.05$ in a Students' T-test, ** $p < 0.001$ in a Students' T-test.

Nevertheless, $DgcA^{WT}$ expressing cells were still partially motile what could be due to c-di-GMP affecting T4P retraction process. In order to test this hypothesis, we expressed $DgcA^{WT}$ and $DgcA^{D164A}$ in a Δ*pilT* background strain. PilT is the ATPase responsible for T4P retraction. The Δ*pilT* mutant has a strong motility defect and is hyperpiliated (Black *et al*., 2006, Wu *et al.*, 1997), (Figure 21A,B). $DgcA^{WT}$ expressed in the Δ*pilT* strain caused stronger motility defect and less PilA than the background Δ*pilT* strain in both total cell extract and sheared-off fraction. Nevertheless, Δ*pilT* expressing $DgcA^{WT}$ strain still assembled T4P on the cell surface (Figure 21B). Thus, high level of c-di-GMP does not affect T4P retraction, but *pilA* gene transcription.

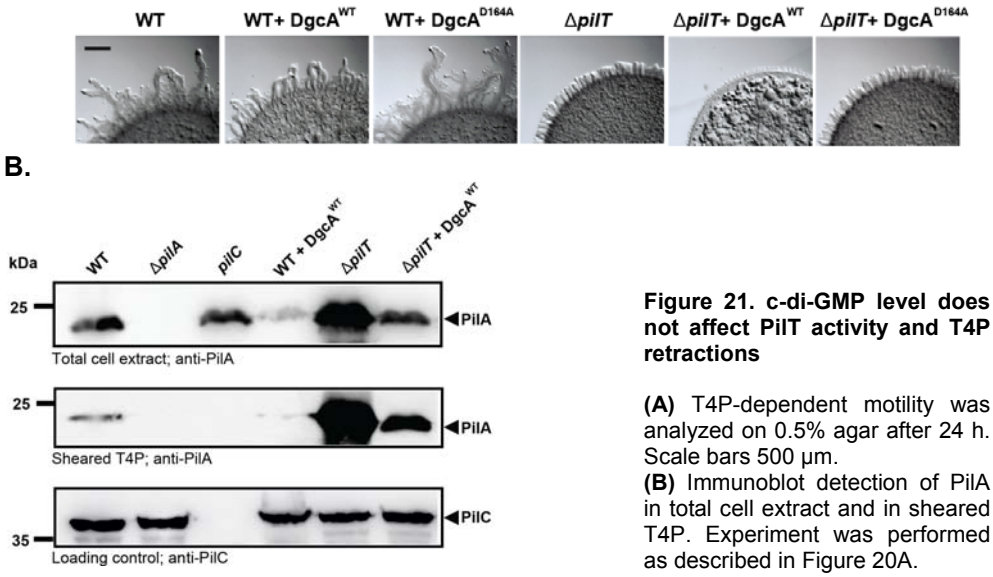


Figure 21. c-di-GMP level does not affect PilT activity and T4P retractions

(A) T4P-dependent motility was analyzed on 0.5% agar after 24 h. Scale bars 500 µm.
(B) Immunoblot detection of PilA in total cell extract and in sheared T4P. Experiment was performed as described in Figure 20A.

2.4. c-di-GMP regulates cell-cell interactions

T4P-dependent motility in *M. xanthus* also depends on EPS (exopolysaccharide) accumulation. Therefore, we determined the EPS accumulation in the strains expressing DgcA or PA5295 variants by using a colorimetric assay. Cells were grown in rich medium in liquid culture or on solid medium (0.5% agar) and trypan blue binding was determined. Trypan blue is a dye capable of binding to EPS produced by *M. xanthus* cells (Dana & Shimkets, 1993). Under both conditions, no significant differences in EPS accumulation were observed for these four strains compared to WT (Figure 22A). In contrast, the negative control strain with a mutation in the *difE* gene, which encodes a component of the Dif chemosensory system that is important for EPS accumulation (Yang *et al*., 2000), was strongly reduced in EPS accumulation under both conditions.

Because assembled T4P have been suggested to function upstream of the Dif chemosensory system to stimulate EPS accumulation (Black *et al*., 2006), we also determined EPS accumulation in the Δ*pilA* mutant. As expected, this mutant also displayed strongly reduced EPS accumulation under both conditions (Figure 22A). It is then surprising and important to notice that although in both Δ*pilA* and DgcA^{WT} mutants T4P are undetectable, those strains dramatically differ in terms of EPS accumulation (Figure 22A), which suggest that simply lack of T4P is not the only defect in DgcA^{WT} expressing cells. It is possible that high level of c-di-GMP stimulates EPS accumulation but this effect is nivelated by the lack of a T4P in DgcA^{WT} expressing cells resulting in WT-like EPS accumulation.

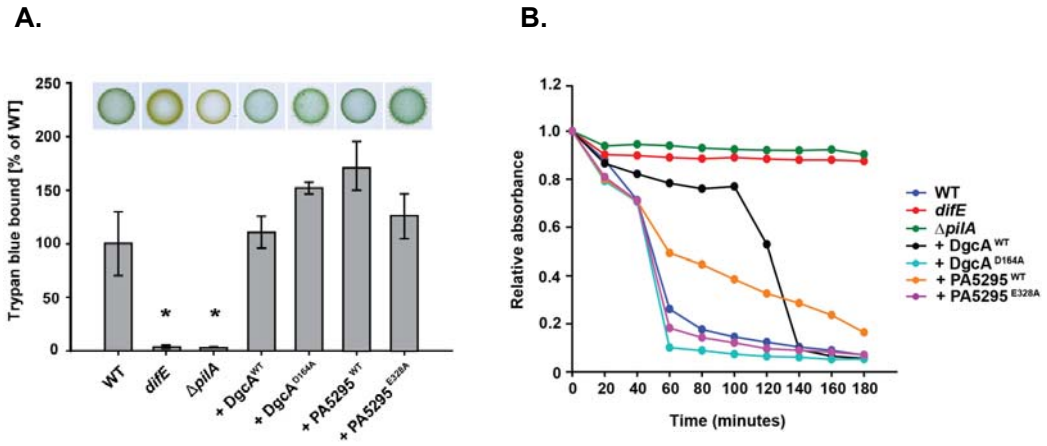


Figure 22. c-di-GMP level does not regulate EPS accumulation but is important for cell-cell interactions

(A) Quantification of EPS accumulation. Exponentially growing WT, *difE* or WT cells expressing the indicated proteins were assayed for EPS accumulation using a colorimetric assay. The percentage of trypan blue bound by a strain is indicated relative to trypan blue bound be WT (100%). Levels of trypan blue binding are shown as mean ± SD from three biological replicates. * $p < 0.05$ in a Students' T-test. For the plate-based assay, aliquots of 20 µl cell suspensions at 7×10^9 cells/ml were spotted on 0.5% agar supplemented with 0.5% CTT and 20 µg/ml trypan blue and incubated at 32 °C for 24 h.
(B) Cell agglutination assay. Agglutination was monitored by measuring the decrease in absorbance at 550 nm for a suspension of cells in agglutination buffer. The relative absorbance was calculated by dividing the absorbance measured at each time point by the initial absorbance for each strain. The graphs show data from one representative experiment.

M. xanthus cells agglutinate in the presence of the divalent cations Mg^{2+} and Ca^{2+} (Shimkets, 1986a). Agglutination depends on T4P (Shimkets, 1986a, Wu & Kaiser, 1997) and EPS (Arnold & Shimkets, 1988). In order to further examine the cell surface properties of the strains with altered levels of c-di-GMP, we determined their agglutination properties. As previously reported, WT cells started to agglutinate shortly after addition of Mg^{2+} and Ca^{2+}, but *difE* and Δ*pilA* mutants did not (Figure 22B). The Dgc^{WT}-expressing strain as well as the $PA5295^{WT}$-expressing strain showed delayed agglutination compared to WT. In agreement with the observation that the Dgc^{WT}-expressing strain is reduced in PilA and thus in T4P assembly, this strain had a more severe defect than the $PA5295^{WT}$-expressing strain. Importantly, the $DgcA^{D164A}$ and $PA5295^{E328A}$ expressing strains agglutinated similarly to WT (Figure 22B).

Together, these data suggest that c-di-GMP has a role in T4P-dependent motility in *M. xanthus*. On the one hand, the defect in T4P-dependent motility in the $DgcA^{WT}$ expressing strain is caused by reduced *pilA* expression resulting in reduced PilA accumulation and reduced T4P assembly. On the other hand, our data suggest that the defect in T4P-dependent motility in the $PA5295^{WT}$ expressing strain is likely not due to a difference in the level of assembled T4P or in the level of EPS

accumulation. Because cell-cell cohesion is reduced among cells of the PA5295^{WT} expressing strain, we suggest that reduced c-di-GMP level results in changes in cell surface properties that are neither reflected in the level of assembled T4P nor in the level of EPS accumulation. Moreover, we suggest that these changes in cell surface properties negatively affect T4P-dependent motility.

2.5. c-di-GMP level is important for fruiting body formation and sporulation

To determine if c-di-GMP level is important for development, we used the strains that express the heterologous DGC, PDE and their active site variants in WT cells, since we previously showed that expression of DgcA^{WT} and PA5295^{WT} causes a significant increase and decrease respectively in the c-di-GMP level during vegetative growth (whereas the two active site variants do not affect the c-di-GMP level). During starvation, c-di-GMP level was significantly increased up to 36h in cells expressing DgcA^{WT} and significantly decreased in cells expressing PA5295^{WT} compared to WT cells (Figure 23A). This experiment was performed by Dr. Tobias Petters.

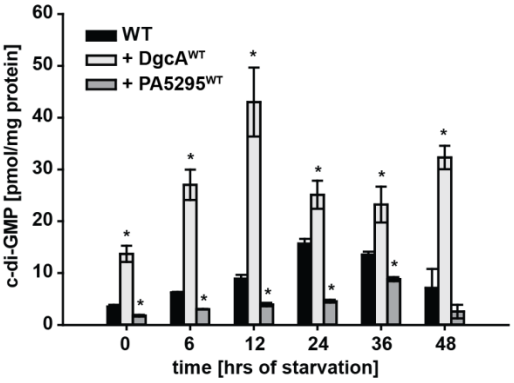


Figure 23. c-di-GMP level in *M. xanthus* can be antagonistically manipulated during starvation

Level of c-di-GMP in cells expressing the indicated proteins during starvation in suspension for the indicated periods of time. Level of c-di-GMP is shown as mean ± SD from three biological replicates. At indicated time points, the c-di-GMP levels in cells expressing DgcA^{WT} or PA5295^{WT} are significantly different from the level in WT cells. (*) indicates $p < 0.05$ in a Students' T-test.

To assess the importance of the c-di-GMP level during fruiting body formation and sporulation, strains expressing DgcA or PA5295 variants were exposed to starvation under two different conditions (TPM starvation agar and MC7 buffer submerged cultures) (Figure 24B). Under both conditions, WT cells aggregated to form nascent fruiting bodies after 24 h and had formed darkened spore-filled fruiting bodies after 120 h. Strains expressing DgcA^{WT}, DgcA^{D164A} or PA5295^{E328A} behaved similarly to WT. In contrast, PA5295^{WT} expressing cells displayed delayed fruiting body formation on TPM-agar and did not form fruiting bodies in submerged culture even after 120 h. Moreover, sporulation in this strain was strongly reduced. We

conclude that decreased level of c-di-GMP interfere with fruiting body formation and reduces sporulation whereas increased level of c-di-GMP does not affect these two processes. This experiment was performed by Dr. Tobias Petters.

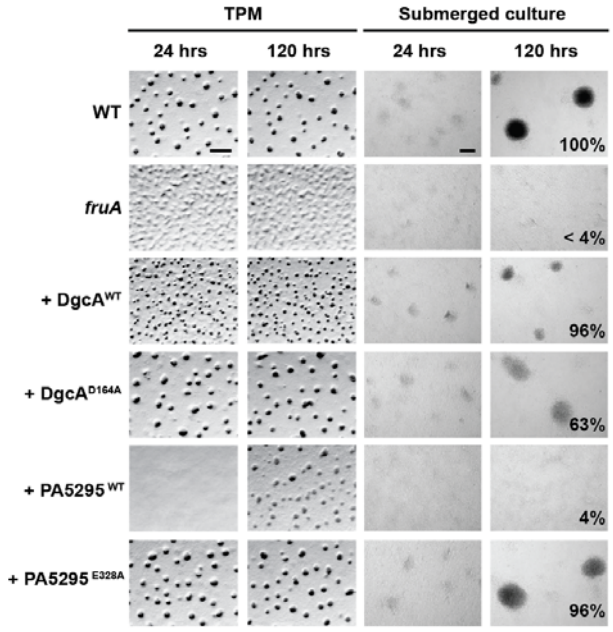


Figure 24. c-di-GMP level is important for fruiting body formation and sporulation

Fruiting body formation was assayed under two different conditions as indicated. Numbers after 120 h of starvation in submerged culture indicate heat- and sonication resistant spores formed relative to WT. *fruA* is deficient in fruiting body formation and strongly reduced in sporulation and was used as a negative control. Scale bars: TPM agar 500 µm; submerged culture 100 µm.

To investigate if sporulation *per se* is affected in strains with altered c-di-GMP level, we tested glycerol-induced sporulation of those strains. It is known that after adding 0.5 M glycerol to an exponentially growing culture WT cells differentiate into spores (Müller *et al.*, 2010). By using this assay, we could not observe any difference when comparing WT cells and strains expressing DgcA and PA5295 variants. All the strains formed heat and sonication resistant spores (Figure 25). This result allowed us to hypothesize that c-di-GMP is essential for proper aggregation during initial stages of starvation induced development, and not for the sporulation process during the later stages of development. This experiment was performed by Dr. Tobias Petters.

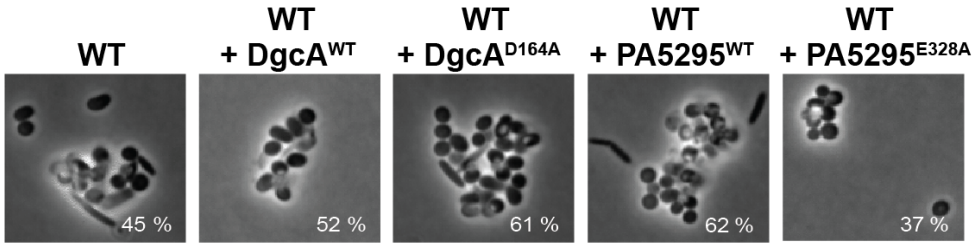


Figure 25. c-di-GMP level is not important for glycerol induced sporulation

Cell morphology 4 h after adding 0.5 M glycerol. Numbers represent the % of heat- and sonication-resistant spores relatively to the initial number of cells.

2.6. *M. xanthus* genome encodes proteins containing GGDEF, EAL and HD-GYP domains

Petters *et al.* identified in *M. xanthus* genome 24 genes encoding proteins containing either a GGDEF, EAL or HD-GYP domain (Petters *et al*., 2012). 17 proteins contain a GGDEF domain, two proteins an EAL domain, and five proteins an HD-GYP domain (Figure 26). Proteins that contain a GGDEF as well as an EAL domain have been identified in many bacteria; however, among the 24 proteins identified in *M. xanthus*, none contain more than a single GGDEF, EAL or HD-GYP domain. With the exception of the two proteins containing an EAL domain, all 24 proteins contain additional domains that are typically involved in signal sensing and signal transduction in bacteria (Figure 26). Only two of the 24 proteins are predicted to be membrane proteins (MXAN3705 and MXAN2061) suggesting that most of these proteins are not directly sensing extracellular signals.

Based on the conservation of amino acid residues important for catalytic activity, 11 of the 17 GGDEF domain-containing proteins are predicted to have DGC activity (Figure 26). Among the remaining six DGCs predicted to be catalytically inactive, four contain residues for c-di-GMP binding and may function as c-di-GMP receptors. The two EAL domain proteins and four of the HD-GYP domain proteins contain all the residues important for catalytic activity while the remaining protein (MXAN2807) lacks several of these conserved residues (Figure 26). This analysis was performed by Dr. Tobias Petters.

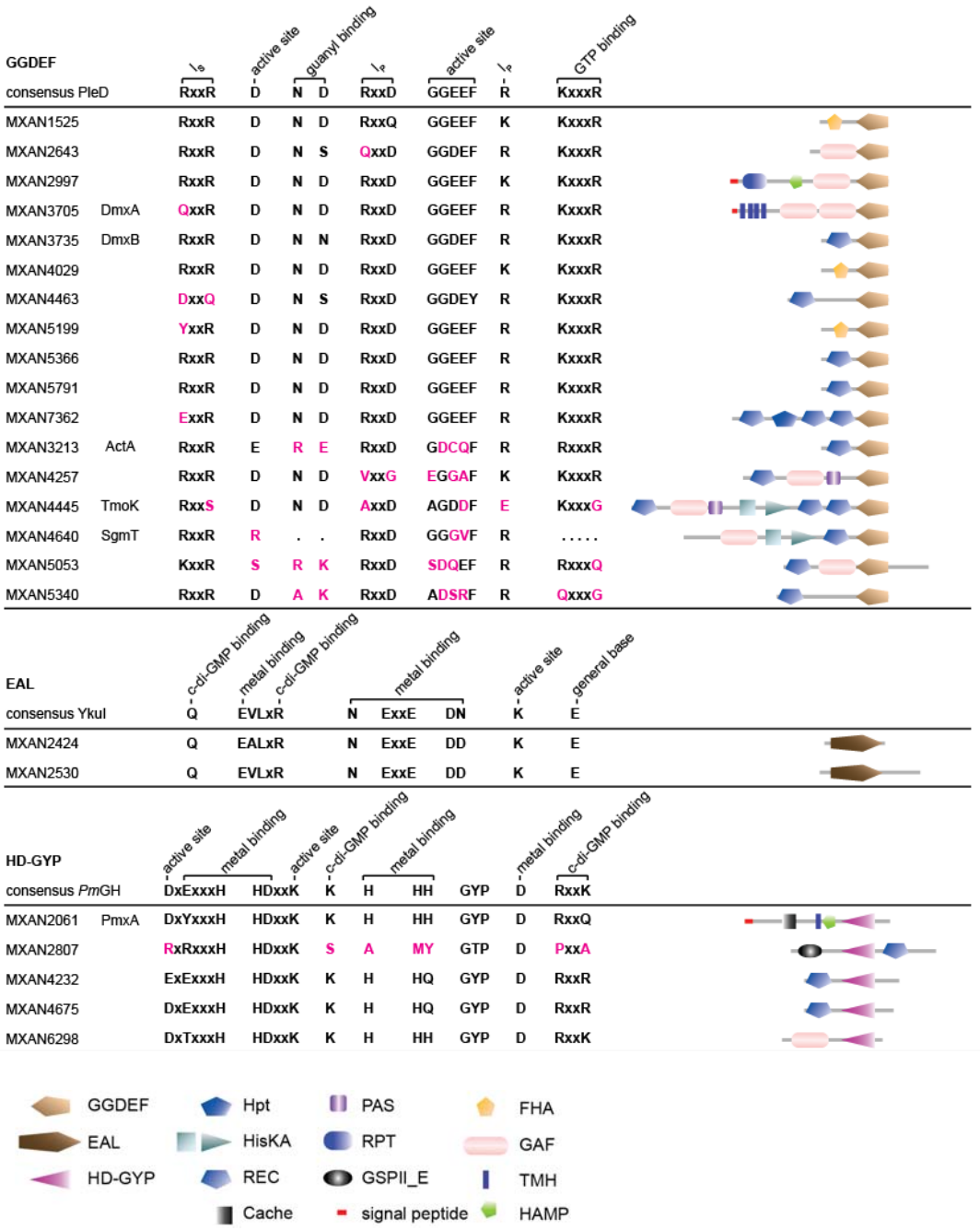


Figure 26. Domain organization of *M. xanthus* proteins containing a GGDEF, EAL or HD-GYP domain

Locus tags and protein names are listed on the left. Domain composition is shown on the left. See the legend at the bottom of the figure for the annotation of domains. Domains are not drawn to scale. Domain annotation was performed using the SMART web tool (Letunic *et al.*, 2015). For GGDEF domains, the GGDEF domain of PleD from *C. crescentus* is used as a reference for residues important for catalytic activity and allosteric inhibition with their function listed (Wassmann *et al.*, 2007, Vorobiev *et al.*, 2012), for EAL domains, the EAL domain of YkuI from *B. subtilis* is used as a reference for important residues and with their function indicated (Minasov *et al.*, 2009, Rao *et al.*, 2008), and for HD-GYP domains, the HD-GYP domain of *Pm*GH from *Persephonella marina* EX-H is used as a reference for important residues and with their function indicated (Bellini *et al.*, 2014, Lovering *et al.*, 2011). For *M. xanthus* proteins, the corresponding amino acid residues are indicated in black if conserved or replaced by a conservative substitution and in pink for non-conserved residues.

The 24 *M. xanthus* proteins containing a GGDEF, EAL or HD-GYP domain are highly conserved in closely related fruiting Myxobacteria (*M. fulvus*, *M. stipitatus*, *Corallococcus coralloides* and *Stigmatella aurantiaca*). However, they are neither highly conserved in four isolates of the non-fruiting myxobacterium *Anaeromyxobacter dehalogenans* nor in the more distantly related fruiting Myxobacteria *Sorangium cellulosum* and *Haliangium ochraceum* (Figure 27). This analysis was performed by Dr. Kristin Wuichet.

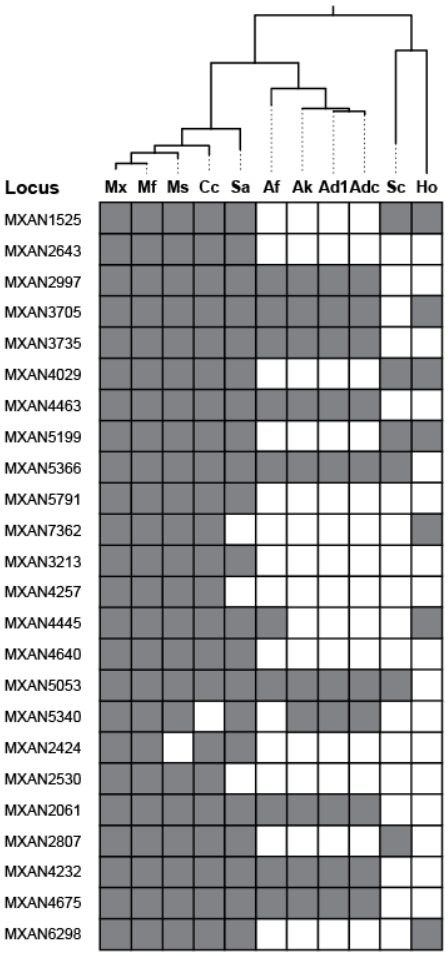
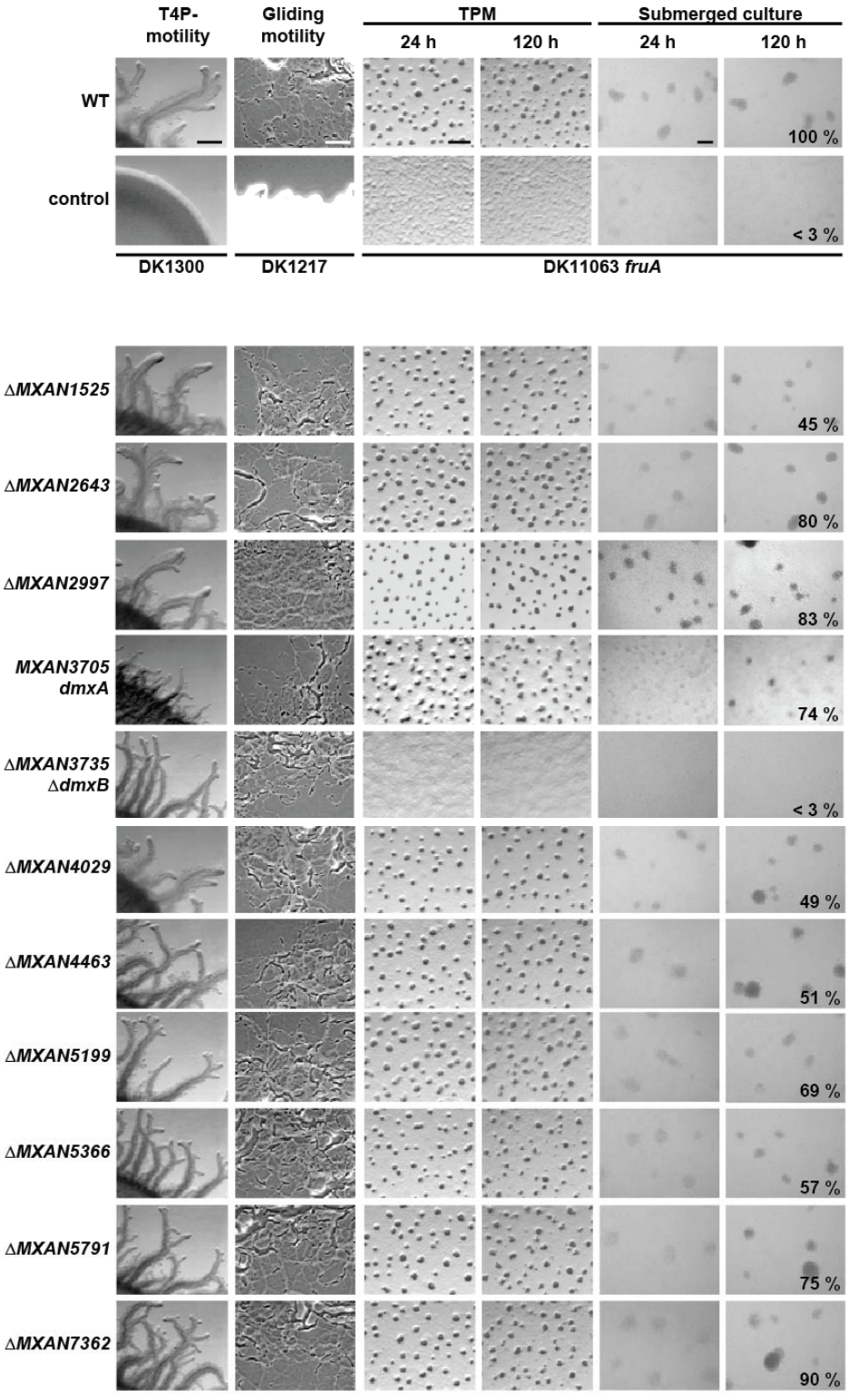


Figure 27. **Conservation of *M. xanthus* proteins with a GGDEF and EAL/HD-GYP domain in Myxobacteria**

The genomic distribution of *M. xanthus* proteins containing a GGDEF, EAL or HD-GYP domain in other Myxobacteria in sequenced myxobacterial genomes. The phylogenetic tree was generated from 16S rRNA sequences. Abbreviations in phylogenetic tree: Mx, *M. xanthus* DK1622; Mf, *M. fulvus* HW-1; Ms, *M. stipitatus* DSM 14675, Cc, *Corallococcus coralloides* DSM 2259; Sa, *Stigmatella aurantiaca* DW 3/4-1; Af, *Anaeromyxobacter* sp Fw109-5; Ak, *Anaeromyxobacter* sp K; Ad1, *A.dehalogenans* 2CP-1; Adc, *A.dehalogenans* 2CP-C; Sc, *Sorangium cellulosum* So ce 56; Ho, *Haliangium ochraceum* DSM 14365.

2.7. Identification of GGDEF, EAL and HD-GYP domain proteins important for T4P-dependent motility and development

To test whether any of the 24 proteins containing a GGDEF, EAL or HD-GYP domain have a function in motility or fruiting body formation, we systematically generated in-frame deletion mutations in 23 of the 24 genes. For one of the genes (*MXAN3705*), we were not able to generate a deletion mutant and, therefore an insertion mutant was generated (Figure 28).


T4P-motility
Gliding motility
TPM
Submerged culture
24 h
120 h
24 h
120 h
WT
control
100 %
< 3 %
DK1300
DK1217
DK11063 fruA
ΔMXAN1525
45 %
ΔMXAN2643
80 %
ΔMXAN2997
83 %
MXAN3705 dmxA
74 %
ΔMXAN3735 ΔdmxB
< 3 %
ΔMXAN4029
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ΔMXAN5366
57 %
ΔMXAN5791
75 %
ΔMXAN7362
90 %

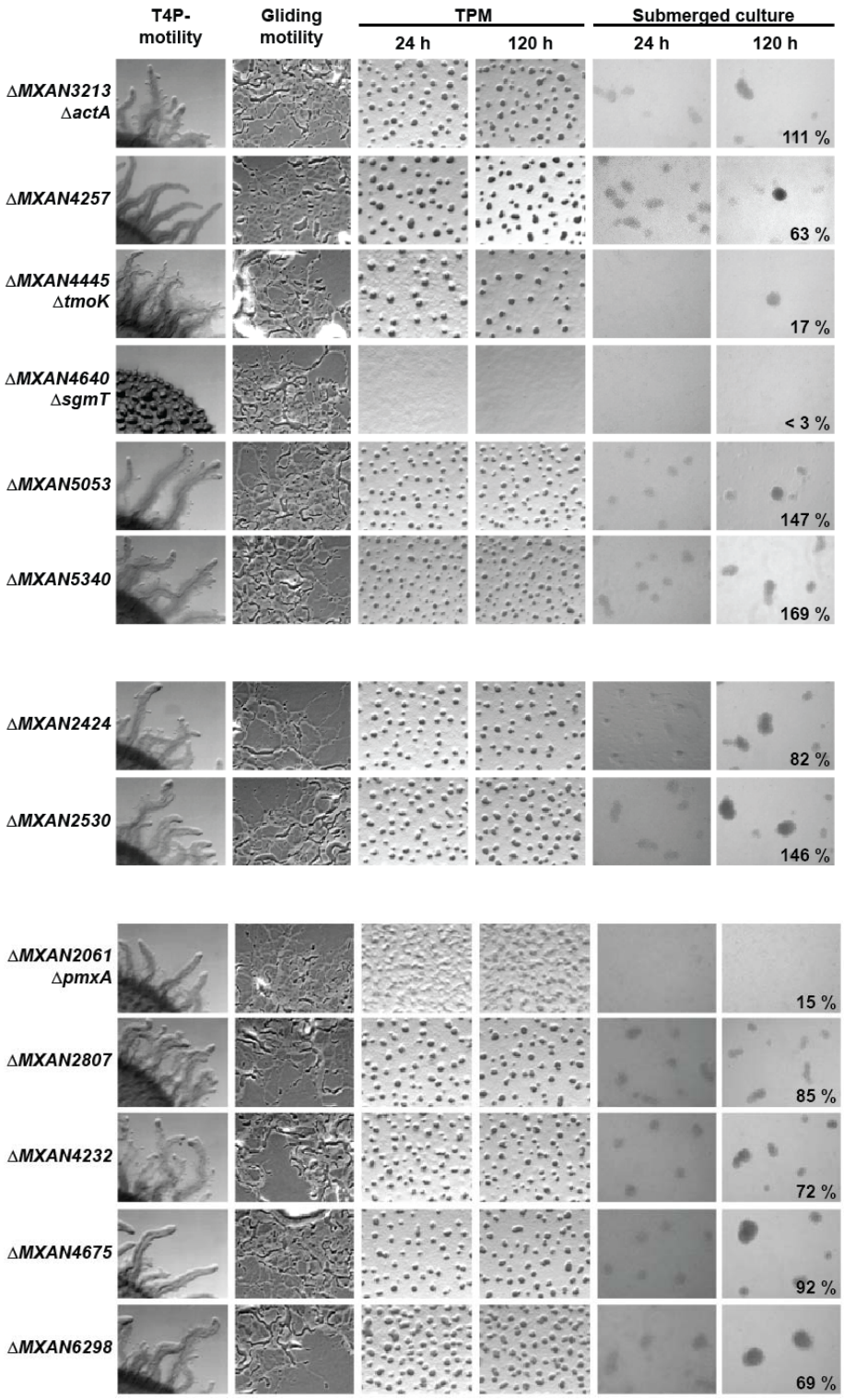


Figure 28. Mutagenesis of *M. xanthus* c-di-GMP related genes

Gliding and T4P-dependent motility were assessed as described in Figure 17 and Figure 18. Scale bars: T4P-dependent motility 500 µm, gliding motility 50 µm. Development was assessed as described in Figure 24. Numbers after 120 h of starvation in submerged culture indicate heat- and sonication resistant spores formed relative to WT (100%). Scale bars: TPM agar 500 µm, submerged culture 100 µm.

Mutation of 21 of the 24 genes did not affect growth, gliding motility or T4P-dependent motility (Figure 28). We did not identify any mutant with a defect in gliding motility, which supports our hypothesis that this type of motility is not regulated by c-di-GMP.

Interestingly, we identified three mutants with defects in T4P-dependent motility. As previously reported, lack of the cytoplasmic hybrid histidine protein kinase SgmT, which contains a C-terminal GGDEF domain that binds c-di-GMP but is catalytically inactive, causes strong defects in T4P-dependent motility (Petters *et al*., 2012). MXAN3705, which we have named *dmxA* (diguanylate cyclase from *M. xanthus* A), encodes a predicted integral membrane protein with a C-terminal GGDEF domain that is predicted to be catalytically active and to bind c-di-GMP (Figure 27). Mutation of *dmxA* caused a reduction in T4P-dependent motility with the formation of shorter flares than in WT (Figure 28). MXAN4445, which we have named TmoK (T4P-motility kinase) encodes a predicted cytoplasmic hybrid histidine protein kinase with a C-terminal GGDEF domain, which lacks residues important for catalytic activity and c-di-GMP binding. Δ*tmoK* mutation caused a subtle defect in T4P-dependent motility with the formation of flares of similar length as in WT but not so well defined and separated (Figure 28).

To investigate the function of those 24 proteins in fruiting body formation and sporulation, we tested the strains with single in-frame deletions or an insertion mutation in the case of *dmxA* for development under two different conditions: TPM agar and submerged culture in MC7 buffer. Single mutations in 20 of the 24 genes did not affect fruiting body formation or sporulation (Figure 28). These 20 genes included *actA*, which has been suggested to be important for development (Gronewold & Kaiser, 2001) and *dmxA*. To generate the in-frame deletion mutant of *actA*, we reannotated *actA* taking into account the GC content in the third position of codons, which is high in *M. xanthus* open reading frames due to the high GC content of the genome, and by comparisons to orthologous genes (Heering, 2013). From these analyses, we suggest that the originally proposed start codon of *actA* is incorrect and instead the start codon maps 123 bp downstream. With this re-annotation, the original Δ*actA* mutation extends into the promoter region of the *act* operon. *actA* is the first gene of this operon and located upstream of the *actB* gene, which codes for a NtrC-like transcriptional regulator that is important for development (Giglio *et al.*, 2011, Gronewold & Kaiser, 2001). Because the original Δ*actA* mutant phenocopies a Δ*actB* mutant (Gronewold & Kaiser, 2001), we speculate that the developmental defects observed for the original Δ*actA* mutant are caused by a polar effect on *actB* (Heering, 2013). We conclude that neither DmxA

nor ActA are required for development. As previously reported, lack of SgmT caused defects in fruiting body formation and sporulation and the Δ*sgmT* mutant did not form fruiting bodies and did not sporulate under any of the conditions tested (Petters *et al*., 2012). Lack of TmoK caused delayed aggregation and reduced sporulation in submerged culture while aggregation was normal on TPM agar (Figure 28).

Mutations in two genes caused developmental defects without affecting growth or motility in vegetative cells. Those genes encode for the proteins: MXAN3735, henceforth DmxB (diguanylate cyclase from *M. xanthus* B) and MXAN2061, henceforth PmxA (phosphodiesterase from *M. xanthus* A) (Figure 28).

Importantly, we were able to complement both motility and developmental defects in all of the mutants by ectopic expression of the relevant WT gene from its native promoter on a plasmid integrated at the Mx8 *attB* site (Figure 29A,B and (Petters *et al*., 2012)). We concluded that among the 24 genes analyzed, one codes for proteins that is important only for T4P-dependent motility (DmxA), two code for proteins that are important for both T4P-dependent motility and development (SgmT, TmoK), and two code for proteins that are important only for development (DmxB, PmxA).

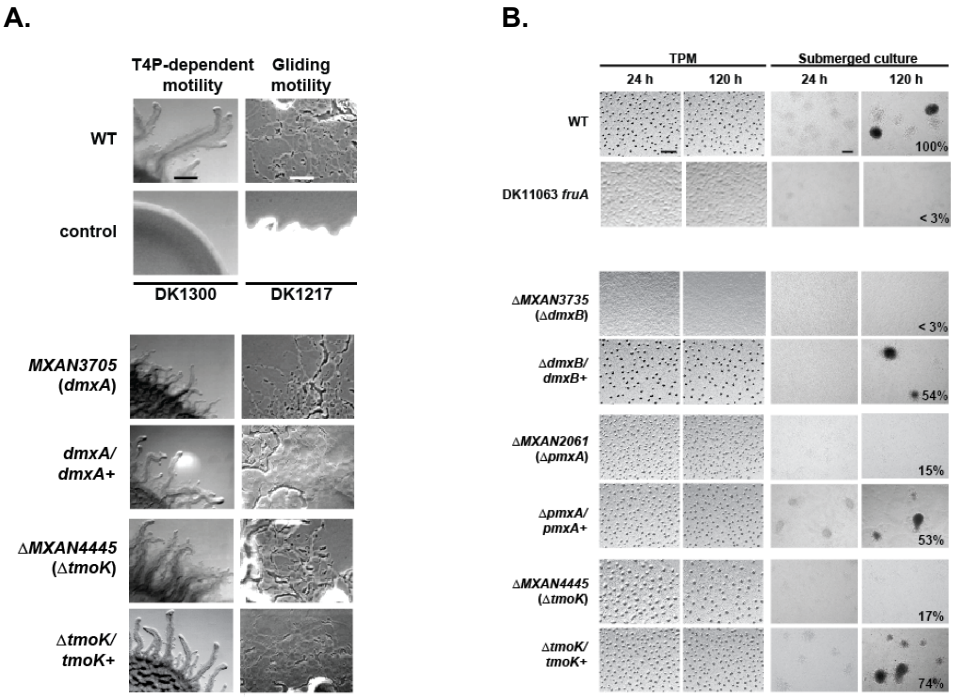


Figure 29. Defects in c-di-GMP related gene mutants can be complemented

(A) Complementation of mutants affected in T4P-motility. Gliding and T4P-motility were assessed as described in Figure 17 and Figure 18. Scale bars: T4P-dependent motility 500 µm, gliding 50 µm.
(B) Complementation of mutants affected in development. Development was assayed as described in Figure 24. Numbers after 120 h of starvation in submerged culture indicate heat- and sonication resistant spores formed relative to WT. Scale bars, TPM agar 500 µm, submerged culture 100 µm.

2.8. DmxA has enzymatic activity and binds c-di-GMP *in vitro*

In order to determine if the proteins regulating T4P-motility are actively involved in c-di-GMP metabolism, we tested SgmT, DmxA and TmoK for enzymatic activity. For this purpose, we overexpressed His6-tagged full-length or truncated variants of these proteins (Figure 30) in *E. coli* and purified them as soluble proteins. As positive and negative controls for enzyme activity, we purified full-length His6-tagged DgcA^{WT} and SgmT, which we previously predicted not to have DGC activity (Petters *et al*., 2012).

DgcA^{WT} as well as DmxA$^{223-722}$ produced c-di-GMP when incubated with [α-^{32}P]-GTP as detected after separation of nucleotides by thin layer chromatography (Figure 30). TmoK$^{654-1109}$ and SgmT did not detectably produce c-di-GMP (Figure 30). DGCs function as dimers with two juxtaposed GGDEF domains forming the active site (Chan *et al*., 2004). In PleD of *C. crescentus* dimer formation is mediated by the two receiver domains that are located N-terminal to the GGDEF domain (Paul *et al*., 2007). For that reason, the truncated TmoK variant analyzed *in vitro* also contained the two receiver domains N-terminal to the GGDEF domain. Although it cannot be ruled out that TmoK$^{654-1109}$ may not form a dimer under the conditions of the DGC assay, the observation that the GGDEF domain lack several residues important for DGC activity (Figure 26) taken together with the *in vitro* data suggest that TmoK does not have DGC activity.

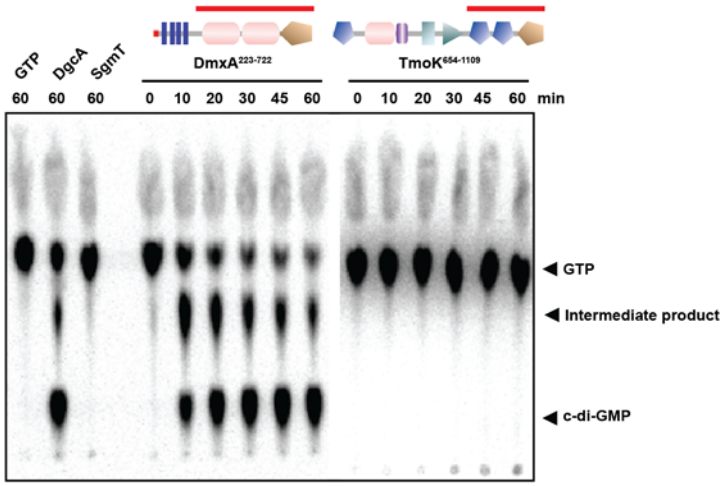


Figure 30. ***In vitro*** **assay for DGC activity**

DGC *in vitro* assay of DmxA and TmoK variants. The indicated DmxA and TmoK variants were incubated with [α-^{32}P]-GTP for the indicated periods of time followed by separation of nucleotides by thin layer chromatography. Full-length DgcA^{WT} and SgmT were used as positive and negative controls, respectively. Domain architectures are shown as SMART images as in Figure 26 with the red bars indicating the part of each protein used in the assay. GTP and c-di-GMP are indicated. The intermediate product indicated was described as a product formed during the DGC-dependent synthesis of c-di-GMP (Bharati *et al*., 2012).

To test whether DmxA or TmoK bind c-di-GMP we used a DraCALA (Differential Radial Capillary Action of Ligand Assay) with [α-^{32}P]-labeled c-di-GMP generated in an enzymatic reaction by mixing purified DgcA from *C. crescentus* with [α-^{32}P]-labeled GTP as described (Sultan *et al.*, 2011) and using SgmT as a positive control for c-di-GMP binding (Petters *et al*., 2012). DRaCALA allows detection of specific interactions between ligands and their cognate binding proteins. When a mixture of protein and radiolabeled ligand is spotted onto a nitrocellulose membrane, protein and bound ligand are immobilized at the site of contact while free ligand is mobilized by capillary action with the liquid phase (Roelofs *et al.*, 2011).

Purified SgmT incubated with with radiolabeled c-di-GMP and spotted on nitrocellulose membrane showed an intense signal in the middle of the spotting area suggesting binding of radiolabeled c-di-GMP (Figure 31). This is in agreement with results published by (Petters *et al*., 2012). The same was true for DmxA$^{223\text{-}722}$ (Figure 31). For both proteins binding could be competed by non-labeled c-di-GMP indicating specific interaction. In contrast, purified TmoK$^{654\text{-}1109}$ showed only background signal, suggesting that this protein does not bind c-di-GMP *in vitro* (Figure 31). These results are in agreement with the predictions from sequence analyses (Figure 26).

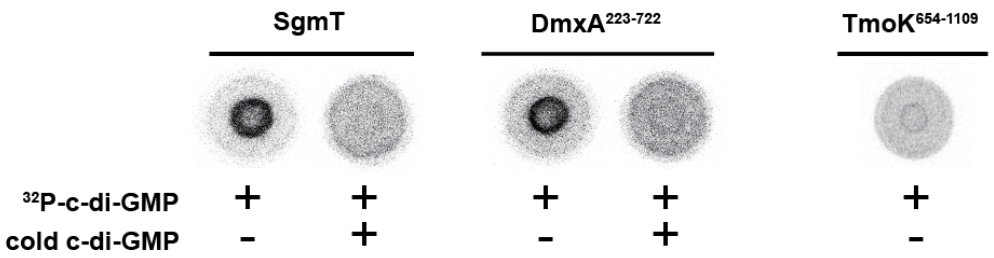


Figure 31. *In vitro* assays for c-di-GMP binding

DRaCALA assay to detect specific c-di-GMP binding by purified proteins. Full-length SgmT or the DmxA and TmoK variants described in Figure 30 were incubated with [α-^{32}P]-labeled c-di-GMP with or without unlabelled c-di-GMP as a competitor.

2.9. Lack of SgmT and DmxA causes an increase in the c-di-GMP level in vegetative *M. xanthus* cells

To test if lack of DmxA, TmoK and SgmT involves changes in c-di-GMP level, the c-di-GMP level was determined in *dmxA,* Δ*tmoK* and Δ*sgmT* mutants. Unexpectedly, *dmxA* and Δ*sgmT* mutants had slightly (approximately 1.5 fold) but

significantly higher c-di-GMP levels than WT in exponentially growing cells whereas Δ*tmoK* mutant had c-di-GMP level similar to that of WT (Figure 32).

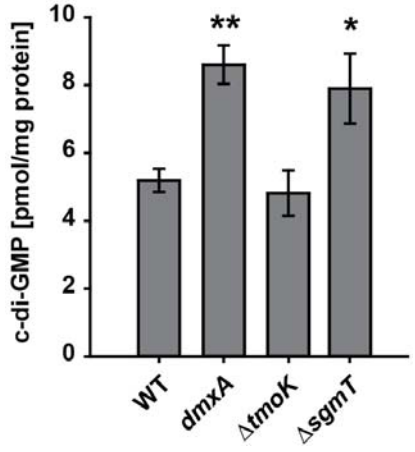


Figure 32. Lack of SgmT and DmxA causes an increase in the c-di-GMP level in vegetative cells

c-di-GMP level in exponentially growing cells of the indicated mutants. Levels of c-di-GMP are shown as mean ± SD from three biological replicates. * $p < 0.05$ in a Students' T-test, ** $p < 0.001$ in a Students' T-test.

To understand the mechanism underlying the reduced T4P-dependent motility in the *dmxA* mutant and the subtle defect in T4P-dependent motility in the Δ*tmoK* mutant, T4P formation and EPS accumulation were quantified in these two mutants. The *dmxA* mutant assembled T4P similarly to WT (Figure 33A) and accumulated ~4-fold more EPS than WT in suspension cultures as well as on solid medium (Figure 34A). The Δ*tmoK* mutant also assembled T4P similarly to WT (Figure 33A) and accumulated ~3-fold more EPS than WT in suspension cultures as well as on solid medium (Figure 34A). As previously reported, the Δ*sgmT* mutant assembled slightly more T4P than WT and also accumulated ~3-fold more EPS than WT in suspension cultures as well as on solid medium (Figure 33A, Figure 34A; (Petters *et al*., 2012). In support of these data, we observed that all three mutants assembled the same total level of PilA as WT; however, the Δ*sgmT* mutant had slightly more PilA in the sheared T4P fraction (Figure 33B).

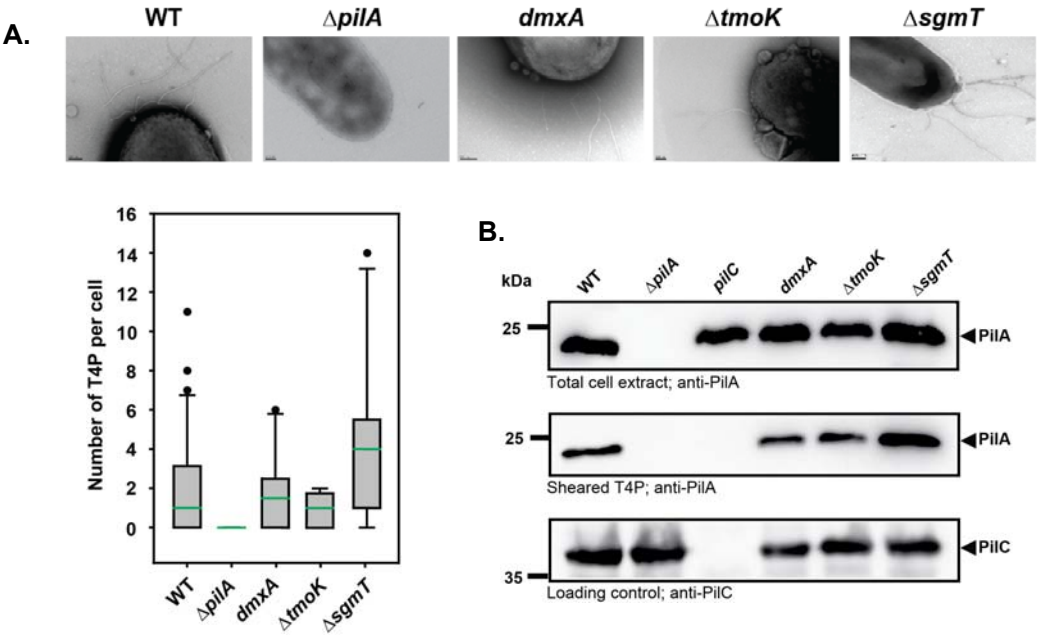


Figure 33. DmxA, TmoK and SgmT are not required for T4P formation and PilA accumulation

(A) T4P formation by exponentially growing cells of the indicated mutants. The experiment was performed as described for Figure 19. Note that data for WT and Δ*pilA* mutant are the same as in Figure 19.
(B) Immunoblot detection of PilA in total cell extract and in sheared T4P. The experiment was performed as described for Figure 20A.

Moreover, all three mutants exhibited differences in agglutination. Δ*sgmT* mutant displayed delayed agglutination and *dmxA* and the Δ*tmoK* mutants displayed slightly faster agglutination when compared to WT (Figure 34B).

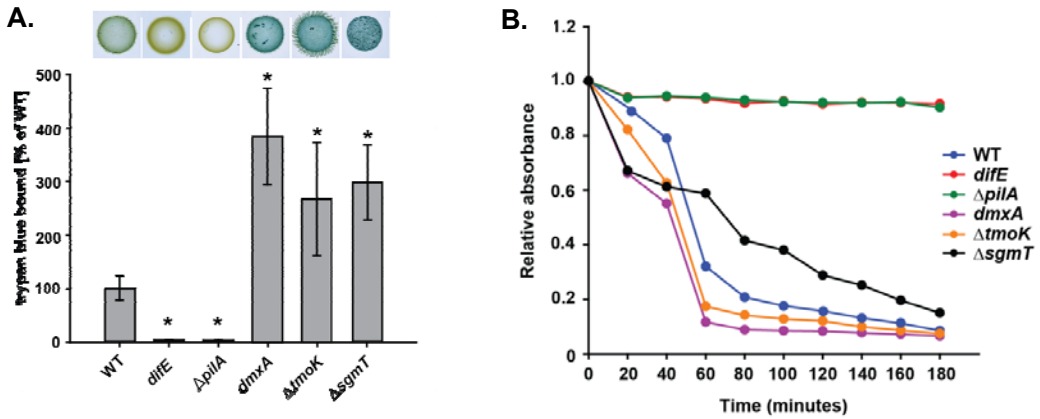


Figure 34. DmxA, TmoK and SgmT are involved in cell-cell interactions and EPS accumulation

(A) Quantification of EPS accumulation. Strains were analyzed as described for Figure 22A. The percentage of trypan blue bound by a strain is indicated relative to WT (100%). Levels of trypan blue bound are shown as mean ± SD from three biological replicates. * $p < 0.001$ in a Students' T-test.
(B) Cell agglutination assay. The experiment was performed as described for Figure 22B

2.10. Analysis of DmxB in *M. xanthus*

2.10.1. DmxB is a predicted diguanylate cyclase important for development

DmxB is a predicted cytoplasmic protein with an N-terminal receiver domain, which contains a conserved phosphorylatable Asp residue (D60), and a C-terminal GGDEF domain with an A-site that contains the conserved residues important for catalytic activity (G^{219}GDEF) and an I-site with residues important for c-di-GMP binding (R^{210}ESD) (Figure 35A). A mutant lacking DmxB had an early developmental defect, neither aggregated on TPM agar nor in submerged culture, and did not sporulate (Figure 35B, Figure 28).

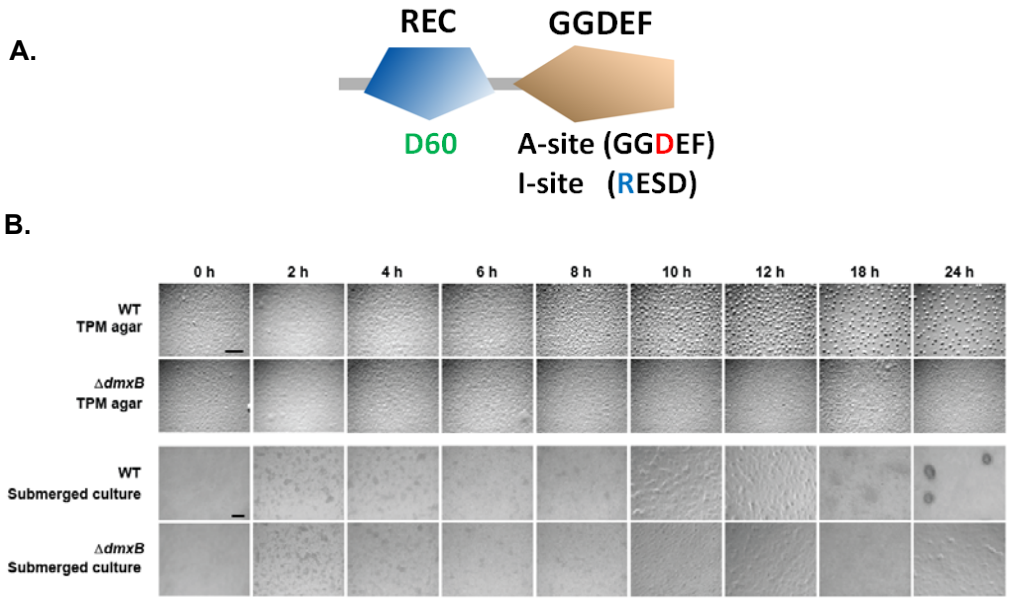


Figure 35. DmxB is a predicted diguanylate cyclase important for development

(A) Domain structure of DmxB protein. Domain annotation was performed using the SMART web tool, domains are not drawn to scale. Coloured are residues targeted by site-directed mutagenesis in the later experiments. Green - conserved aspartate in the Rec domain; red - conserved aspartate in the GGDEF domain essential for DGC activity; blue - conserved arginine in I-site of GGDEF domain essential for c-di-GMP binding and feedback inhibition.
(B) Time course experiment following the phenotype of WT and Δ*dmxB* mutant on TPM agar and in MC7 submerged culture during the first 24h of development. Development was assayed as described in Figure 24. Scale bars, TPM agar 500 μm, submerged culture 100 μm.

To differentiate between DmxB being important for development and/or sporulation, we tested glycerol-induced sporulation in Δ*dmxB* mutant. We did not observe any difference between WT and Δ*dmxB* mutant, and we concluded that DmxB in important for aggregation process during development but not for sporulation *per se* (Figure 36).

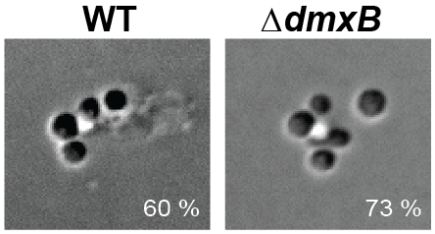


Figure 36. DmxB is not required to form glycerol induced spores

Cell morphology 4 h after adding 0.5 M glycerol. Numbers represent the amount of heat- and sonication-resistant spores relatively to intial number of cells.

2.10.2. DmxB has enzymatic activity and binds c-di-GMP *in vitro*

To test enzymatic activity of DmxB *in vitro*, we overexpressed His6-tagged full-length variant of DmxB in *E. coli* and purified it as a soluble protein.

DmxB^{WT}, similarly to the positive control DgcA^{WT}, produced c-di-GMP in a time dependent manner, when incubated with [α-^{32}P]-GTP, as detected after separation of nucleotides by TLC (Figure 37A). In contrast, the A-site variant DmxB^{D221A} did not produce c-di-GMP. As previously mentioned, DmxB possesses an intact I-site motif and is predicted to bind c-di-GMP. To test this hypothesis we used DRaCALA assay with [α-^{32}P]-labeled c-di-GMP. In agreement with the predictions from sequence analyses, DmxB specifically bound [α-^{32}P]-c-di-GMP whereas the I-site mutant DmxB^{R210A} did not (Figure 37B).

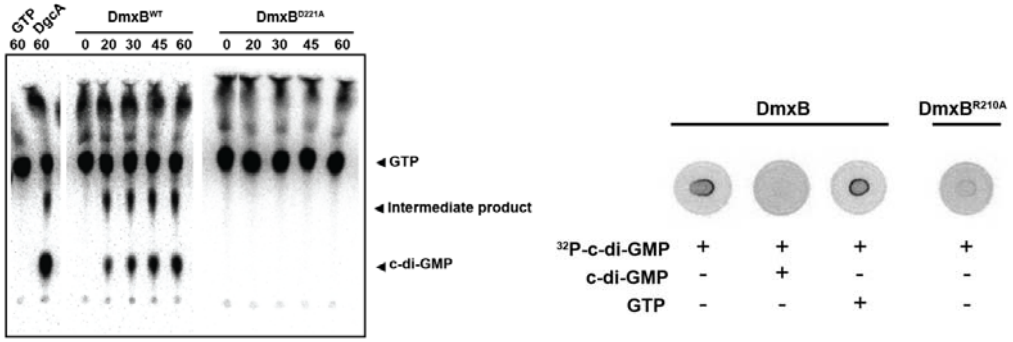


Figure 37. Enzymatic activity and c-di-GMP binding

(A) DGC *in vitro* assay of DmxB variants. The indicated DmxB variants were incubated with [α-^{32}P]-GTP for the indicated periods of time followed by separation of nucleotides by thin layer chromatography. Full-length DgcA^{WT} was used as a positive control. GTP and c-di-GMP are indicated.
(B) DRaCALA assay to detect specific c-di-GMP binding by purified proteins. Full-length DmxB variants were incubated with [α-^{32}P]-labeled c-di-GMP with or without unlabelled c-di-GMP and GTP as competitors added.

2.10.3. Lack of DmxB causes change in c-di-GMP level during starvation

To determine the effect of DmxB on c-di-GMP levels *in vivo*, we determined c-di-GMP level in the Δ*dmxB* mutant strain. In the Δ*dmxB* mutant, c-di-GMP level in vegetative cells (time-point 0 h) was similar to that in WT and it essentially remained constant throughout the entire time course of starvation without showing the approximately 4-fold increase observed in WT (Figure 38). In the Δ*dmxB/dmxB*^{WT} complementation strain, but not in the Δ*dmxB/dmxB*^{D221A} strain containing A-site variant of DmxB, c-di-GMP level during starvation was restored to WT levels (Figure 38). Importantly, the Δ*dmxB/dmxB*^{D221A} strain phenocopied the Δ*dmxB* mutant, did not aggregate and was strongly reduced in sporulation (Figure 40A). In the two complementation strains, the DmxB variants accumulated at the same level, which was lower than in WT (Figure 40B). This data strongly suggests that DmxB is responsible for the 4-fold increase in the c-di-GMP level in WT during starvation, which is essential for the proper development.

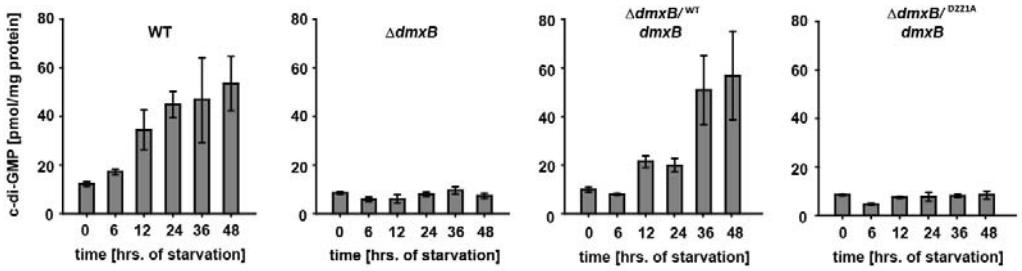


Figure 38. c-di-GMP level in WT starving cells and indicated mutants

Level of c-di-GMP in WT cells, Δ*dmxB* and complementation strains is shown as mean ± SD from three biological replicates.

2.10.4. DmxB specifically accumulates during development

In order to answer the question why Δ*dmxB* mutant only shows defect during development, we determined the expression pattern of *dmxB* by using qRT-PCR. *dmxB* transcription increased more than 100-fold during the first 24 h of development in comparison to vegetative cells (Figure 39A).

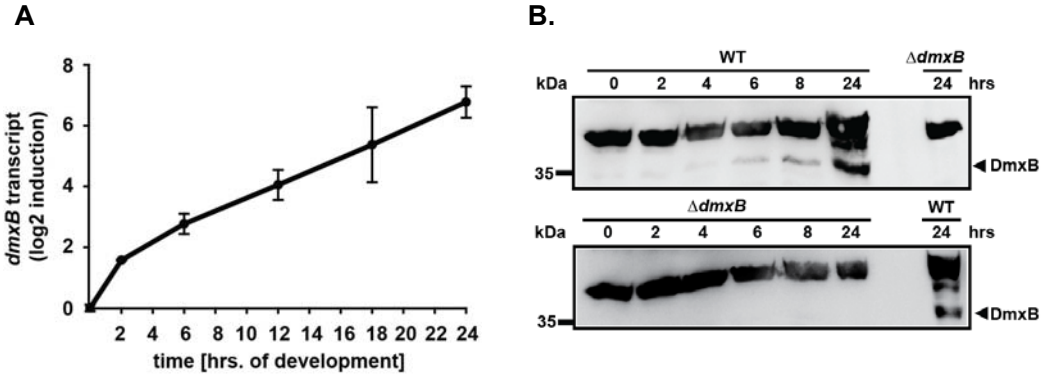


Figure 39. *dmxB* transcription and protein accumulation

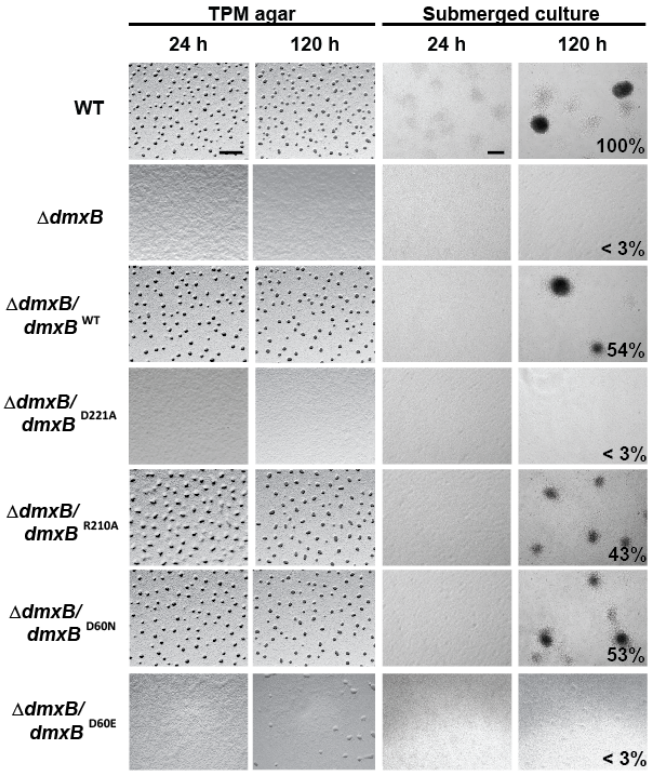
(A) qRT-PCR analysis of *dmxB* expression. RNA was isolated from WT cells developing in submerged cultures by using a hot-phenol extraction. *dmxB* transcript level is shown as mean from two biological replicates, each of them in three technical replicates, relative to WT at time point 0h, in a $\log_2$ scale.
(B) Immunoblot detection of DmxB in total cell extracts of WT and Δ*dmxB* strain. Total cell lysates from cells harvested from starvation agar plates at indicated time points of development were separated by SDS-PAGE and probed with anti-DmxB antibodies. Protein from the same number of cells was loaded per lane.

Consistently, immunoblot analysis performed in a timecourse experiment during development revealed that DmxB was undetectable in vegetative cells and accumulated in a pattern similar to the *dmxB* transcript during the first 24 h of development (Figure 39B). We conclude that DmxB accumulation is induced in response to starvation and regulated at the level of transcription of *dmxB.*

2.10.5. DGC activity of DmxB is regulated by phosphorylation and feedback inhibition

To further understand how DmxB is regulated, we ectopically expressed mutant *dmxB* alleles in the Δ*dmxB* mutant, including *dmxB*^{D60N}, which encodes a DmxB variant in which the phosphorylatable Asp in the receiver domain has been substituted with the non-phosphorylatable Asn, *dmxB*^{D60E}, which encodes a DmxB variant predicted to mimic the phosphorylated state of the receiver domain, and *dmxB*^{R210A}, which encodes a DmxB variant with a substitution of the conserved Arg in the I-site, and which is unable to bind c-di-GMP *in vitro* (Figure 37B). All three variants accumulated at lower levels that native DmxB expressed in WT, but similar to the levels of DmxB^{WT} expressed in Δ*dmxB* (Figure 40B). DmxB^{D60N} and DmxB^{R210A} complemented the developmental defects in the Δ*dmxB* mutant while DmxB^{D60E} did not (Figure 40A).

A.



B.

Figure 40. **Complementation with different alleles of *dmxB***

(A) Developmental assay of indicated Δ*dmxB* complementation strains. Development was assayed as described in Figure 24. Numbers after 120 h of starvation in submerged culture indicate heat- and sonication resistant spores formed relative to WT (100%). Scale bars: TPM agar 500 µm, submerged culture 100 µm.
(B) Detection of DmxB in total cell extracts of WT, Δ*dmxB* and different complementation strains by immunoblot. Total cell lysates from cells harvested from starvation agar plates at different time points of development were separated by SDS-PAGE and probed with anti-DmxB antibodies. Protein from the same number of cells was loaded per lane.

In vitro, all three DmxB variants were able to produce c-di-GMP and displayed DGC activity similar to $DmxB^{WT}$ (Figure 41).

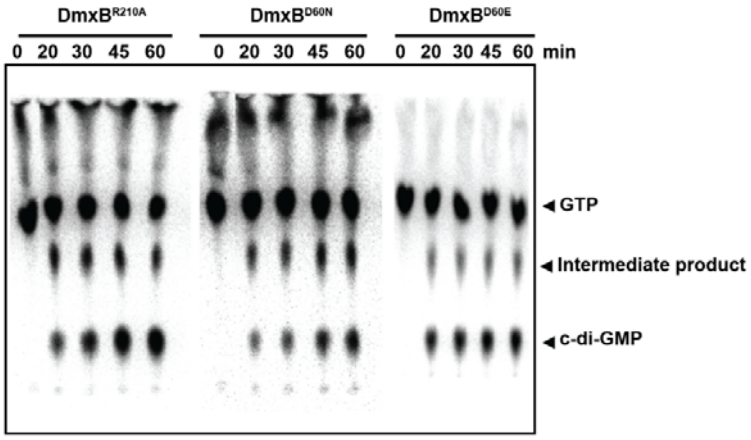


Figure 41. *In vitro* assay for enzymatic activity of the indicated variants of DmxB

DGC *in vitro* assay of DmxB variants. Experiment was performed as described in Figure 30. GTP and c-di-GMP are indicated.

In vivo, the strain expressing DmxB^{D60N} accumulated c-di-GMP similarly to WT whereas the strain expressing DmxB^{D60E} did not display the starvation induced increase in the c-di-GMP level observed in WT (Figure 42). Finally, the strain expressing DmxB^{R210A} had a c-di-GMP level that was approximately 10-fold higher than WT (Figure 42) consistent with the idea that this DmxB variant is no longer subject to feedback inhibition by c-di-GMP *in vivo*. In contrast, *in vitro* DmxB^{R210A} had the same DGC activity as the WT protein (Figure 41) suggesting that under these conditions the [c-di-GMP] *in vitro* was not sufficiently high to result in feedback inhibition.

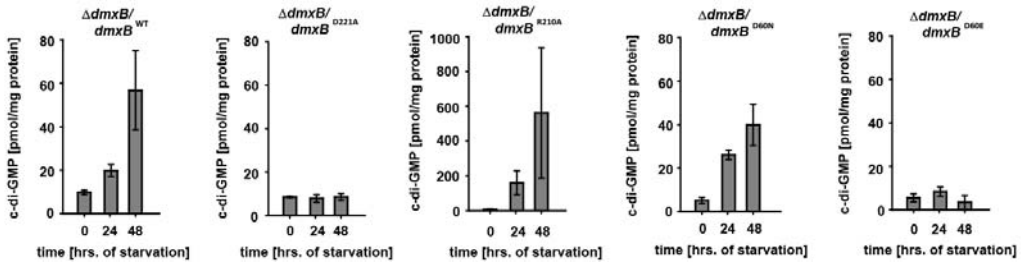


Figure 42. c-di-GMP level in starving cells of the indicated complementation strains

Levels of c-di-GMP in complementation strains of Δ*dmxB.* Note that graphs for Δ*dmxB/dmxB*^{WT} and Δ*dmxB/dmxB*^{D221A} are the same as in Figure 38 and are shown again for comparison. Levels of c-di-GMP are shown as mean ± SD from three biological replicates. Note the different scale in Δ*dmxB/dmxB*^{R210A}.

To additionally confirm the results from *in vitro* activity assay, we performed an *in vivo* activity assay in *E. coli.* It is a phenotypic assay that correlates c-di-GMP level with cellulose production in *E. coli* stained by Congo Red (CR) and can be used as an indicator for DGC activity (Zogaj *et al.*, 2001). *E. coli* transformed with a vector control (pMALc2x) or the inactive DmxB^{D221A} remained uncoloured when plated on CR-containing plates. In contrast, expression of DmxB^{WT}, DmxB^{R210A,} DmxB^{D60N} or DmxB^{D60E} resulted in a red colony phenotype (Figure 43). This was in agreement with the *in vitro* DGC activity of these variants. Therefore, DmxB^{D60E} is active *in vitro* and *in vivo* in *E. coli* but not *in vivo* in *M. xanthus,* suggesting that in *M. xanthus* DmxB^{D60E} is kept in an inactive state.

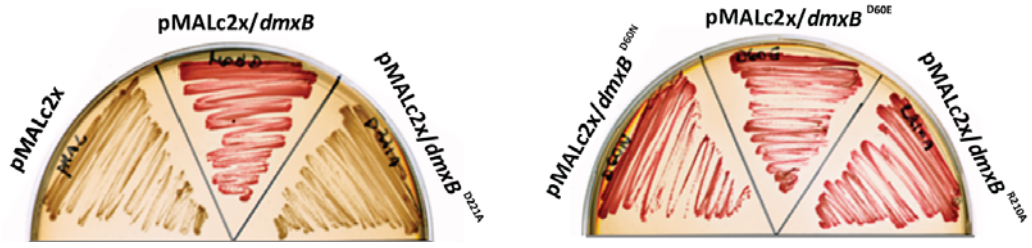


Figure 43. DGC activity assay of different DmxB variants *in vivo* in *E. coli*, based on Congo Red binding

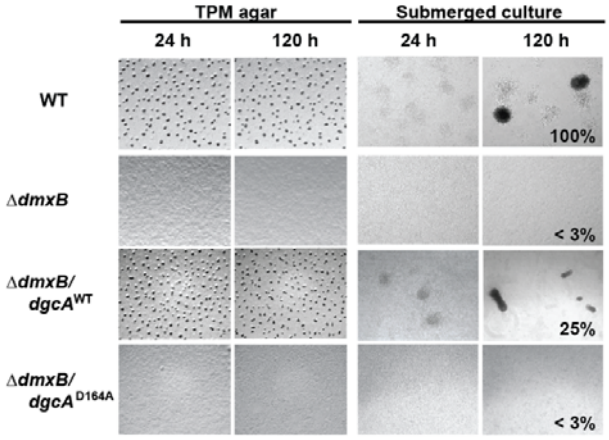
E. coli cells transformed with indicated expression plasmids were grown on LB plates supplemented with 50 µg/ml Congo Red at 30° C overnight. Red colour of the colonies indicates DGC activity of the indicated protein variant.

These data, together with the observation that WT/DgcA^{WT} strain (which accumulates c-di-GMP at a significantly higher level than WT) develops normally, whereas the WT/PA5295^{WT} strain (that accumulates significantly less c-di-GMP) does not develop, strongly suggest that a minimal threshold level of c-di-GMP is essential for development and demonstrate that a c-di-GMP level even 10-fold higher than WT level does not interfere with development. Moreover, our data suggest that DmxB^{WT} is subject to two levels of post-translational control: feedback inhibition by c-di-GMP and phosphorylation-dependent regulation by a mechanism in which phosphorylation of the receiver domain results in inhibition of DGC activity.

2.10.6. The Δ*dmxB* mutant can be partially complemented by heterologous DGC

Our data suggest that DmxB is the diguanylate cyclase responsible for the increase in the c-di-GMP level during development and that this increase is essential for development. To test whether the function of DmxB is to contribute to a global pool of c-di-GMP, allowing it to reach a minimal threshold level, we expressed the heterologous DGC ($DgcA^{WT}$) or its active site variant $DgcA^{D164A}$ in the Δ*dmxB* mutant. Interestingly, fruiting body formation and sporulation were partially restored in the Δ*dmxB* mutant by $DgcA^{WT}$ but not by $DgcA^{D164A}$ (Figure 44A) and in the $DgcA^{WT}$ containing strain the level of c-di-GMP was similar to that in WT during starvation (Figure 44B). These results corroborate that the major function of DmxB is to contribute to the global pool of c-di-GMP in developing *M. xanthus* cells.

A.



B.

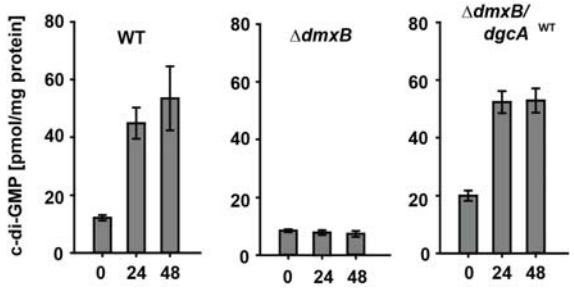


Figure 44. Δ*dmxB* mutant is complemented by expression of a heterologous DGC

(A) Developmental assay of indicated Δ*dmxB* complementation strains. Development was assessed as described in Figure 24. Numbers after 120 h of starvation in submerged culture indicate heat- and sonication resistant spores formed relative to WT (100%). Scale bars: TPM agar 500 µm, submerged culture 100 µm.
(B) Levels of c-di-GMP in indicated Δ*dmxB* complementation strains. Levels of c-di-GMP are shown as mean ± SD from three biological replicates. Note that the data for WT and Δ*dmxB* mutant are the same as those in Figure 42 and in Figure 38.

2.10.7. Δ*dmxB* developmental defects are due to reduced EPS accumulation

We previously showed that in vegetative cells an increase in the c-di-GMP level interfere with T4P function by either causing a reduction in *pilA* transcription, and therefore T4P formation, or a change in cell-cell interactions and EPS accumulation. To deduce the mechanism underlying the developmental defects of the Δ*dmxB* mutant, we tested it for PilA accumulation and T4P formation by quantifying the total amount of cellular PilA as well as PilA assembled into T4P during development. In WT cells as well as in the Δ*dmxB* mutant the total level of PilA increased from 0 to 24 h of development as previously reported for WT (Wu & Kaiser, 1997). Similarly, the level of PilA incorporated into T4P increased significantly in both strains, and even more in the Δ*dmxB* mutant than in the WT (Figure 45A). As expected, PilA was not detected in the Δ*pilA* mutant and also not in the sheared T4P fraction of the *pilC* mutant (Figure 45A).

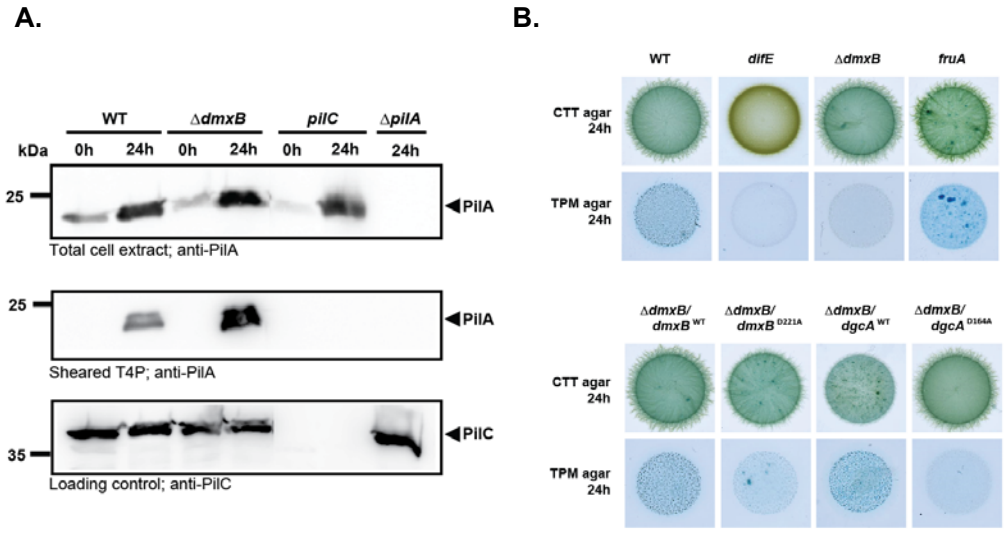


Figure 45. Phenotypic characterization of Δ*dmxB* mutant

(A) Detection of PilA in total cell extract and in sheared T4P fraction of the cells developing in MC7 submerged cultures by immunoblot. The experiment was performed as described for Figure 20A.
(B) EPS accumulation. Aliquots of 20 µl cell suspensions at 7 × 10^9 cells/ml were spotted on 0.5% agar supplemented with 0.5% CTT and 20 µg/ml trypan blue or TPM agar supplemented with 20 µg/ml trypan blue and incubated at 32 °C for 24 h.

EPS accumulation was determined by using a colorimetric assay. For this purpose, cells were inoculated on solid medium containing trypan blue either in the presence of nutrients or in the absence of nutrients. In the presence of nutrients, no differences in trypan blue binding were observed between WT, *fruA* which is

strongly affected in development and the Δ*dmxB* mutant complemented with *dmxB*^{WT}, *dmxB*^{D221A}, *dgcA*^{WT} or *dgcA*^{D164A} whereas the negative control *difE* did not bind trypan blue (Figure 45B). In the absence of nutrients, WT, *fruA*, Δ*dmxB*/*dmxB*^{WT} and Δ*dmxB*/*dgcA*^{WT} strains all had the same high level of EPS as indicated by their dark blue colour, while Δ*dmxB,* Δ*dmxB*/*dmxB*^{D221A} and Δ*dmxB*/*dgcA*^{D164A} strains, similarly to the *difE* negative control, bound trypan blue at a much reduced level (Figure 45B). We conclude that DmxB-dependent increase in the c-di-GMP level during development is responsible for EPS accumulation during development.

2.10.8. Developmental defects caused by the lack of DmxB are rescued by extracellular complementation

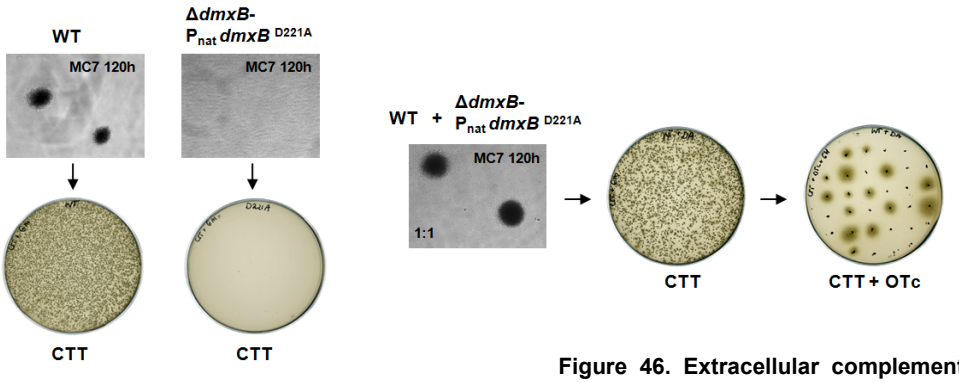
It was demonstrated already many years ago, that certain developmental mutants that are unable to form fruiting bodies can be rescued by co-developing with wild type cells (Hagen *et al.*, 1978). The phenotype of the Δ*dmxB* mutant is similar to that of the *difE* mutant, which is strongly reduced in EPS accumulation and unable to aggregate and sporulate (Yang *et al*., 2000). Because the developmental defects of a *difE* mutant can be rescued by extracellular complementation by WT or by addition of purified EPS (Yang *et al*., 2000, Shimkets, 1986b), we reasoned that the Δ*dmxB* mutant would also be rescued if the main defect in this mutant is the reduced EPS accumulation during development.

To this end, cells of a tetracycline resistant Δ*dmxB* mutant (Δ*dmxB/dmxB*^{D221A}) were mixed with tetracycline sensitive WT cells in a 1:1 ratio and co-developed in submerged culture (Figure 46A). Subsequently, spores formed by the two strains were counted. In this experiment, 53% of the germinating spores derived from the Δ*dmxB/dmxB*^{D221A} strain (Figure 46B). Importantly, and in agreement with the EPS accumulation profile, the Δ*dmxB* mutant was not rescued by co-development with the *difE* mutant, suggesting that the mechanism underlying the Δ*dmxB* developmental defects is indeed the reduced EPS accumulation during starvation. As a control, we successfully complemented *difE* mutant by co-development with WT cells as previously reported in the literature (Yang *et al*., 2000, Shimkets, 1986b).

Inspired by the finding that in *Dictyostelium discoideum* development could be restored by adding c-di-GMP to a DGC mutant (Chen & Schaap, 2012) we decided to test if exogenous c-di-GMP restores development of the Δ*dmxB* mutant. Fruiting body formation was not restored upon addition of c-di-GMP to the Δ*dmxB* mutant in

submerged culture. We used 1mM c-di-GMP as reported for *D. discoideum* (Chen & Schaap, 2012) and added it at 0 h or at 24 h of development (data not shown).

A.



B.

Extracellular complementation assay		
Strain	% spores, donor	% spores, recipient
WT	100	NA
Δ*dmxB/dmxB*^{D221A}	< 2	NA
difE	< 2	NA
WT + Δ*dmxB/dmxB*^{D221A}	47	53
difE + Δ*dmxB/dmxB*^{D221A}	< 2	< 2
WT + *difE*	62	38

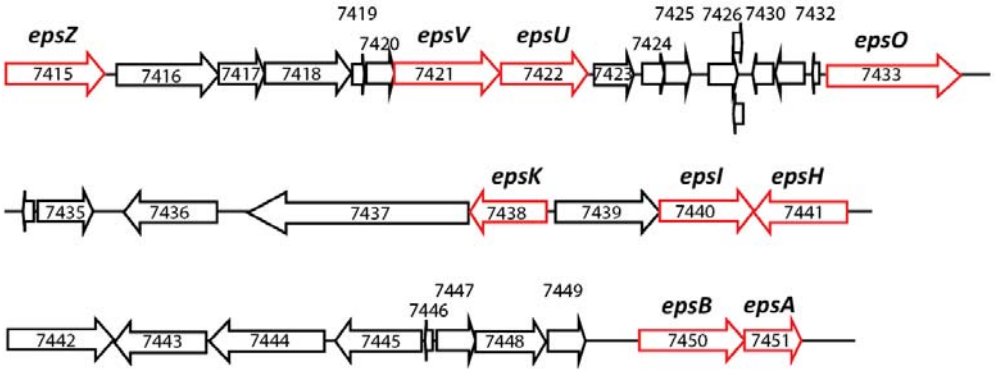
Figure 46. Extracellular complementation assay of the Δ*dmxB* mutant

(A) Example of extracellular complementation assay. Δ*dmxB/dmxB*^{D221A} mutant was mixed with WT in a ratio 1:1 and developed in submerged culture. Cells after 120 h of starvation were harvested, incubated for 2 h at 55 °C, sonicated to disperse fruiting bodies and dilutions were plated on 1% CTT agar plates supplemented with relevant antibiotics. **(B)** Summary of the results of extracellular complementation assay for Δ*dmxB/dmxB*^{D221A} and other strains.

2.10.9. Transcription of the genes from *eps* locus is affected in the Δ*dmxB* mutant

The *eps* locus encode proteins involved in EPS synthesis and transport and at least 10 of the genes in this locus are essential for fruiting body formation and sporulation (Lu *et al*., 2005). To determine if DmxB is important for the expression of the *eps* genes, we determined the expression profile of nine of the *eps* genes in WT and in Δ*dmxB* mutant during development by using qRT-PCR. These nine genes encode for proteins with different functions in EPS synthesis and transport (Figure 47A). Eight of them have been shown to be essential for development, only *epsB* mutant does not show developmental defect (Lu *et al*., 2005). For seven of the nine genes we did not observe significant differences in their expression profile in the two strains; however, two genes (*epsA* and *epsB*) were transcribed at significantly lower level in the Δ*dmxB* mutant than in the WT strain at the late time points of development (Figure 47B). *epsA* encodes a predicted glycosyl transferase and *epsB* encodes a predicted glycosyl hydrolase (Lu *et al*., 2005).

A.



PROTEIN	PUTATIVE FUNCTION
MXAN7451 (EpsA)	UDP-*N*-acetyl-mannosamine transferase
MXAN7450 (EpsB)	Endo-1,4-β-glucanase precursor
MXAN7449 (EpsC)	Serine acetyltransferase
MXAN7448 (EpsD)	Glycosyltransferase, family 2
MXAN7445 (EpsE)	Glycosyltransferase, family 1
MXAN7444 (EpsF)	Response regulator/sensory box histidine kinase
MXAN7442 (EpsG)	Mg^{2+} transporter
MXAN7441 (EpsH)	Glycosyltransferase, family 1
MXAN7440 (EpsI)	Nla24, σ^{54}-dependent DNA-binding response regulator
MXAN7439 (EpsJ)	Sensory box histidine kinase
MXAN7438 (EpsK)	Membrane fusion protein
MXAN7437 (EpsL)	CzcA family heavy metal efflux protein
MXAN7436 (EpsM	Outer membrane efflux protein
MXAN7435 (EpsN)	Hydrolase alpha/beta fold family
MXAN7433 (EpsO)	von Willebrand factor type a domain protein
MXAN7431 (EpsP)	Transposase
MXAN7426 (EpsQ)	Hypothetical protein
MXAN7425 (EpsR)	Hypothetical protein
MXAN7424 (EpsS)	Hypothetical protein
MXAN7423 (EpsT)	Hypothetical protein
MXAN7422 (EpsU)	Glycosyltransferase, family 2
MXAN7421 (EpsV)	Chain length determinant family protein
MXAN7420 (EpsW)	Signal transduction histidine kinase
MXAN7418 (EpsX)	Hypothetical protein
MXAN7417/6 (EpsY)	Polysaccharide biosynthesis/export protein
MXAN7415 (EpsZ)	Glycosyltransferase domain protein

B.

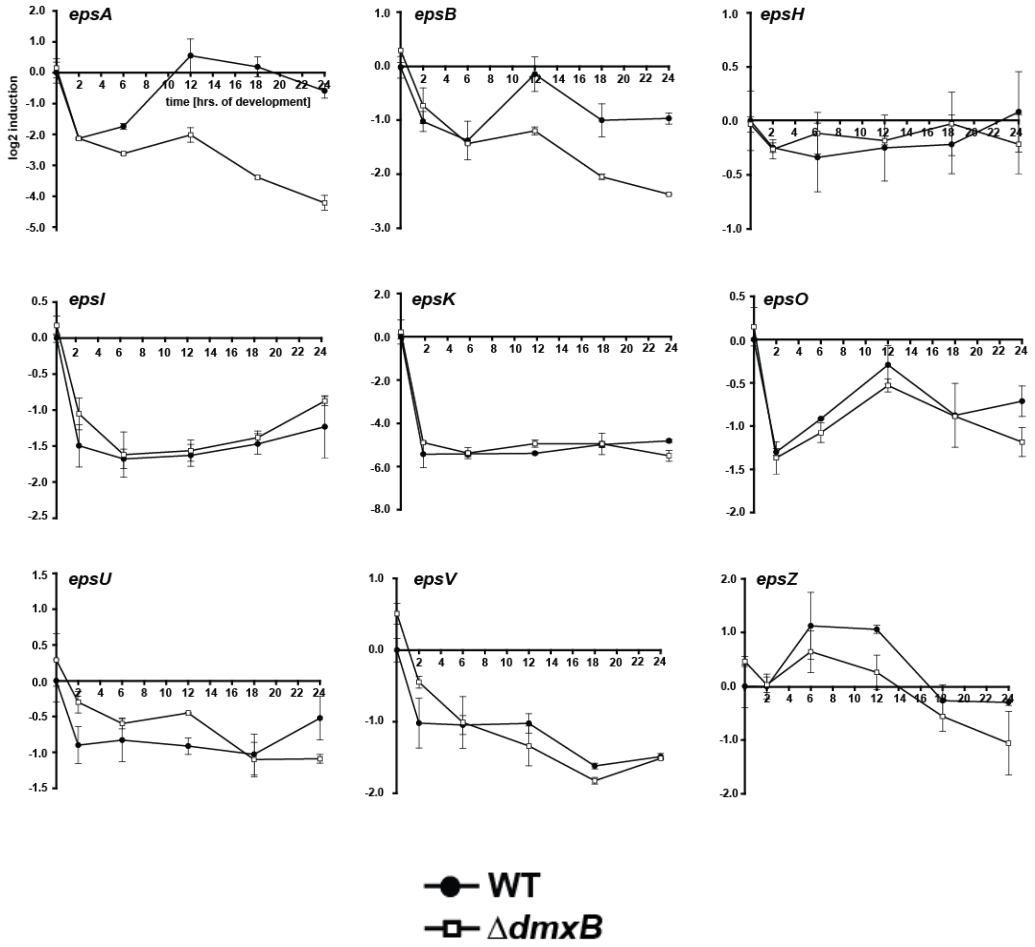


Figure 47. **qRT-PCR analysis of the *eps* genes transcription in WT (closed circles) and Δ*dmxB* strain (opened squares) during development**

(A) Organization of the *eps* locus and the predicted functions of the proteins encoded in this locus. Genes marked in red were tested for expression level in WT and Δ*dmxB* mutant during development.
(B) Result of the qRT-PCR experiment. WT (closed circles) and Δ*dmxB* strain (opened squares) RNA was isolated from cells developing in MC7 submerged cultures by using a hot-phenol extraction. Transcripts levels are shown as mean ± SD from two biological replicates, each of them with three technical replicates, relative to WT at time point 0h, in a $\log_2$ scale. Analyzed genes are indicated in panel A, in red.

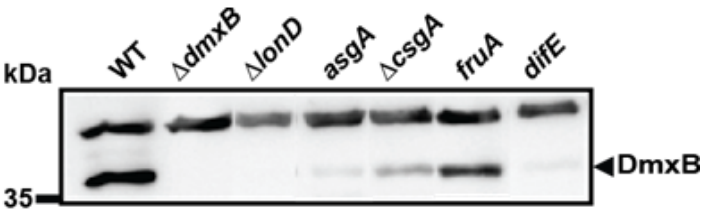
2.10.10. DmxB accumulation is altered in developmental mutants

To identify regulators involved in *dmxB* expression, we determined DmxB accumulation in a set of mutants that are arrested in development at different time points. DmxB accumulation was almost undetectable after 24 h of development in a Δ*lonD* mutant (also referred to as Δ*bsgA*), which is arrested at the onset of development (Kroos & Kaiser, 1987, Gill *et al.*, 1993), in a *asgA* mutant, which is arrested at 1-2 h of development (Kuspa *et al.*, 1986), and in a *difE* mutant, which is

arrested early in the aggregation phase (Yang *et al*., 1998b) (Figure 48A) whereas in Δ*csgA* mutant, which is arrested in development after 4-6 h (Kroos & Kaiser, 1987, Ellehauge *et al*., 1998, Ogawa *et al*., 1996) reduced levels of DmxB were detected after 24 h of development. In *fruA* mutant, which is arrested later in development, DmxB accumulated on WT level. Among these mutants, Δ*lonD, asgA* and Δ*csgA* mutants are deficient in synthesis of intercellular signals required for development, suggesting that intercellular signaling is required for the stability or for the expression of *dmxB*.

This conclusion was further confirmed by determination of EPS accumulation in the Δ*lonD, asgA,* Δ*csgA,* and *fruA* mutants. As shown in Figure 48B, EPS accumulation in these four mutants correlates with the level of DmxB accumulation in these mutants (Figure 48A).

A.



B.

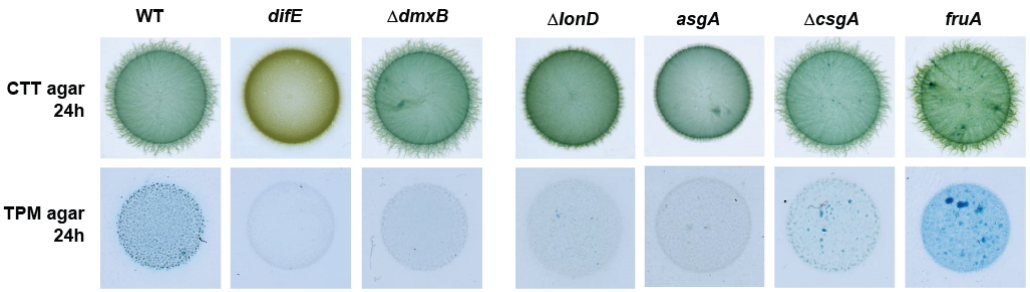


Figure 48. DmxB accumulation and EPS accumulation are altered in developmental mutants

(A) Detection of DmxB in total cell extract of different mutants with developmental phenotypes by immunoblot. Total cell lysates from cells harvested from starvation agar plates after 24h of development were separated by SDS-PAGE and probed with anti-DmxB antibodies. Protein from the same number of cells was loaded per lane.
(B) EPS accumulation in mutants affected in development. Aliquots of 20 µl cell suspensions at 7×10^9 cells/ml were spotted on 0.5% agar supplemented with 0.5% CTT and 20 µg/ml trypan blue or TPM agar supplemented with 20 µg/ml trypan blue and incubated at 32 °C for 24 h.

For *difE* mutant, we confirmed the immunoblot results by measuring c-di-GMP level. Consistently, *difE* mutant was found to be strongly reduced in c-di-GMP accumulation during starvation (Figure 49).

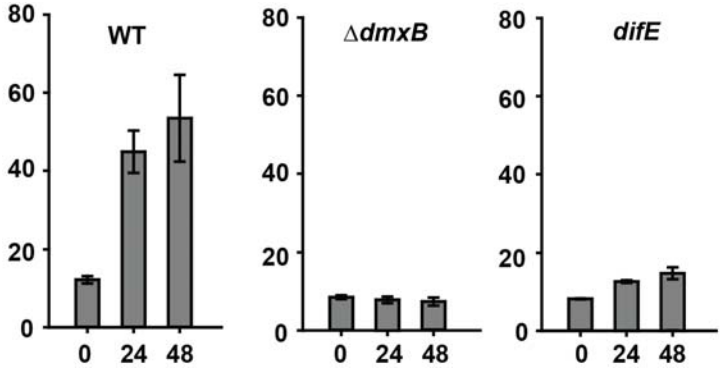


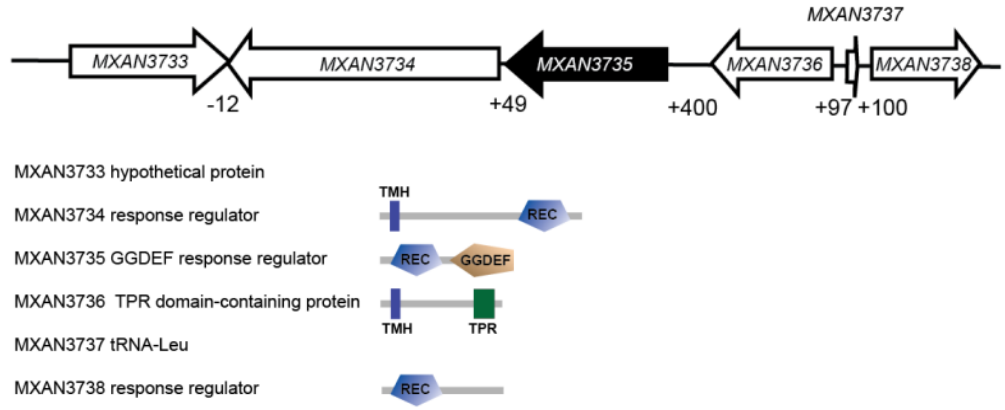
Figure 49. c-di-GMP level is reduced in *difE* mutant

Levels of c-di-GMP in the indicated strains. Levels of c-di-GMP are shown as mean ± SD from three biological replicates. Note that the data for WT and Δ*dmxB* mutant are the same as those in Figure 38, Figure 42 and Figure 44 and are shown here for comparison.

2.10.11. MXAN3734 is not involved in development

In order to identify the potential interacting partners of DmxB we investigated the genetic neighbourhood of the *dmxB* gene. In close proximity to *dmxB*, in the same operon, we identified a gene coding for a response regulator, MXAN3734. The MXAN3734 protein is predicted to have a transmembrane domain and a receiver domain (Figure 50). Following the hypothesis that MXAN3734 could be an interacting partner of DmxB, we created an in frame deletion of *MXAN3734* and examined the strain for development. We did not observe any developmental defects in Δ*MXAN3734* mutant (Figure 50). We did not focus on the other neighbouring genes as they are in different transcriptional units.

A.



B.

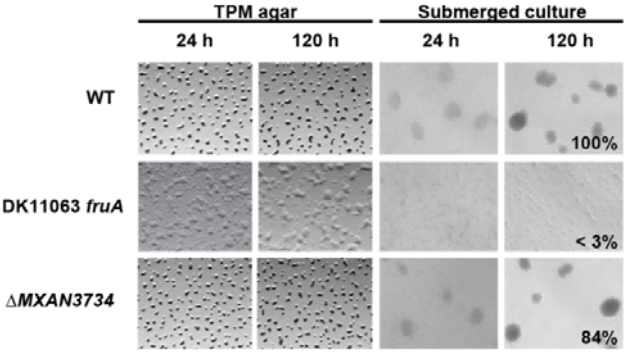


Figure 50. MXAN3734 in not involved in development

(A) Genetic neighbourhood of *dmxB* (*MXAN3735*). On the left, the arrows representing genes indicate the direction of transcription and are drawn to scale. Numbers on the bottom indicate distances between genes in bp. On the right, domain annotation was performed using the SMART web tool; domains are not drawn to scale. TMH – transmembrane helix.
(B) Phenotype of Δ*MXAN3734* mutant during development. Development was assessed as described in Figure 24. Numbers after 120 h of starvation in submerged culture indicate heat- and sonication resistant spores formed relative to WT (100%). Scale bars, TPM agar 500 μm, submerged culture 100 μm.

2.11. Analysis of PmxA in *M. xanthus*

2.11.1. PmxA is a predicted phosphodiesterase important for development

PmxA (MXAN2061) is a predicted integral membrane PDE of the HD-GYP type and contains a Cache domain, a HAMP domain and a C-terminal HD-GYP domain with all the residues for catalytic activity ($H^{424}D$-$G^{485}YP$) (Figure 51A, Figure 26). The proposed function of the extracellular Cache domain is binding different small molecules and converting this signal into diverse responses depending on the

intracellular effector domains (Anantharaman & Aravind, 2000). Cache and HAMP domains are often found in bacterial sensor and chemotaxis proteins. The Δ*pmxA* mutant aggregated to form slightly irregular fruiting bodies on TPM agar and did not aggregate in submerged culture (Figure 51B) and only sporulated at 15% of the WT level (Figure 51B).

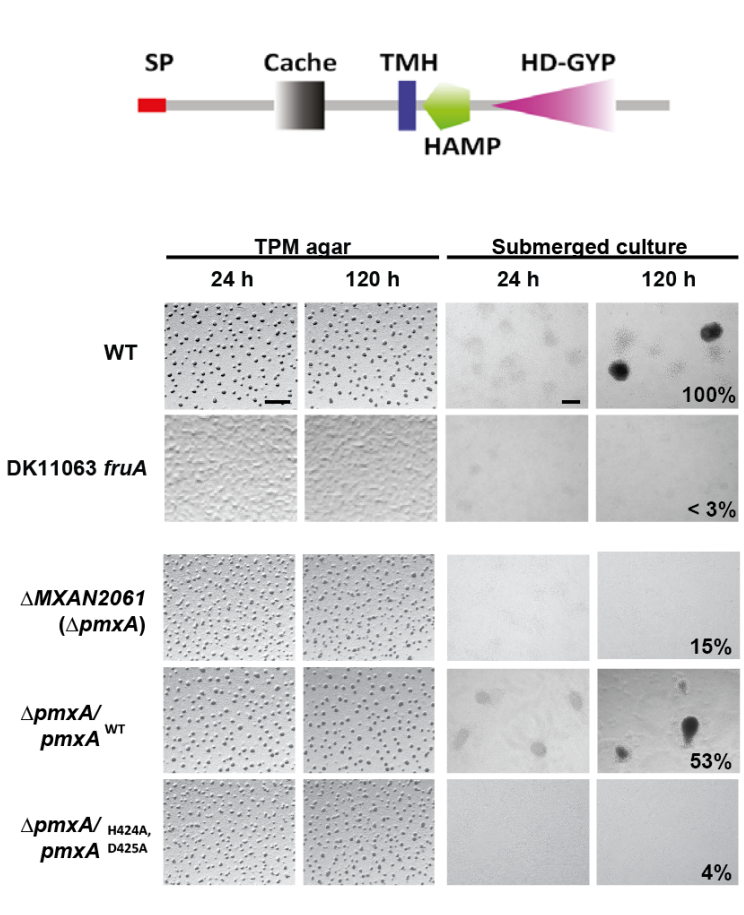


Figure 51. PmxA is a predicted phosphodiesterase important for development

(A) Domain structure of PmxA protein. Domain annotation was performed using the SMART web tool, domains are not drawn to scale. TMH – transmembrane helix; SP – signal peptide.
(B) Phenotype of WT, Δ*pmxA* mutant and complementation strains on TPM agar and in MC7 submerged culture. Development was performed as described previously in Figure 24. Scale bars, TPM agar 500 μm, submerged culture 100 μm.

We also tested Δ*pmxA* strain for glycerol-induced sporulation and we could not observe any difference when compared to WT (Figure 52). We conclude that, like all the previously tested mutants, Δ*pmxA* strain is able to sporulate but is impaired in aggregation during fruiting body formation.

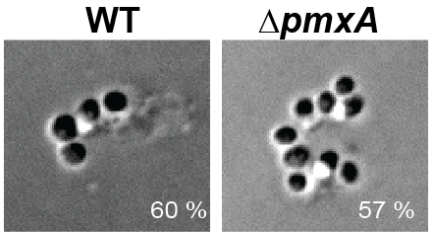


Figure 52. PmxA is not important for glycerol induced sporulation

Cell morphology 4 h after adding 0.5 M glycerol. Numbers represent the amount of heat- and sonication-resistant spores relatively to intial number of cells.

2.11.2. PmxA has enzyme activity *in vitro*

To test *in vitro* for enzymatic activity of PmxA, we overexpressed His6-tagged truncated variants of PmxA in *E. coli* and purified them as soluble proteins. In order to obtain soluble proteins, we used PmxA$^{384\text{-}568}$ variants which correspond to the isolated HD-GYP domain. It was previously described that isolated HD-GYP domains, in contrast to GGDEF domains, still possess enzymatic activity (Ryan *et al*., 2006). To test PmxA$^{384\text{-}568}$ for PDE activity, [α-^{32}P]-labeled c-di-GMP was generated in an enzymatic reaction by purified DgcA^{WT} as described (Roelofs *et al*., 2011). PmxA$^{384\text{-}568}$ displayed PDE activity and degraded [α-^{32}P]-labeled c-di-GMP to linear pGpG, whereas the active site variant PmxA$^{H424A,\ D425A}$ did not (Figure 53). Thus, PmxA is an active enzyme *in vitro*.

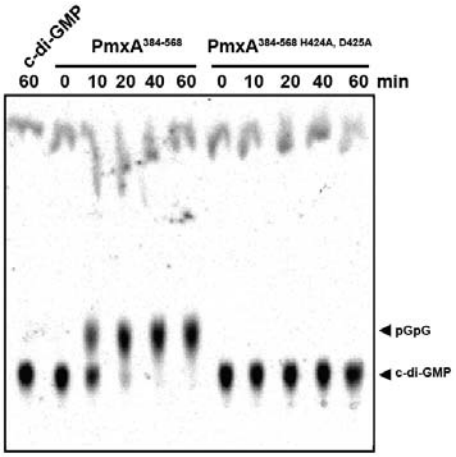


Figure 53*. In vitro* c-di-GMP degradation by PmxA

The indicated PmxA variant was incubated with [α-^{32}P]-labeled c-di-GMP for the indicated periods of time followed by separation of nucleotides by thin layer chromatography. pGpG and c-di-GMP are indicated. The calculated retention factors (R_f) of c-di-GMP ~0.18 and pGpG ~ 0.29 are in agreement with previous reports (Christen, 2007).

2.11.3. Lack of PmxA does not change the c-di-GMP level during starvation

To determine if lack of PmxA caused changes in c-di-GMP levels *in vivo*, we determined the c-di-GMP level in the Δ*pmxA* mutant. In the Δ*pmxA* mutant, the c-di-GMP level in vegetative cells was similar to that in WT (Figure 54). Surprisingly,

during starvation, the level was slightly lower at 12 h and 24 h than in WT but reached WT level at 36 h and 48 h (Figure 54).

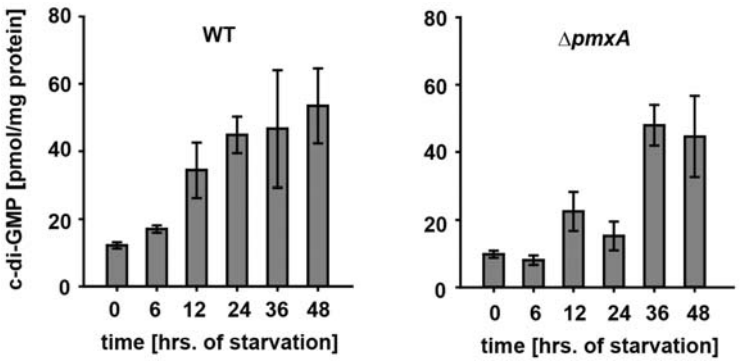


Figure 54. c-di-GMP level in starving cells of the indicated strains

Levels of c-di-GMP in WT and Δ*pmxA* strains. Levels of c-di-GMP are shown as mean ± SD from three biological replicates. Note that the data for WT is the same as in Figure 38, Figure 42 and Figure 44 and is shown here for comparison.

2.12. c-di-GMP receptors in *M. xanthus*.

2.12.1. Development of an assay based on capture compound

The c-di-GMP Capture Compound (cdG-CC) is a powerful tool to identify novel c-di-GMP effectors (Nesper *et al*., 2012). The compound is based on a chemical scaffold harbouring specificity, reactivity and sorting determinants (Figure 55) (Nesper *et al*., 2012).

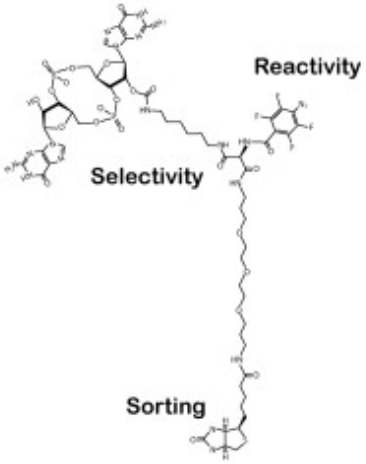


Figure 55. Chemical structure of cdG-CC

Upon UV irradiation, the photo-reactivity group forms a highly reactive nitrene that interacts with proteins bound by c-di-GMP, forming a covalent crosslink. The sorting function of biotin allows for the efficient isolation of the captured proteins by binding of the compound to streptavidin coated magnetic beads. Figure was reproduced from (Nesper *et al*., 2012).

cdG-CC can enrich c-di-GMP binding proteins directly from whole cell extracts in a highly specific manner and is suited for a global isolation procedure of c-di-GMP binding proteins (Nesper *et al*., 2012). It was successfully used to identify c-di-GMP effectors in *P. aeruginosa*, *S. enterica* (Nesper *et al*., 2012)*, B. bacteriovorus* (Rotem *et al*., 2015) and *S. venezuelae* (Tschowri *et al*., 2014) Taking advantage of this technique, we decided to identify c-di-GMP effectors in *M. xanthus.* Therefore, we performed the capture experiments combined with the analysis of isolated proteins by LC–MS/MS.

For these experiments, we used soluble fractions of cell lysate from *M. xanthus* cells growing exponentially or starved for 24h in suspension. In parallel, we prepared two negative controls: a competition control and a binding control (Nesper *et al*., 2012). In the first control, c-di-GMP was added as a competitor to all protein extracts to a final concentration of 1 mM and incubated before cdG-CC was added. This allowed us to exclude proteins, which are binding unspecifically to the capture compound. In second control experiment no cdG-CC was added to the reaction. This allowed us to exclude the proteins which are binding unspecifically to the magnetic beads.

2.12.2. Candidates

As a result from this experiment, we obtained a list of candidates for c-di-GMP specific effectors. We decided to initially consider only the proteins which were not unspecifically binding to the magnetic beads and were significantly enriched in comparison to the competition control. In this way, we were able to specifically pull-down four GGDEF domain proteins (MXAN1525, MXAN5199, MXAN4029 and MXAN4463). These four proteins posses all the conserved residues in the I-site required for c-di-GMP binding. This was an indication, that the cdG-CC can be used to detect c-di-GMP effectors in *M. xanthus cell* lysates.

We also detected several proteins that do not possess GGDEF, EAL or HD-GYP domains and those proteins became our priority candidates for receptors (Table 1). With the exception of MrpC, we did not find any protein that would appear only in the sample where the lysate from starving cells was used (Table 1).

Table 1. c-di-GMP effector candidates in *M. xanthus* based on a capture compound pull-down experiment

Candidate protein	Description	Detected in
MXAN1525	GGDEF domain protein with I-site	veg./starv.
MXAN5199	GGDEF domain protein with I-site	veg./starv.
MXAN4029	GGDEF domain protein with I-site	veg.
MXAN4463	GGDEF domain protein with I-site	veg.
MXAN5707	PilZ domain protein	veg./starv.
MXAN0415	PilT paralog	veg./starv.
MXAN5787 (PilT)	Pilus retraction ATPase	veg./starv.
MXAN3993 (LonD)	ATP-dependent Lon protease	veg.
MXAN5125 (MrpC)	Transcriptional regulator	starv.
MXAN7043	Oxidoreductase	veg./starv.
MXAN6605	PilZ-DnaK domain protein	veg.
MXAN4361	Small hypothetical protein	veg./starv.
MXAN4362	Small hypothetical protein	veg./starv.

2.12.3. Candidates verification

As a first test for the c-di-GMP receptor candidates, we decided to verify their binding using a DRaCALA assay. In order to do so, we overexpresed (or purified) soluble proteins and used them for the binding assay. As a control, we used the soluble fractions of cell lysate from: Rosetta 2 (DE3) cells (negative control) and Rosetta 2 (DE3) cells overexpressing the GGDEF domain of DmxB (positive control). As expected, extract from Rosetta 2 (DE3) cells did not bind c-di-GMP while extract from the cells overexpressing the GGDEF domain of DmxB did show binding of c-di-GMP (Figure 56).

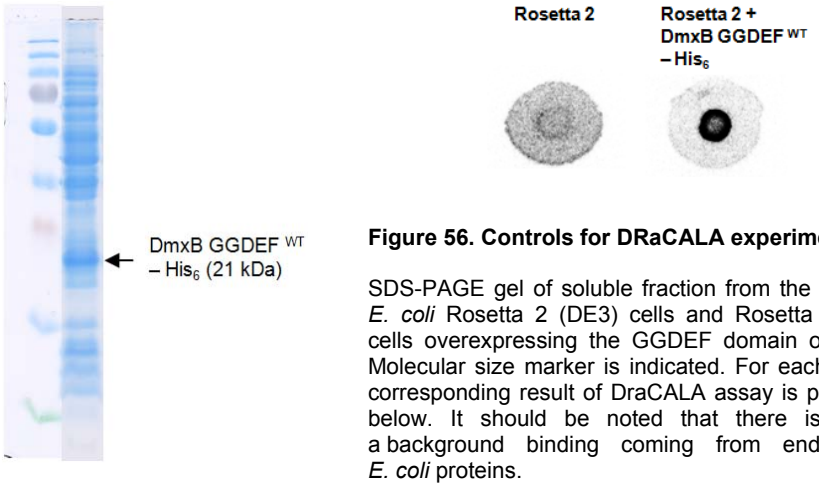


Figure 56. Controls for DRaCALA experiment

SDS-PAGE gel of soluble fraction from the lysate of *E. coli* Rosetta 2 (DE3) cells and Rosetta 2 (DE3) cells overexpressing the GGDEF domain of DmxB. Molecular size marker is indicated. For each gel the corresponding result of DraCALA assay is presented below. It should be noted that there is always a background binding coming from endogenous *E. coli* proteins.

Having those controls established, we decided to test the candidates. For the proteins where we could clearly see overexpression on the SDS-PAGE gels (MXAN5707, MXAN0415, PilT, LonD, MrpC) we used the soluble fraction of the cell lysate. From this list we were able to confirm c-di-GMP binding by two of the candidates: MXAN5707 and MXAN0415 (Figure 57). We considered a result as positive (+) when the signal in the centre of the spot was visibly stronger than for the negative control (Figure 57).

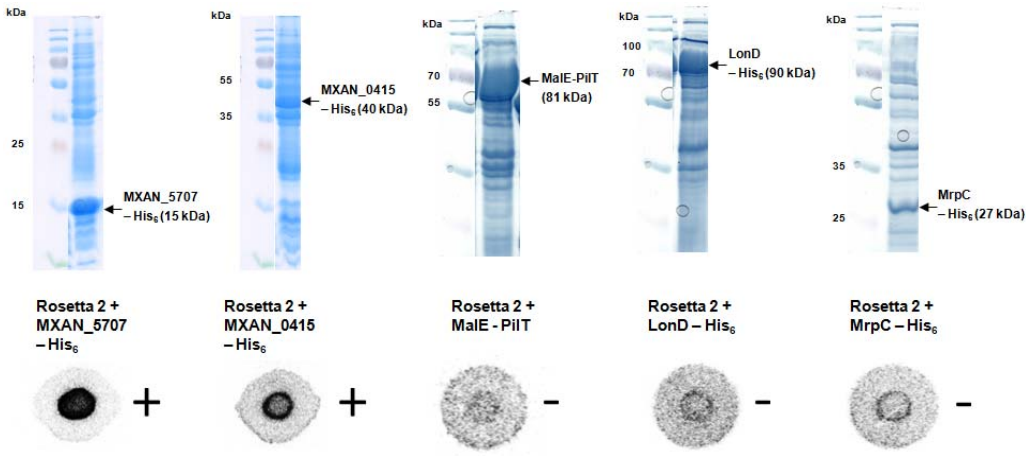


Figure 57. Candidates verification (cell lysate)

SDS-PAGE gels of the soluble fractions from the lysate of *E. coli* Rosetta2 (DE3) cells overexpressing the indicated proteins. Molecular size marker is indicated. For each gel the corresponding result of DraCALA assay is presented below. **(+)** indicates result considered as positive (binding); **(-)** indicates result considered as negative (no binding).

For some of the candidates (MXAN7043, MXAN4361 and MXAN4362) overexpression was not clearly visible on the SDS-PAGE gel. To be sure that the protein amount was high enough to perform the experiment, we decided to first purify those proteins and then test for binding. The result of protein purification as well as DRaCALA assay is shown in Figure 58. We were able to successfully detect binding for MXAN4362 and MXAN4362.

Despite several attempts, we were not able so far to successfully overexpress or purify MXAN6605 protein (data not shown).

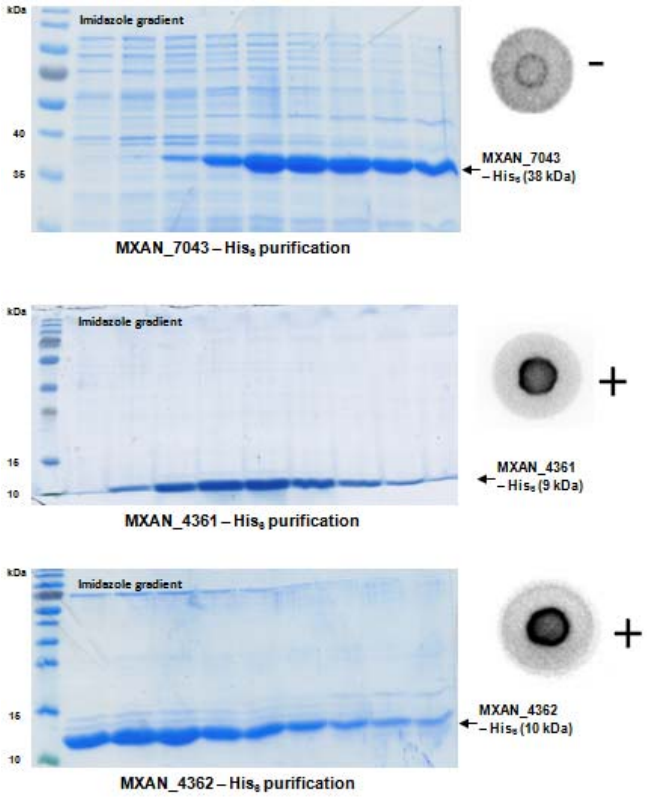


Figure 58. **Candidates verification (purified prioteins)**

SDS-PAGE gels with the purified indicated proteins. Molecular size marker is indicated. For each gel the corresponding result of DraCALA assay is presented below. **(+)** indicates result considered as positive (binding). **(-)** indicates result considered as negative (no binding).

Additionally, we used DRaCALA to test c-di-GMP binding of Nla24 (EpsI). This protein was not detected in the pull down but was promising candidate to be c-di-GMP effector based on our previous results (c-di-GMP in *M. xanthus* regulates EPS accumulation). Nla24/EpsI is encoded by a gene located within the *eps* locus, it is essential for both gliding and T4P-dependent motility systems as well as for EPS accumulation (Lu *et al*., 2005, Lancero *et al*., 2004), and it shares domain structure and 39% amino acid identity with FleQ, a c-di-GMP receptor from *P. aeruginosa*. Despite those similarities, Nla24/EpsI was not able to bind c-di-GMP under the conditions tested (Figure 59).

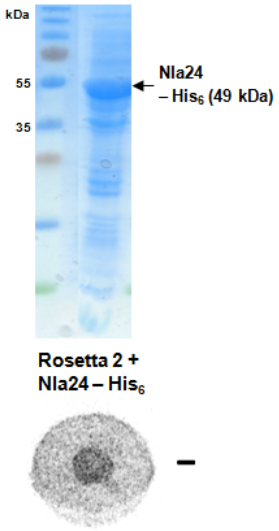


Figure 59. Candidate verification (cell lysate)

SDS-PAGE gels with the soluble fractions from the lysate of *E. coli* Rosetta2 (DE3) cells overexpressing indicated protein. Molecular size marker is indicated.
The corresponding result of DraCALA assay is presented below.
(+) indicates result considered as positive (binding).
(-) indicates result considered as negative (no binding).

2.12.4. Characterization of MXAN0415

MXAN0415 is a paralog of the pilus retraction ATP-ase PilT (Clausen *et al.*, 2009). Interestingly, during analysis of T4P-motility in *M. xanthus*, Clausen *et al*. observed PilT-independent retractions at a low frequency, suggesting the existence of an additional PilT-independent T4P retraction motor(s) (Clausen *et al*., 2009).

Genome analyses of *M. xanthus* revealed the presence of four PilT paralogs (MXAN0415, MXAN1995, MXAN6705 and MXAN6706). Those proteins share sequence identities from 37% to 49% and similarities between 43% and 70% with PilT protein encoded in the *pil* gene cluster (MXAN5787). MXAN0415 shares 43% identity and 65% similarity with PilT (Figure 60). Like PilT, it posses all the conserved residues required for ATP binding and hydrolysis (Figure 60). Interestingly, in the sequence of MXAN0415 we found several motifs described previously to be involved in c-di-GMP binding: RxxD (Christen *et al*., 2006) and RxxxR (Shin *et al*., 2011). Some of them are not conserved within PilT paralog and can be found only in MXAN0415 (Figure 60).

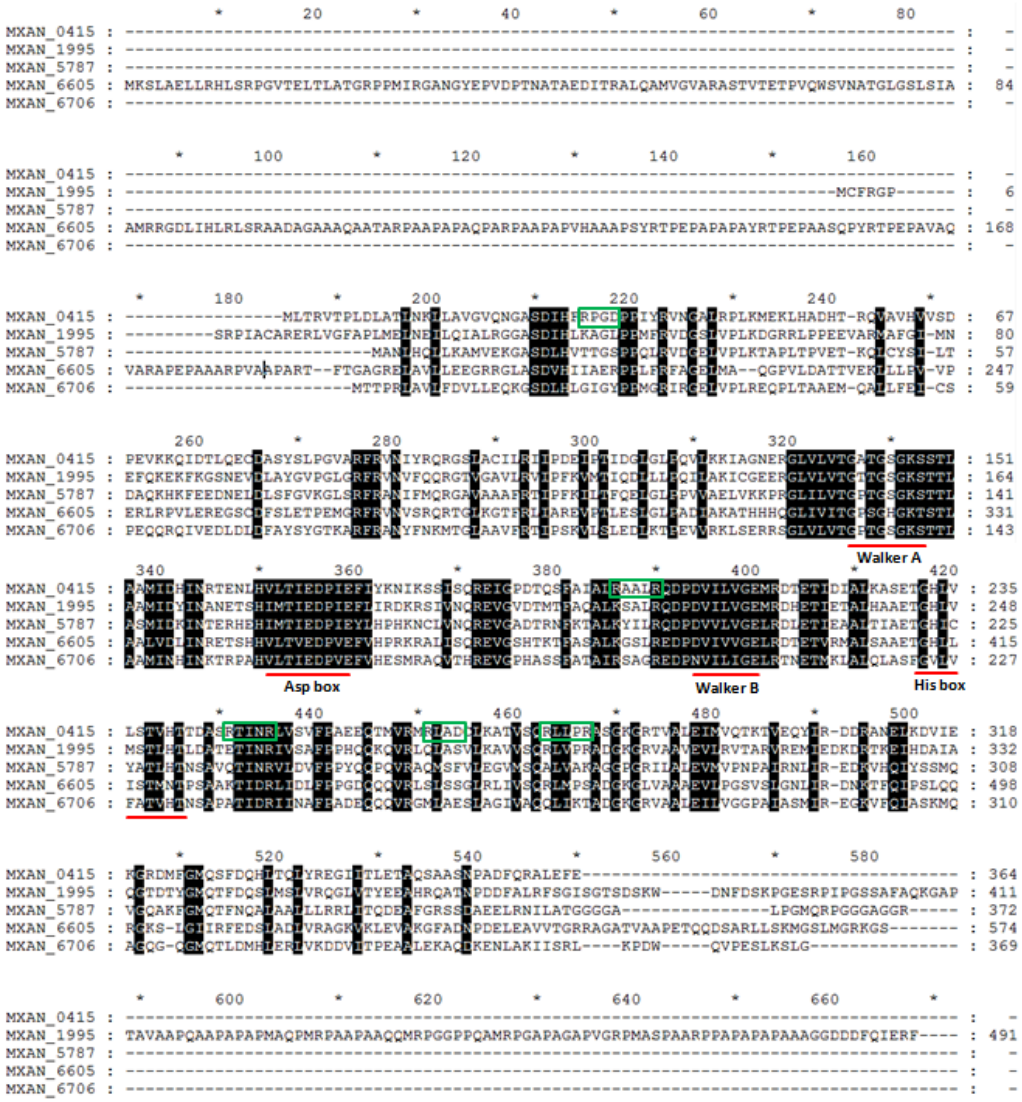


Figure 60. Alignment of amino-acid sequence of full-length PilT paralogs

The conserved Walker a and B motif, Asp and His boxes are indicated in red. Putative c-di-GMP binding motifs in MXAN0415 are indicated in green. White-on-black residues are 100% conserved.

In order to determine the function of MXAN0415 we created an in-frame deletion mutant for *MXAN0415* gene. The deletion mutant had the same growth rate as the WT strain and did not show any defect in terms of motility, fruiting body formation and sporulation (Figure 61). As the double mutant Δ*MXAN0415* Δ*pilT* did not show any additional defect to those observed for Δ*pilT* (Figure 61), the function of MXAN0415 remains unknown.

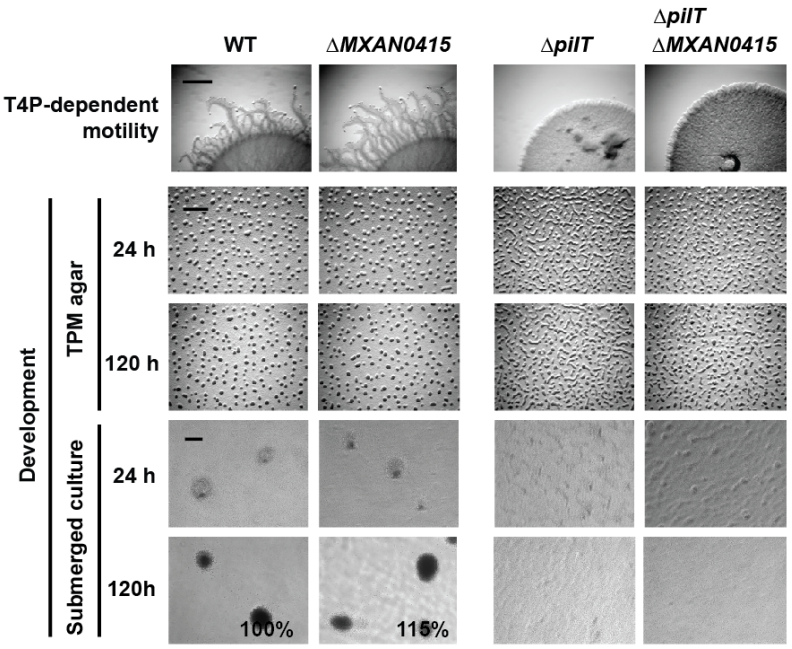


Figure 61. Motility and development assays of Δ*MXAN0415*, Δ*pilT* and Δ*MXAN0415*Δ*pilT* strains

T4P-dependent motility was performed as described in Figure 18. Scale bar: 500 μm. Development was performed as described in Figure 24. Scale bar: TPM agar 500 μm, submerged culture 100 μm.

2.12.5. Characterization of MXAN5707

MXAN5707 is a protein with only PilZ domain identified. Bioinformatic analysis of *M. xanthus* genome predicted the existence of 22 PilZ domain containing proteins, 15 of them with all the residues described to be required for c-di-GMP binding (Table 2). Most of them, like in MXAN5707, function as single domain proteins. Such proteins were reported to be involved mostly in protein-protein interactions (see introduction). In the others, PilZ domain is linked to other regulatory domains. One of them (MXAN1467, Pkn1) has been studied in detail and it has been shown to be a serine-threonine kinase involved in development (Muñoz - Dorado *et al.*, 1991). Nevertheless, the PilZ domain of Pkn1 is degenerated in terms of residues important for c-di-GMP binding (Table 2). This analysis was performed by Dr. Tobias Petters.

Table 2. *M. xanthus* proteins possessing PilZ domain. Two conserved motifs: RxxxR and D/NxSxxG important for c-di-GMP binding are listed

Gene	RxxxR motif	D/NxSxxG motif	Domain structure
MXAN0063	RRNGR	DLSEGG	PilZ
MXAN0614	-	ELSRGG	Pkn-PilZ
MXAN0833	RRFPR	DASLGG	PilZ
MXAN0961	RRGRR	NISNGG	PilZ
MXAN1087	RQHPR	NLSHEG	PilZ-REC
MXAN1467 (*pkn1*)	RLAPA	GLSRGG	Pkn-PilZ
MXAN2528	RQNGR	NISKGG	PilZ
MXAN2649	RHFPR	NVSVSG	PilZ
MXAN3585	RKNKR	DISQEG	PilZ
MXAN3721	RKSTR	NLSEGG	PilZ
MXAN3778	-	NVSRGG	PilZ-DnaK
MXAN3788	PRAPR	NLSKGG	PilZ
MXAN4328	RSHLR	NISARG	PilZ
MXAN4567	RADER	NISAGG	PilZ
MXAN5615	RRFPR	DISRGG	PilZ
MXAN5655	RFHPR	DVSMAG	PilZ
MXAN5707	RDSPR	DLSLGG	PilZ
MXAN5804	-	NVARGG	PilZ-DnaK
MXAN6013	RSDDR	NLSSGG	DnaJ-PilZ
MXAN6605	RTTDR	NLSPGG	PilZ-DnaK
MXAN6957	RVEAR	ALSPGG	PilZ
MXAN7024	RAAER	DAGPGA	REC-REC-PilZ

Sequence alignment of MXAN5707 and Alg44, c-di-GMP binding protein from *P. aeruginosa* harbouring a PilZ domain (Merighi *et al*., 2007) indicates that MXAN5707 possesses both conserved motifs required for c-di-GMP binding, in agreement with the binding detected *in vitro* (Figure 62).

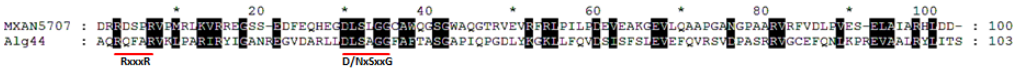


Figure 62. Alignment of amino-acid sequence of PilZ domain from MXAN5707 (*M. xanthus)* and Alg44 (*P. aeruginosa)*

Conserved residues required for c-di-GMP binding are indicated. White-on-black residues are 100% conserved.

In order to determine the function of MXAN5707 we created an in-frame deletion mutant for *MXAN5707* gene. This deletion mutant had the same growth rate as the WT strain and did not show any defect in terms of motility, fruiting body formation and sporulation (Figure 63).

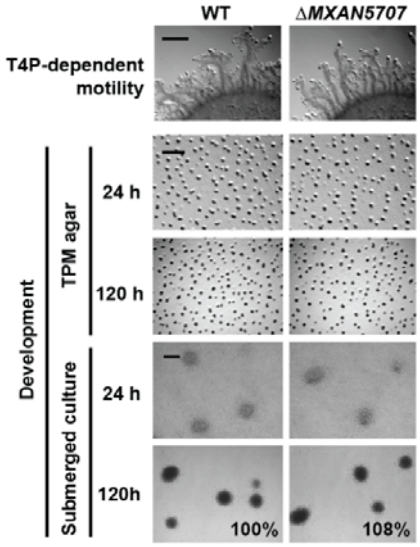


Figure 63. Motility and developmental assay of Δ*MXAN5707* strain

T4P-dependent motility was performed as described in Figure 18. Scale bar: 500 µm. Development was performed as described in Figure 24. Scale bars: TPM agar 500 µm, submerged culture 100 µm.

2.12.6. Characterization of MXAN4361 and MXAN4362

MXAN4361 and MXAN4362 were found in the c-di-GMP capture compound experiment. Genes encoding for those proteins are located next to each other in the genome and the proteins are paralogs. They share 77% of sequence identity and 87% of similarity (Figure 64).

```
                  *         20        *         40        *         60        *         80
MXAN4361 : MAGTDRRKQSLYFPEDMLKEIQREATRQDRSLSWVVQQAWKIAERRIKSPEAVNDVTGDERQDPREE------------------- : 67
MXAN4362 : MATTDRRKQSLYFPEDMLEEIQREATRQDRSLSWIVQQAWKVARGDIRKMESVNDVLSPPPRPAAPAPAPAQPVTAVAVAPSEEPK : 86
```

Figure 64. Alignment of amino-acid sequence of full-length MXAN4361 and MXAN4362

White-on-black residues are 100% conserved.

Those proteins are conserved and encoded next to each other in the genomes of closely related fruiting Myxobacteria (*M. xanthus*, *M. fulvus, M. stipitatus*, *C. coralloides*, *S. aurantiaca*) but they do not have close orthologs within other organisms. Both proteins belong to the ribbon-helix-helix (RHH) Pfam protein family (Punta *et al.*, 2012). Those proteins usually function as transcriptional regulators (Schreiter & Drennan, 2007). There is an interesting RHH family member from *Pseudomonas* (AmrZ – previously AlgZ). AmrZ has been implicated in the transcriptional regulation that involves c-di-GMP dependent phenotypes such an alginate synthesis, polysaccharide Psl production, twitching motility and pili biogenesis, flagella mediated motility (Martinez-Granero *et al.*, 2014) although it was never shown to bind c-di-GMP directly.

Our attempts to create single or double in frame deletion mutants were so far unsuccessful.

3. Discussion

3.1. c-di-GMP in regulation of T4P-motility in *M. xanthus*

Here, we show that the second messenger c-di-GMP is a regulator of T4P-dependent motility in *M. xanthus*. c-di-GMP accumulates in growing *M. xanthus* cells and its level stays stationary during all the growth phases. Our conclusion is based on following lines of evidence. First, expression of a heterologous DGC (DgcA) or a heterologous PDE (PA52959) but not the corresponding variants with substitutions in the active sites allowed the manipulation of the c-di-GMP level in growing cells. In these cells, an increase as well as a decrease in the c-di-GMP level caused defects in T4P-dependent motility without affecting the gliding motility. Because enzymatically inactive variants of DgcA and PA5295 did not interfere with T4P-dependent motility, these effects are caused by changes in the c-di-GMP level. Second, in an approach in which all 24 genes potentially encoding active DGCs or PDEs in *M. xanthus* were systematically inactivated, it was observed that lack of the active DGC DmxA caused a defect in T4P-dependent motility.

T4P-dependent motility in *M. xanthus* depends on T4P and EPS. A ~7-fold increase in the c-di-GMP level in otherwise WT cells caused by expression of the heterologous $DgcA^{WT}$ resulted in a significant reduction in transcription of the *pilA* gene (which codes for the major pilin), in PilA accumulation and in assembled T4P. It has previously been shown that there is a correlation between cell-cell cohesion and T4P-dependent motility in *M. xanthus* and that this cohesion requires T4P and EPS (Arnold & Shimkets, 1988, Shimkets, 1986b, Shimkets, 1986a). Consistent with the significantly reduced formation of T4P in $DgcA^{WT}$ expressing cells, they displayed delayed agglutination. Elevated c-di-GMP levels are often associated with increased EPS synthesis (Römling *et al*., 2013, Hengge, 2009, Jenal & Malone, 2006, Krasteva *et al*., 2012, Boyd & O'Toole, 2012). However, the $DgcA^{WT}$ expressing cells did not exhibit differences in EPS accumulation in comparison to the WT. In *M. xanthus*, assembled T4P have been suggested to function upstream of the Dif chemosensory system to stimulate EPS accumulation (Black *et al*., 2006) whereas lack of EPS does not affect T4P assembly (Yang *et al*., 2000).

Taken together, these observations suggest, that the primary defect caused by a highly increased c-di-GMP level in otherwise WT cells during growth is reduced *pilA* transcription leading to reduced PilA accumulation and as a result reduced T4P formation. Increased c-di-GMP level may stimulate EPS accumulation in *M. xanthus* but possibly this effect is confounded by the lack of assembled T4P in $DgcA^{WT}$ expressing cells. Previously, *pilA* expression has been shown to depend on the

transcriptional regulator PilR, which is an NtrC-like transcription regulator (Wu & Kaiser, 1997). How c-di-GMP regulates *pilA* transcription remains to be shown; however, it is interesting to note that NtrC-like transcriptional regulators have been identified as c-di-GMP effectors (Srivastava *et al*., 2011, Hickman & Harwood, 2008).

A ~2-fold reduction during growth in the c-di-GMP level by expression of the heterologous $PA5295^{WT}$ in otherwise WT cells did not cause significant differences in PilA accumulation, T4P assembly or EPS accumulation. Nevertheless, these cells had reduced T4P-dependent motility. Interestingly, the cells displayed delayed agglutination. This suggests that a reduced c-di-GMP level results in changes in cell surface properties that are neither reflected in the level of assembled T4P nor in the level of EPS accumulation. We speculate that these changes in cell surface properties cause the defect in T4P-dependent motility.

Manipulation of the c-di-GMP level in otherwise WT cells proved, that the c-di-GMP level is important for T4P-dependent motility. By systematically inactivating the 24 genes encoding GGDEF, EAL or HD-GYP domain containing proteins, we identified three such proteins involved in T4P-dependent motility.

TmoK is a hybrid histidine protein kinase with a catalytically inactive C-terminal GGDEF domain that based on sequence analysis and experimental data using the DRaCALA assay does not bind c-di-GMP. Lack of TmoK caused a subtle defect in T4P-dependent motility but did not affect the level of c-di-GMP and T4P while EPS accumulation was increased and cells agglutinated faster than WT. Because the c-di-GMP level was unchanged in the Δ*tmoK* mutant, the effects of lack of TmoK on EPS, T4P and agglutination are likely independent of c-di-GMP and connected with its kinase activity.

SgmT is a hybrid histidine protein kinase with a catalytically inactive C-terminal GGDEF domain that binds c-di-GMP (Petters *et al*., 2012) and DmxA contains a catalytically active GGDEF domain that also binds c-di-GMP. Unexpectedly, lack of either SgmT or DmxA caused a slight but significant ~1.5-fold increase in the c-di-GMP levels. Lack of SgmT or DmxA also caused a 3-4-fold increase in EPS accumulation, had no or only a small effect on T4P assembly, and also altered cell-cell cohesion as measured in the agglutination assay. Thus, the increased c-di-GMP levels in otherwise WT cells caused by expression of $DgcA^{WT}$ and in cells lacking SgmT or DmxA correlate with reduced T4P-dependent motility. Clearly, however, the underlying mechanisms leading to this defect are different, i.e. in the $DgcA^{WT}$ expressing cells our data suggest that this defect is caused by lack of T4P and in *dmxA* and Δ*sgmT* cells this defect is caused by increased EPS accumulation.

EPS biosynthesis depends on proteins encoded by the *eps* locus (Lu *et al*., 2005); however, little is known about the function of the corresponding proteins. Also little is known about the function of the several regulators of EPS synthesis that have been identified, including the Dif chemosensory system, T4P, NtrC-like transcription regulators, the MasK tyrosine protein kinase, the FrzS response regulator and DnaK homologs (Yang *et al*., 2000, Caberoy *et al*., 2003, Berleman *et al*., 2011, Thomasson *et al*., 2002, Lancero *et al*., 2004, Overgaard *et al*., 2006, Petters *et al*., 2012, Weimer *et al*., 1998, Dana & Shimkets, 1993, Lu *et al*., 2005, Lancero *et al*., 2005) (Figure 65).

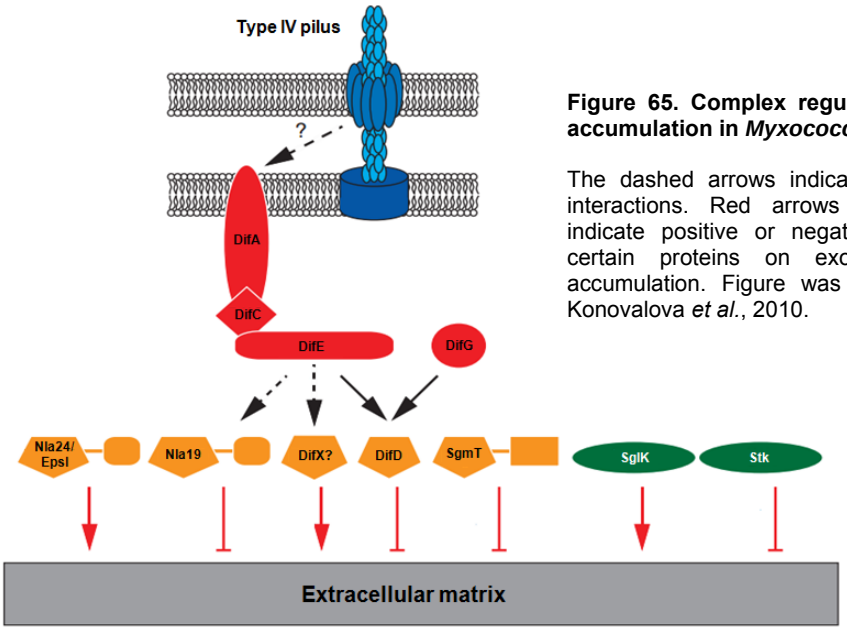


Figure 65. Complex regulation of ECM accumulation in *Myxococcus xanthus*

The dashed arrows indicate hyphotetical interactions. Red arrows or inhibitions indicate positive or negative effects on certain proteins on exopolysaccharide accumulation. Figure was modified from Konovalova *et al*., 2010.

Nla19 belongs to NtrC-like transcriptional regulators which have been shown to be c-di-GMP effectors (Krasteva *et al*., 2010, Srivastava *et al*., 2011, Hickman & Harwood, 2008). Thus, it remains a possibility that c-di-GMP may directly regulate the activity of one or more of the regulators previously identified as important for correct EPS accumulation.

It is an open question how c-di-GMP produced by different DGCs can elicit different responses. In one model for c-di-GMP-dependent regulation, distinct signaling systems with dedicated functions regulate c-di-GMP levels in highly localized and insulated pools rather than contributing to a shared cellular pool of c-di-GMP (Hengge, 2009). Based on the observation that the mechanism(s) underlying the defects in T4P-dependent motility in the *dmxA* and Δ*sgmT* mutants are different from those in DgcA^{WT} expressing cells, we suggest that DmxA and SgmT are embedded in signaling systems that contribute to local c-di-GMP pools. Because SgmT does not have DGC activity

and DmxA does, the higher c-di-GMP levels in the mutants lacking one or the other of these two proteins is not simply caused by the lack of either protein but likely involve indirect effect(s) on other DGCs or PDEs. We previously showed that SgmT is sequestered in one or more clusters localized along the cell length in a manner that depends on c-di-GMP binding by the GGDEF domain and suggested that catalytically active DGC(s) are present in these clusters and would function to sequester SgmT (Petters *et al*., 2012). Thus, in the case of the Δ*sgmT* mutant, it is possible that lack of SgmT may cause an increase in the activity of this hypothetical DGC(s). In several other bacteria low c-di-GMP levels are associated with reduced EPS accumulation and high levels with increased EPS accumulation (Römling *et al*., 2013) as reported here for the *dmxA* and Δ*sgmT* mutants. Also, c-di-GMP-dependent inhibition of motility is commonly observed. A well-understood example involves the PilZ domain protein YcgR in *E. coli* and *S. enterica*, which upon c-di-GMP binding interacts with the flagella basal body to interfere with flagella rotation (Boehm *et al*., 2010). The *M. xanthus* genome encodes at least 15 PilZ domain proteins but their function is unknown. c-di-GMP has also been reported to regulate gliding motility in *B. bacteriovorus* (Hobley *et al*., 2012). Finally, c-di-GMP has been implicated in regulation of T4P-dependent motility in *P. aeruginosa* and *X. campestris* by binding to the catalytically inactive EAL domain of the FimX protein that stimulates T4P assembly (Kazmierczak *et al*., 2006, Navarro *et al*., 2009, Guzzo *et al*., 2013, Guzzo *et al*., 2009). In *X. campestris* FimX interacts with a PilZ domain protein that in turn interacts with the PilB ATPase that is required for T4P assembly (Guzzo *et al*., 2013, Guzzo *et al*., 2009). The *M. xanthus* genome does not encode a FimX homolog. We have reported here that high levels of c-di-GMP inhibit *pilA* transcription, thus, introducing a novel mechanism for how c-di-GMP may regulate T4P-dependent motility.

3.2. c-di-GMP role during development in *M. xanthus*

In this study, we have shown for the first time that the second messenger c-di-GMP is a regulator of starvation-induced development in *M. xanthus*. In response to starvation, c-di-GMP level increased in a time dependent manner ~4-fold in WT cells. In otherwise WT cells, further increase of c-di-GMP level by expression of $DgcA^{WT}$ neither interfered with aggregation of cells to form fruiting bodies nor with sporulation, whereas reducing the c-di-GMP level by expression of $PA5295^{WT}$ caused defects in aggregation as well as in sporulation. These data document that c-di-GMP level is important for aggregation into fruiting bodies and sporulation and suggest a mechanism in which a threshold level of c-di-GMP is essential for these two processes to occur.

We identified two catalytically active proteins that are specifically required for aggregation into fruiting bodies and sporulation: DmxB and PmxA. DmxB has DGC activity and binds c-di-GMP via its I-site and PmxA has PDE activity.

Inactivation of PmxA did not have an effect on c-di-GMP level during starvation, nevertheless it is an active phosphodiesterase *in vitro* and mutation in the conserved HD-GYP domain abolished protein function *in vivo*. This suggests that this protein does not contribute to the global cellular pool of c-di-GMP but rather acts locally and its precise function remains to be identified. Along these lines, the HD-GYP domain protein RpfG from *X. campestris* was found to interact directly with several GGDEF domain proteins. This interaction was independent on phosphodiesterase activity of RpfG and diguanylate cyclase activity of GGDEF domain proteins. These results suggest that c-di-GMP signalling occurs in “microcompartments” - multiprotein complexes that contain a specific DGC and/or PDE as well as specific effector and target components, which associate by specific protein–protein or protein–DNA interactions (Ryan *et al*., 2010, Hengge, 2009, Ryan *et al*., 2012a).

In case of DmxB, our data demonstrate that this protein is a developmentally induced DGC responsible for increasing c-di-GMP level upon nutrient limitation. DmxB and c-di-GMP stimulate transcription of a subset of *eps* genes that encode enzymes involved in EPS metabolism, and by increasing EPS accumulation allows cells to aggregate to form fruiting bodies.

c-di-GMP has been shown to be important in response to the level of nutrients. *E. coli* cells swim exploring the environment and the average swimming speed decreases when cells enter stationary phase. Swimming is powered by the rotary flagellar motor. It has been shown that during exponential growth PDE activity is favoured, keeping the c-di-GMP level low. In contrast during starvation, the level of c-di-GMP increases activating the receptor protein YcgR, which in turn binds directly to the motor and slows it down (Boehm *et al*., 2010) (Figure 66). *M. smegmatis* possesses two genes encoding GGDEF–EAL (MSDGC-1) and GGDEF (MSDGC-2) domain proteins but only MSDGC-1 was shown to be active *in vitro*. MSDGC-1 is a bifunctional protein and its inactivation, resulting in c-di-GMP-null strain, affects long-term survival under nutrient starvation. The authors suggested that increased c-di-GMP level is required in the *M. smegmatis* stationary phase under nutrient-depleted conditions (Bharati *et al*., 2012). These examples show that increasing the level of c-di-GMP may be a widespread response to stress and nutrients limitation, although the mechanism of this regulation differs between species.

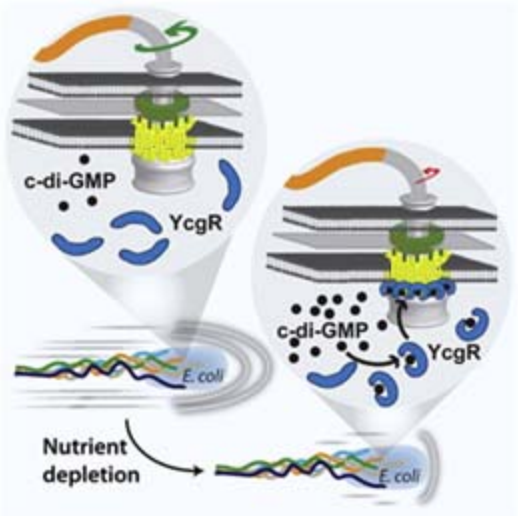


Figure 66. Model for c-di-GMP-mediated regulation of swimming velocity in *E. coli*

E. coli can fine-tune its swimming speed with the help of a molecular brake (YcgR) that, upon binding of cyclic di-GMP, interacts with the motor protein to interfere with flagellar motor function. Activation of this network is connected to nutrient depletion and might represent an adaptation to starvation. Figure was modified from Boehm *et al.*, 2010.

Most bacterial genomes encode DGCs and PDEs, but the numbers vary dramatically (Römling *et al.*, 2013). The presence of large numbers of enzymes that synthesize or degrade c-di-GMP raises the question how these enzymes are regulated to obtain specific output responses. It has been suggested that specificity in c-di-GMP signaling could be obtained by temporal and/or spatial sequestration of these proteins or by effectors having different binding affinities (Hengge, 2009). Temporal sequestration would ensure that specific proteins are only available under certain conditions, eliminating unwanted cross-talk between signaling modules; spatial sequestration sequester partner proteins to distinct subcellular locations where they would contribute to making and breaking local pools of c-di-GMP that would only be available to the relevant partner proteins. We observed that DmxB can, at least partially, be functionally replaced by a heterologous DGC. Because it is unlikely that this DGC would be able to replace protein-protein interactions involving DmxB, these data suggest that DmxB may contribute to a global cellular pool of c-di-GMP. Consistently, preliminary data have provided no evidence that DmxB localizes to a particular subcellular location. However, simply increasing the level of c-di-GMP in vegetative cells is not sufficient to initiate fruiting body formation. Starvation signals are still required, suggesting that those signals might regulate accumulation or the activity of a specific effector.

In total, our data are consistent with a model in which a threshold of c-di-GMP is essential for the proper progression of the developmental program in *M. xanthus*. In WT cells, this threshold level of c-di-GMP is generated by DmxB. c-di-GMP in excess does not interfere with development and once the threshold level has been reached, cells can form fruiting bodies; however, lack of c-di-GMP blocks the developmental program. Interestingly, a DmxB variant with a mutated I-site accumulating ~10-fold

more c-di-GMP than the WT strain developed normally, suggesting that an allosteric feedback inhibition of DGC activity by DmxB is not essential. We speculate that this feedback minimizes “wasteful” c-di-GMP synthesis during starvation.

Among the 17 GGDEF domain proteins in *M. xanthus*, 11 are predicted to have DGC activity. We could show that DmxA has DGC activity and is involved in regulating EPS accumulation in vegetative cells. Lack of DmxA causes a slight but significant increase in the level of c-di-GMP and a 4-fold increase in EPS accumulation and in that way also a defect in T4P-dependent motility. Our finding that DmxB is exclusively synthesized in developing cells provide evidence that *M. xanthus* deploys functionally distinct DGCs at different stages of its life cycle. Similarly, it was recently demonstrated that *B. bacterivorus* uses different DGCs at different stages of its predatory life cycle (Hobley *et al*., 2012).

There are only a few examples documenting the role of c-di-GMP in cell cycle control and multicellular development. In *C. crescentus,* phosphotransfer signaling integrates with c-di-GMP signaling to regulate cell cycle progression and cell differentiation (Aldridge *et al.*, 2003, Paul *et al.*, 2004, Curtis & Brun, 2010, Abel *et al.*, 2011). High c-di-GMP level is important for the transition from a swarmer cell to a stalked cell, whereas low c-di-GMP levels seem to be required for the correct development of swarmer cells. Altogether, a body of evidence suggests that c-di-GMP is asymmetrically distributed between the dividing swarmer cell and stalked cell (Aldridge *et al*., 2003, Paul *et al*., 2004, Christen *et al*., 2010).

In *S. venezuelae*, c-di-GMP also regulates development, specifically the formation of aerial hyphae. However, in this organism, a high level of c-di-GMP inhibits development by binding to the master regulator BldD, which inhibits expression of sporulation genes, while a decrease level of c-di-GMP stimulates development (Tschowri *et al*., 2014) (Figure 67). Since low level of c-di-GMP in *M. xanthus* inhibits progression of development*,* this observation suggests that c-di-GMP has opposite effects on development in *S. venezuelae* and *M. xanthus*.

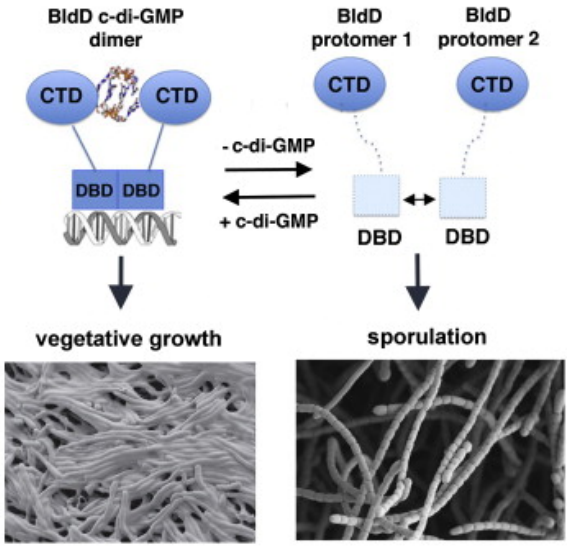


Figure 67. Schematic model of c-di-GMP-activated DNA Binding by BldD and its influence on *S. venezuelae* development

CTD - C-terminal domain of BldD
DBD – DNA binding domain of BldD
The DBDs interact only weakly *in vivo* (indicated by the double-headed arrow). Increased c-di-GMP levels lead to CTD dimerization, resulting in a significant increase in the local concentration of the DBDs, allowing them to dimerize in the presence of cognate DNA to effect high affinity DNA binding. This leads to repression of the BldD regulon and blocks multicellular differentiation. Figure was reproduced from Tschowri *et al*., 2014.

In *Dictyostelium discoideum* (the only eukaryote where c-di-GMP has been identified) the DGC DgcA produces c-di-GMP and is responsible for stalk cell differentiation during fruiting body formation. Lack of DgcA blocks the transition from slug migration to fruiting body formation, as well as the expression of stalk specific genes. Intriguingly, development and stalk formation is restored by adding c-di-GMP to a *dgcA* mutant (Chen & Schaap, 2012). In this context, it should be noted that we have found no evidence that addition of c-di-GMP to a Δ*dmxB* mutant restores development.

Our data suggest that c-di-GMP regulates *eps* gene transcription during development in *M. xanthus*. c-di-GMP is known from the literature to affect transcription of genes involved in accumulation of ECM components. The c-di-GMP receptor FleQ in *P. aeruginosa* controls expression of not only the flagellar regulon but also the polysaccharide *pel* and *psl* biosynthesis genes (Hickman & Harwood, 2008). In *V. cholerae* the transcriptional regulators VpsR and VpsT, both of which bind c-di-GMP, activate *vps* (*Vibrio* polysaccharide) gene expression (Shikuma *et al.*, 2012). In *E. coli,* a set of c-di-GMP metabolizing proteins regulates expression of the CsgD response regulator, which is a key transcription factor controlling expression of the *csgBAC* operon, encoding the structural subunits of curli fimbriae and expression of *adrA*,

a putative regulatory gene required for cellulose synthesis (Sommerfeldt *et al*., 2009, Chirwa & Herrington, 2003). In this context it has to be mentioned that EPS composition and metabolism in *M. xanthus* are not well studied so the precise functions of *epsA* and *epsB* genes need to be further investigated. *eps* gene cluster described in this study is not the only cluster in *M. xanthus* genome encoding for proteins involved in EPS accumulation. Thus, it is highly probable that c-di-GMP regulates also other *eps* genes.

Interestingly, it was previously reported that aggregating cells accumulate more EPS than non-aggregating cells (Lee *et al*., 2012a) raising the possibility that DmxB may not accumulate in all starving cells or, alternatively, not be active in all starving cells. Future experiments will be aimed for addressing these questions. Several proteins have been described to accumulate differentially in the aggregating and non-aggregating cells during development of *M. xanthus*. The zinc metalloprotease FibA is specific for the aggregated cell fraction (Lee *et al*., 2012a) while protein S (a spore coat protein) is produced in both non-aggregated and aggregated fractions of the population but by 48 h is upregulated at least 2-fold in the aggregated fraction (O'Connor & Zusman, 1991b). Also developmental regulator proteins FruA and MrpC display distinct accumulation patterns in the aggregating and non-aggregating fractions (Lee *et al*., 2012a).

Interestingly, we did not observe that phosphorylation of the receiver domain is required for DmxB function. It was reported that DGCs can be activated by dimerization mediated by phosporylation of the receiver domain. The best-studied examples of this type of regulation are PleD from *C. crescentus* and WspR from *P. aeruginosa* (Paul *et al*., 2007, Hickman *et al*., 2005). However, while the DmxB variant which mimics the phosphorylated state is active *in vitro* and *in vivo* in *E. coli*, it is not active *in vivo* in *M. xanthus*. We speculate, that phosphorylation results in DmxB interacting with other proteins in *M. xanthus* cells and this interaction inhibits DmxB activity. Nevertheless, this hypothesis needs to be further tested by identification of proteins potentially interacting with DmxB. As a starting point, we tested, if the response regulator MXAN3734 encoded next to *dmxB* is involved in development, but this was not the case. Additionally, it would be also interesting to identify the partner kinase that phosphorylates DmxB receiver domain.

We found that DmxB accumulation was significantly affected in all the strains tested that had an early developmental defects (*difE,* Δ*lonD, asgA*), and normal in a mutant affected at the later stage of development (*fruA*). We conclude that in *M. xanthus* c-di-GMP signaling is connected with intercellular signaling pathways required for fruiting body formation and sporulation.

3.3. c-di-GMP effectors *in M. xanthus*

An important question remains open and concerns c-di-GMP effectors in *M. xanthus*. In contrast to c-di-GMP metabolizing proteins, their identification is very challenging due to the high diversity and limited tools for their identification. As part of this work, we aimed to initially identify and characterize c-di-GMP receptors using a c-di-GMP capture compound approach.

Capture Compound Mass Spectrometry (CCMS) is a powerful tool to identify new effectors nevertheless there are several limitations of this technique. The important parameter is the required high protein concentration thus, the cell pellet resuspension should be performed in a low volume of lysis buffer. This is difficult to perform in case of *M. xanthus* cells that possess high amounts of EPS and are difficult to resuspend at the high density. Additionally, during starvation many cells lyse significantly decreasing the protein content and the sample quality. The critical factors are also a clean keratin-free environment, proper negative controls and biological replicates.

As this was the first trial to apply the CCMS technology in *M. xanthus*, the list of candidates obtained was likely not complete and included false positives as well as negatives. Nevertheless, the experiment allowed us to identify several candidates that, based on *in vitro* DRaCALA binding assay are real c-di-GMP binding proteins: MXAN5707, MXAN0415, MXAN4361 and MXAN4362.

We observed that most of the c-di-GMP metabolizing proteins in *M. xanthus* do not show distinct phenotypes upon inactivation. This could be due to high redundancy in c-di-GMP network where proteins with the same functions can compensate for the absence of each other. It is also possible that similar redundancy exists in case of c-di-GMP specific effectors as the deletion of *MXAN5707* or *MXAN0415* did not have any effect on growth, cellular motility or fruiting body formation. This hypothesis is additionally supported by bioinformatic analysis suggesting that MXAN5707 is one of 22 PilZ domain proteins encoded in the *M. xanthus* genome while MXAN0415 is one of five PilT paralogs. Also MXAN4361 and MXAN4362 are paralogs and we suspect that in order to determine their function we would need to inactivate both of them at the same time. We were so far unsuccessful in creating the in-frame deletions mutant for those genes and we suspect that the deletion construct affects the promoter region of the essential genes in the neighbourhood *MXAN4360* (*tilS* – described as essential in *B. subtilis* (Fabret *et al.*, 2011)) and *MXAN4359* (*ftsH* – described as essential in *M. xanthus* (Konovalova *et al.*, 2012)).

Additionally, we investigated by using DRaCALA the c-di-GMP binding of sigma-54 dependent DNA-binding response regulator Nla24 which is located within eps gene

cluster *(epsI).* Nla24 was shown to be important for both motility systems and EPS accumulation and to regulate the expression of *epsY* and A-motility genes *aglU and cglB* (Lancero *et al*., 2004). Although Nla24 shares domain structure and 39% amino acids identity with FleQ (a c-di-GMP receptor from *P. aeruginosa*), we were not able to detect binding under the conditions tested. Also, a PilZ domain in *M. xanthus* can not be recognized within proteins of *eps* locus or within the known proteins involved in EPS metabolism. Future effort will be crucial to identify more of the c-di-GMP effectors in *M. xanthus* during both vegetative growth and development and to investigate their function in motility, fruiting body formation and possibly other cellular processes.

3.4. Future perspectives

The data presented here provide evidence that c-di-GMP is involved in the regulation of T4P-dependent motility as well as development in *M. xanthus*. While identification of the five proteins (among 24 predicted to be involved in c-di-GMP metabolism) important for both of those processes gives a basic insight into their c-di-GMP dependent regulation in *M. xanthus*, it also raises questions about the function of the remaining 19 proteins. Most of these proteins are conserved in related fruiting Myxobacteria suggesting strong selective pressure to maintain these genes. Yet, no phenotypic differences were evident between WT and any of the 19 mutants suggesting that these 19 proteins are not active under the conditions tested or have partially redundant functions. To be able to determine their role we would like to systematically delete all of them creating a c-di-GMP-null strain. This would allow us to answer the question which proteins are involved in maintaining the basic c-di-GMP pools during vegetative growth.

As we were not able so far to assign a role for PmxA during development, we could like to study this protein in more details. It would be especially interesting to investigate if PmxA accumulation and gene transcription is also developmentally regulated. As we suggest that this protein contributes to maintain some local c-di-GMP pool, we would aim as well to investigate its cellular localization.

Also, the screening performed so far did not allow us to identify the specific receptor responsible for c-di-GMP influence on vegetative growth and *pilA* transcription as well as on development and transcriptional regulation of *eps* cluster. We believe that improved capture compound based pull-down in combination with method based on functionalized 2′-aminohexylcarbamoyl-c-di-GMP (2′-AHC-c-di-GMP) covalently coupled to sepharose beads (Duvel *et al.*, 2012), could help us to identify more candidates for c-di-GMP specific effectors in *M. xanthus*.

4. Materials and Methods

4.1. Chemicals and equipment

All the reagents, enzymes, antibiotics and kits used in this work are listed together with their supplier in the Table 3. All the devices, their application and manufacturer as well as software used for data analysis are listed in Table 4.

Table 3. Reagents, enzymes, antibiotics and kits

Reagents	**Supplier**
Chemicals	Roth (Karlsruhe), Merck (Darmstadt), Sigma-Aldrich (Taufkirchen)
Media components, agar	Roth (Karlsruhe), Merck (Darmstadt), Difco (Heidelberg), Invitrogen (Darmstadt)
2-log DNA Ladder	New England Biolabs (NEB) (Frankfurt a. M.)
Oligonucleotides	Eurofins MWG Operon (Ebersberg), Invitrogen (Karlsruhe)
Rabbit antisera	Eurogentec (Seraing, Belgium)
Goat anti-rabbit IgG, goat anti-rabbit IgG DyLight 549	Pierce/Thermo Scientific (Dreieich)
Luminata Western HRP Substrate	Merck Millipore (Darmstadt)
PageRuler Plus Prestained Protein Ladder	Thermo Scientific (Dreieich)
2-log DNA Ladder	New England Biolabs (NEB) (Frankfurt a. M.)
Enzymes	
Antarctic Phosphatase	New England Biolabs (Frankfurt a. M.)
Phusion High-Fidelity DNA Polymerase	Thermo Scientific (Dreieich)
T4 DNA Ligase	Fermentas (St. Leon-Rot)
5 PRIME MasterMix	5 PRIME GmbH (Hamburg)
restriction enzymes	Fermentas (St. Leon-Rot), New England Biolabs (Frankfurt a. M.)
SYBR Green PCR Master Mix	Applied Biosystems (Darmstadt)
Calf intestine alkaline phosphatase	Fermentas (St. Leon-Rot)

Antibiotics

kanamycin sulfate, chloramphenicol, ampicillin sodiumsulfate, gentamycin sulfate, oxytetracycline dehydrate, tetracycline hydrochloride	Roth (Karlsruhe)
Kits	
DNA purification (chromosomal DNA)	Epicentre Biotechnologies (Wisconsin,USA)
DNA purification (plasmid DNA), PCR purification, Gel purification	Zymo Research (Freiburg), Qiagen (Hilden), Macherey-Nagel (Düren)
cDNA Archive kit	Applied Biosystems (Darmstadt)
RNA purification	RNeasy kit (Qiagen)
c-di-GMP caproKit	Caprotec Bioanalytics GmbH (Berlin)

Table 4. Equipment

Application	**Device**	**Manufacturer**
Cell disruption	Branson Sonifier 250, French pressure cell press	G. Heinemann (Schwäbisch Gmünd) SLM instruments (Urbana, IL)
Centrifugation	RC 5B plus, Ultra Pro 80, Multifuge 1 S-R, Biofuge fresco, Biofuge pico, Avanti J-26 XP, Optima L-90K, Centrifuge 5424 R	Sorvall/Thermo Scientific (Dreieich), Heraeus/Thermo Scientific (Dreieich), Beckman Coulter (Krefeld), Eppendorf (Hamburg)
PCR	Mastercycler personal, Mastercycler epgradient	Eppendorf (Hamburg)
Thermomixer	Thermomixer compact	Eppendorf (Hamburg)
DNA illumination and documentation	E-BOX VX2 imaging system	PeqLab (Eberhardzell)
DNA illumination	UVT_20 LE	Herolab (Wiesloch)
Electroporation	GenePulser Xcell	Bio-Rad (München)
Protein electrophoresis	Mini-PROTEAN® 3 cell	Bio-Rad (München)
Western blotting	TransBlot®Turbo™ Transfer System	Bio-Rad (München)
Chemiluminescence detection	Luminescent image analyzer LAS-4000	Fujifilm (Düsseldorf)
Microscopes	MZ75 Stereomicroscope DM IRE2 Inverted microscope EM 301 Electron microscope	Leica Microsystems (Wetzlar), Philips (Eindhoven, The Netherlands)

Application	Device	Manufacturer
Determination of optical densities or nucleic acids absorption	Ultrospec 2100 pro Spectrophotometer Nanodrop ND-1000 UV-Vis spectrophotometer	Amersham Biosciences (München) Nanodrop (Wilmington)
Identification of proteins	4800 plus MALDI TOF/TOF Analyzer	Applied Biosystems (Darmstadt)
UV crosslinking	CaproBox	Caprotec Bioanalytics GmbH (Berlin)
Checking sequences, sequence alignments	Vector NTI advance software, suite 11	Invitrogen (Darmstadt)

4.2. Media

E. coli cells were cultivated in Luria-Bertani (LB) liquid media or on LB agar plates with 1.5% agar concentration. *M. xanthus* cells were cultivated in CTT media or on CTT agar plates with 1.5% agar concentration. Motility assays of *M. xanthus* cells were performed on A- or S-motility plates. Media composition is described in Table 5.

Table 5. Growth media for *E. coli* and *M. xanthus*

Media	Composition
E. coli	
LB medium	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl
LB agar plates	LB medium, 1.5% (w/v) agar
M. xanthus	
CTT	1% (w/v) Bacto casitone, 10 mM Tris-HCl pH 8.0, 1 mM potassium phosphate buffer pH 7.6, 8 mM $MgSO_4$
CTT agar plates	CTT medium, 1.5% agar
CTT soft agar	CTT medium, 0.5% agar
Motility assays	
A-motility plates (Hodgkin & Kaiser, 1977)	0.5% CTT, 1.5% agar
S-motility plates (Hodgkin & Kaiser, 1977)	0.5% CTT, 0.5% agar

Developmental assays

TPM-agar (Kuner & Kaiser, 1982)	10 mM Tris-HCl, pH 7.6, 1 mM KH_2PO_4, pH 7.6, 8 mM $MgSO_4$, 1.5% agar
CF-agar (Shimkets & Kaiser, 1982)	10 mM Tris- HCl, pH 8.0, 1 mM KH_2PO_4, pH 7.6, 8 mM $MgSO_4$, 0.02% $(NH_4)_2SO_4$, 0.1% NaPyruvate, 0.2% NaCitrate, 1.5% agar
MC7 buffer	10 mM MOPS, pH 7.0, 1 mM $CaCl_2$

Table 6. Media for recombinant protein expression in *E. coli*

Media	**Composition**
ZY	10 g tryptone, 5 g yeast extract, 925 ml H_2O
NPS buffer (20x)	0.5 M $(NH_4)_2SO_4$, 1 M KH_2PO_4, 1 M Na_2HPO_4, H_2O to 1 liter, pH 6.75
5052 solution (50x)	250 g glycerol, 25 g glucose, 100 g lactose, H_2O to 1 liter
ZYP-5052 autoinduction medium	929 ml ZY, 1 ml 1 M $MgSO_4$, 50 ml NPS buffer (20x), 20 ml 5052 solution (50x)
2 x TY medium	16 g tryptone, 10 g yeast extract, 5 g NaCl ml H_2O H_2O to 1 liter

The appropriate antibiotics were added to cultures when needed. For the protein induction IPTG was added and for the selection galactose were added (Table 7).

Table 7. Additives used for *E. coli* and *M. xanthus*

Additive	Final concentration	Dissolved in
E. coli		
Ampicillin sodium sulfate	100 µg/ml	H_2O
Chloramphenicol	30 µg/ml	99.99% ethanol
Kanamycin sulfate	50 µg/ml	H_2O
Tetracyclin	15 µg/ml	99.99% ethanol
IPTG	0.1 mM - 0.5 mM	H_2O
M. xanthus		
Kanamycin sulfate	50 µg/ml	H_2O
Oxytetracycline	10 µg/ml	0.1M HCl
Galactose	2.5%	H_2O

4.3. Microbiological methods

4.3.1. *E. coli* strains

Table 8. *E. coli* strains used in this study

Strain	Relevant characteristics	Source or reference
Mach1	Δ*recA*1398 *endA1 tonA* Φ80Δ*lacM15* Δ*lacX74 hsdR*(rK- mK+)	Invitrogen (Darmstadt)
Rosetta 2 (DE3)	F-*ompT hsd*SB(rB-mB-) *gal dcm*(DE3) pRARE2(CmR)	Novagen/Merck (Darmstadt)

4.3.2. *M. xanthus* strains

For strains containing plasmids integrated at the Mx8 *attB* site, the gene expressed including the promoter driving the expression is indicated in brackets.

Table 9. *M. xanthus* strains used in this study

Strain	Relevant characteristics	Source or reference
DK1622	Wild-type	(Kaiser, 1979)
DK1300	*pilC*	(Hodgkin & Kaiser, 1979)
SA3502	Δ*sgmT*	(Petters *et al*., 2012)
DK11063	*fruA::Tn5 lacΩ7540*; kan^R	(Søgaard-Andersen *et al*., 1996)
SA4600	Δ*csgA*	Somasri Dam Pal
DK5057	*asgA*	(Kuspa & Kaiser, 1989)
DK4398	*asgB*	(Kuspa & Kaiser, 1989)
SA6273	Δ*lonD*	Magdalena Polatynska

Strain	Relevant characteristics	Source or reference
SW501	*difE*::kan^{R}	(Yang *et al*., 1998b)
DK10410	Δ*pilA*	(Wu & Kaiser, 1996)
DK10409	Δ*pilT*	(Jakovljevic *et al*., 2008)
SA3535	*attB*::pTP110; (P_{pilA}-PA5295 ^{WT}-strepII)	Tobias Petters
SA3537	*attB*::pTP112; (P_{pilA}-PA5295 ^{E328A}-strepII)	Tobias Petters
SA3543	*attB*::pTP114; (P_{pilA}-*dgcA* ^{WT}-strepII)	Tobias Petters
SA3559	*attB*::pTP131; (P_{pilA}-*dgcA* ^{D164A}-strepII)	Tobias Petters
SA3524	Δ*MXAN2424*	Tobias Petters
SA3525	Δ*MXAN2530*	Tobias Petters
SA3544	Δ*MXAN4232*	Tobias Petters
SA3546	Δ*pmxA*	Tobias Petters
SA3554	Δ*tmoK*	Tobias Petters
SA3533	Δ*MXAN5791*	Tobias Petters
SA3545	Δ*MXAN5199*	Tobias Petters
SA3548	Δ*MXAN4675*	Tobias Petters
SA3555	Δ*MXAN1525*	Tobias Petters
SA3556	Δ*MXAN2643*	Tobias Petters
SA3557	Δ*MXAN4029*	Tobias Petters
SA3558	Δ*MXAN2807*	Tobias Petters
SA3561	*dmxA*::pTP133	Tobias Petters
SA3569	Δ*MXAN4257*	Tobias Petters
SA3599	Δ*actA*	(Heering, 2013)
SA5524	Δ*MXAN2997*	Tobias Petters
SA5600	Δ*MXAN4463*	This study
SA5605	Δ*dmxB*	This study
SA5606	Δ*MXAN7362*	This study
SA5607	Δ*MXAN5366*	This study
SA5525	Δ*MXAN5340*	Tobias Petters
SA5526	Δ*MXAN5053*	Tobias Petters
SA5527	Δ*MXAN6098*	Tobias Petters
SA5619	Δ*dmxB*; *attB*::pDJS27 (P_{nat}-*dmxB*^{WT})	This study
SA5620	Δ*dmxB*; *attB*::pDJS37 (P_{nat}-*dmxB*^{D221A})	This study
SA5621	Δ*dmxB*; *attB*::pDJS33 (P_{nat}-*dmxB*^{D60N})	This study
SA5642	Δ*dmxB*; *attB*::pDJS66 (P_{nat}-*dmxB*^{D60E})	This study
SA5622	Δ*dmxB*; *attB*::pDJS38 (P_{nat}-*dmxB*^{R210A})	This study
SA5636	Δ*dmxB*; *attB*::pTP114 (*dgcA*^{WT}-strepII)	This study
SA5637	Δ*dmxB*; *attB*::pTP131 (*dgcA*^{D164A}-strepII)	This study
SA5629	Δ*pmxA*; *attB*::pDJS56 (P_{nat}-*pmxA*^{WT})	This study

Strain	Relevant characteristics	Source or reference
SA5631	Δ*pmxA*; *attB*::pDJS62 (P_{nat}-*pmxA*$^{H424A, D425A}$)	This study
SA5630	Δ*tmoK*; *attB*::pDJS57 (P_{nat}-*tmoK*^{WT})	This study
SA5634	Δ*MXAN5707*	This study
SA5635	Δ*MXAN0415*	This study
SA5640	Δ*pilT*; *attB*::pTP114 (*dgcA*^{WT}-strepII)	This study
SA5641	Δ*pilT*; *attB*:: pTP131 (*dgcA*^{D164A}-strepII)	This study
SA5646	Δ*pilT* Δ*MXAN0415*	This study

4.3.3. Cultivation and storage of *E. coli* and *M. xanthus*

E. coli strains were grown in liquid LB media with 230 rpm horizontal shaking or on agar plates at 37 °C. Appropriate antibiotics were added when necessary. The optical densities of cultures were determined photometrically at 600 nm. Glycerol stocks were made with overnight culture by adding the glycerol to the final concentration of 10%, freezing in liquid nitrogen and stored at -80 °C.

M. xanthus cells were grown on CTT agar plates at 32 °C in dark with appropriate antibiotics when necessary. For the liquid cultures, cells were harvested from the plate, resuspended in volume of 1 ml of CTT and then transferred to the bigger volume of media. Liquid cultures were incubated with horizontal shaking 220 rpm at 32 °C. The optical densities of *M. xanthus* cultures were determined photometrically at 550 nm. The glycerol stocks were made with the *M. xanthus* culture growing exponentially by adding the glycerol to 4%. The mixtures were fast frozen in liquid nitrogen and stored at -80 °C.

4.3.4. Motility assays for *M. xanthus*

For motility assays, *M. xanthus* cells from exponentially growing cultures were harvested by centrifugation at 4700 rpm for 10 min and resuspended in 1% CTT to a calculated density of 7×10^9 cells/ml. 5 µl aliquots of the suspensions were spotted on 0.5% for S-motility and 1.5% agar for A-motility supplemented with 0.5% CTT and incubated in dark at 32 °C. After 24 h, colony morphology and colony edges were observed using a Leica MZ8 stereomicroscope or a Leica IMB/E inverted microscope and visualized using Leica DFC280 and DFC350FX CCD cameras, respectively. T4P-dependent motility was quantified by the increase in colony diameter in three technical replicates.

4.3.5. Cell agglutination assay

Cell agglutination was measured as described (Shimkets, 1986a) in agglutination buffer (10 mM MOPS, pH 7.0, 1mM $MgCl_2$, 1mM $CaCl_2$). Briefly, exponentially growing cells in 1% CTT were harvested and resuspended in agglutination buffer to a calculated density of 1×10^9 cells/ml and kept at room temperature in the darkness between OD readings. OD_{550} was monitored every 20 min. for 180 min. The relative absorbance was calculated by dividing the absorbance measured at each time point by the initial absorbance for each strain. Experiments were done in three biological replicates.

4.3.6. T4P-shearing assay

T4P were sheared from cells that had been grown on 1% CTT/1.5% agar plates at 32 °C, purified and analyzed by immunoblot with anti-PilA antibodies as described (Wu & Kaiser, 1997). Briefly, 30 mg of cells from fresh CTT plates were harvested and carefully resuspended in 1 ml of Tris-HCl pH 7.6. The suspension was then vortexed 2x 10 min. with maximal speed to shear-off T4P from the cell surface. Whole cells and cell debris were pelleted by centrifugation 2x 13 000 rpm, 20 min, 4 °C and aliquot was used for immunoblot as a whole-cell sample. T4P from the supernatant has been precipitated with 100 mM $MgCl_2$ overnight, at 4 °C, and then harvested by centrifugation 13 000 rpm, 20 min, 4 °C. Sheared-off samples were analyzed *via* immunoblot with α-PilA antibodies. Whole-cell sample was analyzed with α-PilA antibodies and additionally with α-PilC antibodies, as a loading control.

4.3.7. Trypan blue dye-binding assays

To determine the ability of *M. xanthus* cells to bind Trypan blue dye, both liquid as well as plate assays were carried out. For plate-based assays, cells were grown in CTT medium to a density of 7×10^8 cells/ml, harvested and resuspended in 1% CTT or MC7 buffer to a calculated density of 7×10^9 cells/ml. 20 µl aliquots of the cell suspensions were placed on 0.5% agar supplemented with 0.5% CTT and 20 µg/ml trypan blue or on 1.5% TPM agar supplemented 20 µg/ml trypan blue. Plates were incubated at 32 °C for 24 h.

To quantify binding of trypan blue, a liquid binding assay was adapted from (Black & Yang, 2004) except that 5×10^8 cells from exponentially growing cultures were harvested, washed, and resuspended in 900 µl 10 mM MOPS pH 7.5, 1 mM

$CaCl_2$ buffer. The cell suspensions were then mixed with 100 µl of Trypan blue stock solution (150 µg/ml). Control sample containing Trypan blue in MOPS buffer only was included. All samples were mixed and incubated in the dark at room temperature for 30 min. Cell suspensions were then pelleted at 13 000 rpm for 5 min, and the absorbances of supernatants were measured at 585 nm. Percentage of Trypan blue bound by each sample was calculated by dividing the absorbance of each sample by the absorbance of the control. Triplicate assays were performed for all samples.

4.3.8. DGC activity assay *in vivo* in *E. coli* based on Congo Red binding

E. coli Rosetta 2(DE3) cells transformed with respective expression plasmids were grown on LB plates supplemented with 50 µg/ml Congo Red (CR). Plates were incubated at 30 °C overnight.

4.3.9. Development assay and spore assay of *M. xanthus*

M. xanthus development was examined on the following three different conditions: TPM agar plates, CF agar plates and submerged culture in MC7 buffer. The strains were cultivated in parallel to OD_{550} ~ 0.5-0.9. The cells were then harvested and resuspended in MC7 buffer to a calculated density of 5 x 10^9 cells/ml. 20 µl aliquots were spotted on TPM and CF agar. For development in submerged culture, 50 µl of concentrated cells were diluted in 350 µl MC7 and placed in a 15 mm well in a microtiter dish. Aggregation was followed using a Leica MZ8 stereomicroscope and a Leica IMB/E inverted microscope and visualized using a Leica DFC280 CCD camera.

Spore numbers were determined as the number of heat and sonication resistant spores formed after 120 h of starvation by harvesting cells from one of the 15 mm well in a microtiter dish. Cells were incubated for 2 h at 55 °C and sonicated 2x, 30%, output 3 to disperse fruiting bodies. Spores were counted in a counting chamber (Depth 0.02 mm, Hawksley) and presented relatively to WT. To determine the number of germinating spores, spore solutions were diluted and plated on CTT 1.5% agar plates covered with CTT softagar.

4.3.10. Glycerol-induced sporulation assay

Assay was performed as described (Müller *et al*., 2010) with a slightly modified protocol. Briefly, cells were cultivated in CTT media and induced at an OD_{550} of 0.3 with glycerol to a final concentration of 0.5 M. After 4 h, cell morphology was observed by placing cells on a thin TPM agar-pad on a glass slide, immediately covered with a coverslip and imaged. To determine efficiency of glycerol-induced sporulation, cells were harvested after 4 h incubation, pelleted at 4700 rpm, resuspended in sterile water, incubated at 55 °C for 2 h, and then sonicated two times 15 pulses, output 3, 30% duty with a Branson sonifier and microtip. Surviving spores from 5 µl of the treated samples counted in a counting chamber (Depth 0.02 mm, Hawksley).

4.4. Molecular biology methods

4.4.1. Oligonucleotides and plasmids

All oligonucleotides used in this study for cloning and sequencing are listed in Table 10 and Table 11. Oligonucleotides used for qRT-PCR are listed in Table 12. Underlined sequences display restriction sites used for cloning. Sequences in blue show added sequences required for cloning. Nucleotides marked as bold were substituted during site-directed mutagenesis. Sequences in green and red indicate sequences which are complimentary to fuse PCR products. Other part of the sequences represents sequences complementary to the respective genes. All plasmids used in this study are listed in Table 13.

Table 10. Oligonucleotides used in this study

Name	Sequence (5'-3')	Purpose
3735_A	ATCGGGTACCATGAAGCCGTACGAGCCCAC	In frame deletion of *MXAN3735* (*dmxB*)
3735_B	AGGCGAATTCTCGGAGGTCCGGCCGCG	
3735 _C	ACCTCCGAGAATTCGCCTCCGGCGCAG	
3735_D	ATCGTCTAGAAAGCGATCGCCACTTCCCTG	
3735 _E	GCAACTACATGGCCAAGGCC	
3735 _F	ATCAGCACCTGGCACAGGTC	
3735 _G	AGGTTCCTTCCCAGCGGAGA	
3735 _H	TGCTTCCAACGCCGTCTGGT	
3735 Pnat400 forw	ATCGAAGCTTTGACGACCCGTCGCGCGGCCACC	Complementation of Δ*dmxB*
3735 Pnat rev	ATCGTCTAGATCAGTGCCGCTGCGCCGGAG	

Name	Sequence (5'-3')	Purpose
3735 OE forw	ATCGCATATGATGGAACGGCGCGGCCGGACC	Overexpression of full lenght DmxB protein
3735 no stop OE rev	ATCGAAGCTTGTGCCGCTGCGCCGGAGGCG	
3735 OE GGDEF Fw	ATCGCATATGATGCGCCAGAGCGAGCAGCAGCGC	Overexpression of DmxB^{GGDEF}
3735 D60N F	CTCATCCTCCTG**AAC**AGGTTCCTTCCC	*dmxB* site directed mutagenesis
3735 D60N R	GGGAAGGAACCT**GTT**CAGGAGGATGAG	
3735 D221A F	CGCTTCGGTGGA**GCC**GAGTTCGTCGCG	
3735 D221A R	CGCGACGAACTC**GGC**TCCACCGAAGCG	
3735 R210A F	AAGCACGAGCTG**GCG**GAGTCGGACTTC	
3735 R210A R	GAAGTCCGACTC**CGC**CAGCTCGTGCTT	
3735 D60E F	CTCATCCTCCTG**GAG**AGGTTCCTTCCC	
3735 D60E R	GGGAAGGAACCT**CTC**CAGGAGGATGAG	
3735 forw	ATCGTCTAGAATGGAACGGCGCGGCCGGACC	Amplification of full lenght *dmxB*
3735 rev	ATCGAAGCTTTCAGTGCCGCTGCGCCGGAGG	

3734_A	ATCGGGTACCTCGCCAACTTCCGTGCGCTG	In frame deletion of *MXAN3734*
3734_B new	CGCGGCCAGACCCGTCGCCAGTCTGTT	
3734_C new	GCGACGGGTCTGGCCGCGGACCGCTAC	
3734_D new	ATCGTCTAGAGGCAGCCAGCACGTCCCGCT	
3734 _E	CGTCATCTTCCTGACGGGCG	
3734 _F	CACGCTGGTGCGGTGGATCA	
3734 _G	TTCGTTGGTGGTACGCGCCC	
3734 _H	TCGCCCTGACGAAGGCCTCC	

2061 Pnat300 forv	ATCGAAGCTTTGAACCGGGACGGGCACCTGGGC	Complementation of Δ*pmxA*
2061 Pnat rev	ATCGTCTAGATCAGGAGGCGAGCTTCACGGGC	
2061 AA-GYP F	GGCGGCATCCTC**GCGGCG**ATCGGGAAGATT	*pmxA* site directed mutagenesis
2061 AA-GYP R	AATCTTCCCGAT**CGCCGC**GAGGATGCCGCC	
2061 HD-GYP F	ATCGCATATGATGAAGGACGCGTACACCCGTGGC	Overexpression of full lenght PmxA$^{HD\text{-}GYP}$
2061 no stop OE rev	ATCGAAGCTTGGAGGCGAGCTTCACGGGCAG	

4445 Pnat200 forv	ATCGAAGCTTTGAGCGCTCCCTCACTCGTCTGC	Complementation of Δ*tmoK*
4445 Pnat rev	ATCGTCTAGATCATCCGGCCACGGAGCGTA	
4445 OE RRG Fw	ATCGCATATGATGGAGCTGCGCCGGGGAGACGAC	Overexpression of TmoK $^{REC\text{-}REC\text{-}GGDEF}$
4445 no stop OE Rv	ATCGAAGCTTTCCGGCCACGGAGCGTACCAC	

dgcA forw	ATCGCATATGATGAAAATCTCAGGCGCCCGG	Overexpression of full lenght DgcA
dgcA no stop rv	ATCGAAGCTTAGCGCTCCTGCGCTTGCGCAG	

Name	Sequence (5'-3')	Purpose
5707_A	ATCGGGTACCCAGGCACGAGGTGCCCCGAGA	In frame deletion of *MXAN5707*
5707_B	GCTCTGCACGCTCATGCTCGCGCCACC	
5707_C	AGCATGAGCGTGCAGAGCGGCACGGCC	
5707_D	ATCGTCTAGAAGTCAGGAGCAAATCCACGGC	
5707_E	GAACTTCGGACGCGCACTCTA	
5707_F	AGAGCGTCTGACAAGCGTGGA	
5707_G	GACTCGATGAGCGACAAGGCC	
5707_H	GGCCTGGAGCACCTCGCCCTT	
5707 F	ATCGCATATGATGACTGGCGGTGGCGCGAG	Overexpression of full length *MXAN5707*
5707 -stop R	ATCGAAGCTTCGAGCGGGCCGTGCCGCTCT	

0415_A	ATCGGGTACCAATCACGAGTACTACCACGGG	In frame deletion of *MXAN0415*
0415_B	GCGCTGGAAGTGGATGTCCGATGCACC	
0415_C	GACATCCACTTCCAGCGCGCGCTCGAG	
0415_D	ATCGTCTAGAATCGCGCTCCACCAGCCGAGC	
0415_E	AGCCGGACGTGGTGGCGGTGA	
0415_F	CCCATCACGGACTCCACCAGC	
0415_G	GGATGAAATCCCAACCAT	
0415_H	TGGCGTTGGAAATCATGG	
0415 F	ATCGCATATGATGTTGCTGACGAGGGTCACTCCCC	Overexpression of full lenght *MXAN0415*
0415 -stop R	ATCGGCGGCCGCCTCGAACTCGAGCGCGCGCTG	

7440 F	ATCGCATATGATGAGCACTCCCAGAAAGCGC	Overexpression of full lenght *MXAN7440*
7440 -stop R	ATCGAAGCTTCTCGTCCAGGTCCAGCTTGCG	
7043 F	ATCGCATATGATGGGAATGGAATATCGGCAG	Overexpression of full lenght *MXAN7043*
7043 -stop R	ATCGAAGCTTGTCCACCTTCGTCGGGAATGG	
4362 F	ATCGCATATGATGGCTACGACGGACCATCGT	Overexpression of full lenght *MXAN4362*
4362 -stop R	ATCGAAGCTTCTTGGGCTCTTCGGAGGGCGC	
4361 Fw	ATCGCATATGATGGCAGGCACCGACAAGCGC	Overexpression of full lenght *MXAN4361*
4361 -stop Rev	ATCGAAGCTTCTCCTCCCGAGGGTCCTGGCG	
attB right	GGAATGATCGGACCAGCTGAA	Primers used to verify integration at Mx8 phage attachment site
attB left	CGGCACACTGAGGCCACATA	
attP right	GCTTTCGCGACATGGAGGA	
attP left	GGGAAGCTCTGGGTACGAA	

Table 11. Sequencing oligonucleotides

Name	Sequence (5'-3')
M13 uni (-43)	AGGGTTTTCCCAGTCACGACGTT
M13 rev (-49)	GAGCGGATAACAATTTCACACAGG
malE	GGTCGTCAGACTGTCGATGAAGCC
petup	ATGCGTCCGGCGTAGA
T7	TAATACGACTCACTATAGGG
T7 term	CTAGTTATTGCTCAGCGGT
MXAN3735 rev	TCTCCGCTGGGAAGGAAC
MXAN2061 fw	TACACGGTGCTGCCCGAG
MXAN2061 rev	CTCGGGCAGCACCGTGTA
MXAN4445 fw	TGCTGGTGAAGCCCGTGCT
MXAN4445 rev	AGCACGGGCTTCACCAGCA
MXAN4445 fw 2	GCGTCATCACCCGCGTGGC
MXAN4445 rev 2	CCACCAGCACGCGGCCGTG

Table 12. Oligonucleotides used for qRT-PCR

Name	Sequence (5'-3')	Gene
3735 qPCR forw	GGTCCCTTCTGCTCATCATC	*dmxB* (*MXAN3735*)
3735 qPCR rev	AGGAACCTGTCCAGGAGGA	
7415 qPCR forw	GCAAGCCCTTCTACATGCTGA	*epsZ* (*MXAN7415*)
7415 qPCR rev	CGTGCTTCATCTTGAAGACGG	
7441 qPCR forw	CACCAGGAAAGCAAGCAGT	*epsH* (*MXAN7441*)
7441 qPCR rev	ACAGCTCGGCAATCAGAAG	
7440 qPCR forw	GTGGACTTCCTCTGCGAATC	*epsI* (*MXAN7440*)
7440 qPCR rev	GATGACCAGGTCGAAGGACT	
7422 qPCR forw	CTTCGAGTTCAGCCAGCAG	*epsU* (*MXAN7422*)
7422 qPCR rev	CTTCGAACCCTCGACACC	
7421 qPCR forw	CAGCAGAACTTCCTCGACAT	*epsV* (*MXAN7421*)
7421 qPCR rev	CCCTTCTTCTCCTCCTCCTT	
7438 qPCR forw	GGTAAGGGTGACGATGCC	*epsK* (*MXAN7438*)
7438 qPCR rev	ACGTACACCACCGAGTCCTT	
7433 qPCR forw	GTGAGGGCAACTACGCCTAT	*epsO* (*MXAN7433*)
7433 qPCR rev	ATCTGGAGCTTCACGTCCTT	
7451 qPCR forw	CATCGACCAGCTCACCTTC	*epsA* (*MXAN7451*)
7451 qPCR rev	GGAACTGCACGTTGTCCTC	
7450 qPCR forw	CGCATCTCCATTGGTGAGTA	*epsB* (*MXAN7450*)
7450 qPCR rev	GCCAGAAGTAGGCGGAGTAG	

Table 13. Plasmids used in this study

Plasmids	Description	Reference
pBJ114	kan^R, *galK*	(Julien *et al.*, 2000)
pSWU30	tet^R	(Wu & Kaiser, 1997)
pSW105	P_{pilA}, kan^R	(Jakovljevic *et al.*, 2008)
pET24b(+)	kan^R, expression vector	Novagen
pMALc2x	amp^R, expression vector	New England Biolabs
pDJS27	pSWU30; P_{nat}-*dmxB*^{WT}; tet^R	This study
pDJS37	pSWU30; P_{nat}-*dmxB*^{D221A}; tet^R	This study
pDJS33	pSWU30; P_{nat}-*dmxB*^{D60N}; tet^R	This study
pDJS66	pSWU30; P_{nat}-*dmxB*^{D60E}; tet^R	This study
pDJS38	pSWU30; P_{nat}-*dmxB*^{R210A}; tet^R	This study
pDJS56	pSWU30; P_{nat}-*pmxA*; tet^R	This study
pDJS62	pSWU30; P_{nat}-*pmxA*$^{H424A,\ D425A}$; tet^R	This study
pDJS57	pSWU30; P_{nat}-*tmoK*; tet^R	This study
pDJS31	pET24b(+); *dgcA*^{WT}; kan^R	This study
pDJS30	pET24b(+); *dmxB*^{WT}; kan^R	This study
pDJS39	pET24b(+); *dmxB*^{D221A}; kan^R	This study
pDJS42	pET24b(+); *dmxB*^{R210A}; kan^R	This study
pDJS45	pET24b(+); *dmxB*^{D60N}; kan^R	This study
pDJS68	pET24b(+); *dmxB*^{D60E}; kan^R	This study
pDJS71	pET24b(+); *pmxA*$^{384\text{-}568}$; kan^R	This study
pDJS75	pET24b(+); *pmxA*$^{384\text{-}568\ H424A,\ D425A}$; kan^R	This study
pDJS29	pMALc2x; *dmxB*; amp^R	This study
pDJS49	pMALc2x; *dmxB*^{D221A}; amp^R	This study
pDJS74	pMALc2x; *dmxB*^{R210A}; amp^R	This study
pDJS50	pMALc2x; *dmxB*^{D60N}; amp^R	This study
pDJS51	pMALc2x; *dmxB*^{D60E}; amp^R	This study
pTP114	pSW105, *dgcA*^{WT}-strepII, kan^R	Tobias Petters
pTP131	pSW105, *dgcA*^{D164A}-strepII, kan^R	Tobias Petters
pDJS54	pET24b(+); *dmxB*^{GGDEF}; kan^R	This study
pDJS77	pET24b(+); *MXAN0415*; kan^R	This study
pDJS78	pET24b(+); *MXAN5707*; kan^R	This study
pDJS79	pET24b(+); *nla24*; kan^R	This study
pDJS81	pET24b(+); *MXAN7043*; kan^R	This study
pDJS83	pET24b(+); *MXAN4362*; kan^R	This study
pDJS86	pET24b(+); *MXAN4361*; kan^R	This study
pSM31	pET28a(+); *lonD*; kan^R	Magdalena Polatynska
pPH158	pET28a(+); *mrpC*; kan^R	Bhardwaj (PhD thesis, 2013)

Plasmids	Description	Reference
pXS141	pET24b(+); *fruA*; kan^R	Shi (PhD thesis, 2008)
pDJS01	pBJ114; in-frame deletion of *dmxB*	This study
pDJS82	pBJ114; in-frame deletion of *MXAN5707*	This study
pDJS84	pBJ114; in-frame deletion of *MXAN0415*	This study
pDJS40	pBJ114; in-frame deletion of *MXAN3734*	This study

4.4.2. Plasmid construction

Genomic DNA of *M. xanthus* DK1622 was used to amplify DNA fragments. Plasmid constructs were transformed to *E. coli* Mach1 cells. To verify the correct sequence the purified plasmid DNA was sent for sequencing to the Eurofins MWG Operon (Ebersberg) company. Sequences were analyzed using program ContigExpress of the VectorNTI advance suite 11 software (Invitrogen).

The plasmids **pDJS01, pDJS82 and pDJS84** were generated for the construction of the *dmxB, MXAN5707, MXAN0415* in-frame deletion mutants, respectively, as described in 4.4.3. The upstream and downstream regions of *dmxB, MXAN5707* and *MXAN0415,* were amplified using primer pairs “3735A/3735B and 3735C/3735D”, “5707A/5707B and 5707C/5707D”, “0415A/0415B and 0415C/0415D”. The AB and CD DNA fragments were fused by overlap PCR reaction. Resulting AD fragment was digested with *KpnI*/*Xba*I or *EcoRI/XbaI* and ligated to pBJ114.

Plasmids **pDJS27**, **pDJS37, pDJS33, pDJS66 and pDJS38** are the derivatives of pSWU30 and were used to complement Δ*dmxB* mutant with different versions of *dmxB* under the native promoter. To amplify *dmxB* with 400 bp of the native promoter, from the genomic DNA, primers “3735 Pnat400 forw” and “3735 Pnat rev” were used. To introduce the point mutations in *dmxB* the following primers were used: *dmxB*^{D221A} (“3735 D221A F and R”), *dmxB*^{D60N} (“3735 D60N F and R”), *dmxB*^{D60E} (“3735 D60E F and R”), *dmxB*^{R210A} (“3735 R210A F and R”). The products were cloned at the *HindIII/XbaI* sites of pSWU30.

Plasmids **pDJS56** and **pDJS62** are the derivatives of pSWU30 and were used to complement Δ*pmxA* mutant with WT version of *pmxA* and *pmxA*$^{H424A,\ D425A}$ under the native promoter. To amplify *pmxA* with 300 bp of the native promoter primers “2061 Pnat300 forw” and “2061 Pnat rev” were used. To introduce the point mutations in *pmxB* the “2061 AA-GYP F and R” primers were used. The products were cloned at the *HindIII/XbaI* sites of pSWU30.

Plasmid **pDJS57** is the derivative of pSWU30 and was used to complement Δ*tmoK* mutant with WT version of *tmoK* under the native promoter. To amplify *tmoK* with 200 bp of the native promoter primers “4445 Pnat200 forw” and “4445 inner rev” as well as “4445 inner forw” and “4445 Pnat rev” were used. The product was cloned at the *HindIII/XbaI* sites of pSWU30.

Plasmid **pDJS31** is the derivative of pET24b(+) and was used for the overexpression of DgcA from *C. crescentus* with C-terminal His6 tag. DgcA was amplified with the primers “dgcA forw” and “dgcA no stop rev” and cloned at the *NdeI/HindIII* sites of pET24b(+).

Plasmids **pDJS30, pDJS39, pDJS42, pDJS45 and pDJS68** are the derivatives of pET24b(+) and were used for the overexpression of the different versions of full-length DmxB protein ($DmxB^{WT}$, $DmxB^{D221A}$, $DmxB^{D60N}$, $DmxB^{D60E}$ and $DmxB^{R210A}$) with C-terminal His6 tag. DmxB was amplified from the plasmids **pDJS27**, **pDJS37, pDJS33, pDJS66 and pDJS38** with the primers “3735 OE forw” and “3735 no stop rev” and cloned at the *NdeI/HindIII* sites of pET24b(+).

Plasmid **pDJS54** is the derivative of pET24b(+) and was used for the overexpression of GGDEF domain from DmxB with C-terminal His6 tag. GGDEF domain from DmxB was amplified with the primers “3735 OE GGDEF Fw” and “3735 no stop rev” and cloned at the *NdeI/HindIII* sites of pET24b(+).

Plasmids **pDJS71 and pDJS75 are** the derivatives of pET24b(+) and were used for the overexpression of WT HD-GYP domain of the PmxA protein as well as PmxA HD-GYP $^{H424A,\ D425A}$ with C-terminal His6 tag. PmxA was amplified from the plasmids **pDJS56** and **pDJS62** with the primers “2061 HD-GYP F” and “2061 no stop OE rev” and cloned at the *NdeI/HindIII* sites of pET24b(+).

Plasmids **pDJS29**, **pDJS49, pDJS74, pDJS50 and pDJS51** are the derivatives of pMAL-c2x and were used for the overexpression of the different versions of full-length DmxB protein ($DmxB^{WT}$, $DmxB^{D221A}$, $DmxB^{D60N}$, $DmxB^{D60E}$ and $DmxB^{R210A}$) with N-terminal MalE tag. DmxB was amplified from the plasmids **pDJS27**, **pDJS37, pDJS33, pDJS66 and pDJS38** with the primers “3735 forw” and “3735 rev” and cloned at the *XbaI/HindIII* sites of pMAL-c2x.

Plasmids **pDJS77**, **pDJS78, pDJS79, pDJS81, pDJS83 and pDJS86** are the derivatives of pET24b(+) and were used for the overexpression of MXAN0415, MXAN5707, MXAN7440, MXAN7043, MXAN4362 and MXAN4361, respectively, with C-terminal His6 tag. *MXAN0415*, *MXAN5707, MXAN7440, MXAN7043, MXAN4362 and MXAN4361* were amplified form the genomic DNA with the primers “0415 forw” and “0415 -stop rev”, “5707 forw” and “5707 -stop rev”, “0415 forw” and “0415 -stop rev”, “7440 forw” and “7440 -stop rev”, “7043 forw” and “7043 -stop rev”, “4362 forw”

and “4362 -stop rev”, “4361 forw” and “4361 -stop rev” and cloned at the *NdeI/HindIII* or *NdeI/NotI* sites of pET24b(+).

4.4.3. Construction of in-frame deletion mutants

In-frame deletion mutants were constructed by two-step homologous recombination as described (Shi *et al*., 2008) (Figure 68). Briefly, the upstream and downstream flanking regions of gene of interest (approximately 600bp) were amplified using AB and CD primer pairs. AB and CD fragments contain overlapping ends and served as a template to generate the in-frame deletion fragment AD. AD fragment was then cloned into pBJ114 vector. The correct pBJ114_AD construct was transformed into *M. xanthus*. The plasmid integration was checked by PCR reaction using E (binds upstream of a primer) and F (binds downstream of D primer), E and M13forward (binds to pBJ114), F and M13reverse (binds to pBJ114) primer pairs. One clone resulted from an each up- and downstream plasmid integration was used for the second step of homologous recombination.

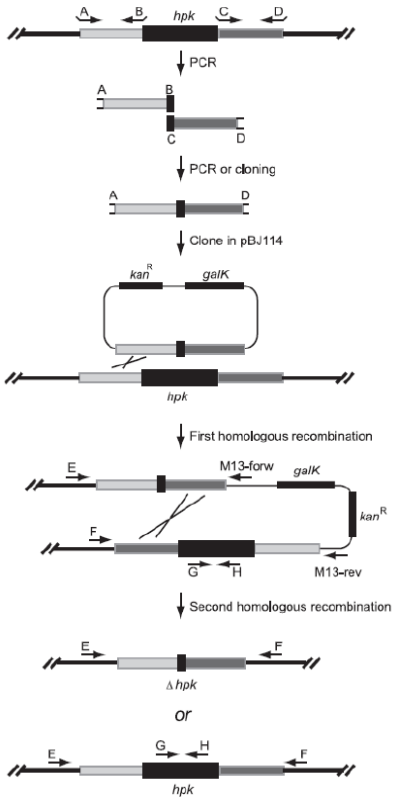


Figure 68. Strategy for in-frame deletion mutants construction

First homologous recombination leads to up- or downstream plasmid integration in the genomic region of interest. Second homologous recombination enables loop out of vector (reconstitution) or vector with the region of interest (in-frame deletion). Details are described in the main text. The figure is reproduced from Shi *et al*., 2008.

To isolate the in-frame deletion mutants the cells were grown in CTT liquid media to reach the exponentially phase. 10µl of cells were mixed with 2 ml of soft agar and

plated on CTT agar plates containing 2.5% galactose. Galactose resistant and kanamycin sensitive clones were checked by PCR reaction using E and F, G (binds downstream of B primer) and H (binds upstream of C primer) primer pairs. The EF fragment was longer for the WT then for the deletion mutant, while GH fragment was amplified only in the WT.

4.4.4. DNA isolation from *E. coli* and *M. xanthus*

Plasmid DNA from *E. coli* was isolated using the QIAprep Spin Miniprep Kit (Qiagen) or the NucleoSpin Plasmid QuickPure kit (Macherey-Nagel) in accordance to the instructions provided by the manufacturer. Genomic DNA from *M. xanthus* was isolated using MasterPure DNA preparation Kit (Epicentre) according to the instructions provided by the manufacturer. Concentration and purity of DNA was determined with the Nanodrop ND-1000 spectrophotometer. Crude genomic DNA for colony PCR was prepared by resuspending cell pellet in 50 µl of ddH_2O, boiling for 5 min and centrifuging the sample for one minute at 13 000 rpm. 1.5 µl from the resulting supernatant were used per PCR reaction.

4.4.5. Polymerase Chain Reaction (PCR)

For the amplification of specific DNA fragments, the Phusion High-Fidelity DNA Polymerase was used in a total reaction volume of 50 µl. The colony PCR was performed in a total volume of 20 µl using 5 PRIME MasterMix. The composition of the PCR reaction mix is described in Table 14.

Table 14. PCR reaction mix

Component	Volume	Final concentration
PCR for cloning		
Template DNA	1 µl	~ 50 ng
10 µM primer (each)	1 µl	0.5 µM
10 mM dNTPs	1 µl	0.2 mM
5 x Phusion GC buffer	10 µl	1x
5 x enhancer	10 µl	1x
Phusion DNA polymerase	0.5 µl	1 unit/50 µl reaction
ddH_2O	to 50 µl	

Colony PCR

Crude genomic DNA	1.5 µl	~ 100 ng
10 µM primer (each)	1 µl	0.5 µM
5 PRIME MasterMix	8 µl	
DMSO	2 µl	10% (v/v)
ddH_2O	to 20 µl	

The PCR programs used in this study are represented in Table 15. PCR conditions were modified depending on the primer annealing temperature and expected product size.

Table 15. PCR programs

Step	Temperature	Time	
Standard/check PCR			
initial denaturation	98 °C	3 min	
denaturation	98 °C	30 sec	35x
primer annealing	5 °C below predicted melting temperature	30 sec	
elongation	72 °C	1 min/kb	
final elongation	72 °C	3 min	
hold	4 °C	∞	
Touch down PCR			
initial denaturation	94 °C	3 min	
denaturation	94 °C	30 sec	10x
primer annealing	65 °C	30 sec	
elongation	72 °C	1 min/kb	
denaturation	94 °C	30 sec	10x
primer annealing	60 °C	30 sec	
elongation	72 °C	1 min/kb	
denaturation	94 °C	30 sec	10x
primer annealing	55 °C	30 sec	
elongation	72 °C	1 min/kb	
final elongation	72 °C	3 min	
hold	4 °C	∞	

4.4.6. RNA preparation from *M. xanthus*

Total RNA was isolated from cell pellets using the hot-phenol method (Overgaard *et al*., 2006). Briefly, *M. xanthus* cells were harvested to a tube containing 1/10 volume of ice-cold stop solution (5% saturated acid phenol (pH <6.0) in 96% ethanol) and spin down (4700 rpm, 10 min, 4 °C). The pellet was resuspended in 600 µl ice cold solution 1 (0.3 M sucrose, 0.01 M NaAc, pH 4.5) and transferred into tubes containing 2.5 ml hot (65 °C) solution 2 (2% SDS, 0.01 M NaAc, pH 4.5). The RNA purification was conducted twice with equal volume hot phenol (saturated acid phenol (pH <6.0) at 65 °C) extraction, once with acid phenol:chloroform (saturated acid phenol, pH 4.5 : chloroform, 5:1) extraction and once with equal volume of chloroform: isoamyl alcohol (24:1) extraction. RNA was precipitated with 1/10 volume of 3 M NaAc pH 4.5 and 2 volume of 96% ethanol for 20 min or over night at -20 °C. The RNA pellet was spin down in microcentrifuge for 5 min. with full speed at 4 °C and washed twice with equal volume of ice cold 75% ethanol. The pellet was dried briefly at room temperature and resuspended in 50 µl RNase-free H_2O. The RNA was stored at -80 °C.

4.4.7. RNA clean up, cDNA synthesis and qRT-PCR

The purified total RNA was treated with 20 U RNase-free DNase I (Ambion) for 3h at 37 °C. RNA was purified using the RNeasy Mini Kit (QIAGEN) according to the manufacturer protocol. The absence of DNA was verified by PCR reaction. The RNA integrity was analyzed by 1% agarose gel electrophoresis. RNA was considered intact, if sharp and clear bands for 23S and 16S rRNA were observed on the gel. 1.0 µg of DNA-free intact total RNA was used as the template to synthesize cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the recommended protocol.

The qRT-PCR reactions were carried out in a total volume of 25 µl containing 2.5 µl Sybr green PCR Master Mix (Applied Biosystems), 1 µl of each primer (10 µM), 1 µl cDNA (diluted 10x) and 11 µl H_2O. AB 7300 Real time PCR detection system was used for qRT-PCR reactions with standard conditions. Experiments were done in two biological replicates, each of them in technical triplicates. Relative gene expression levels were calculated using the comparative Ct method.

4.4.8. Agarose gel electrophoresis

Nucleic acid fragments were separated by size and visualized on 1% agarose gels with 0.01% (v/v) ethidium bromide in TBE buffer (Invitrogen) at 120 V. DNA samples were mixed with 5 x DNA loading buffer (Bioline). As a DNA marker the 2-log DNA ladder (NEB) was used. Agarose gels were imaged using the E-BOX VX2 imaging system from PeqLab.

4.4.9. DNA restriction and ligation

Restriction of DNA fragments (0.5-2 µg) was performed with restriction endonucleases at 37 °C for 2 h. Restricted DNA was purified using the DNA Clean & Concentrator kit (Zymo Research) according to manufacturer protocol.

Ligation reactions were performed with T4 DNA ligase (NEB) with ~50 ng of vector DNA and 3- to 5-fold molar excess of insert DNA. Reaction was performed at room temperature for 1h. Ligation mixtures were used for transformation into *E. coli* Mach1.

4.4.10. Preparation and transformation of chemical *E. coli* cells

To prepare electrocompetent *E. coli* cells, the overnight culture was used to inoculate 50 ml of LB media. The cells were grown at 37 °C to an OD_{600} of 0.5 - 0.7. The cells were harvested by centrifugation at 4700 rpm for 20 min at 4 °C and the cell pellet was resuspended in 25 ml of ice-cold sterile 50 mM $CaCl_2$ solution. The cells were pelleted again at the same conditions and resuspended in 2 ml 50 mM $CaCl_2$ + 16% glycerol solution. The final cell suspension was aliquoted à 50 µl, frozen in liquid nitrogen and stored at -80 °C.

One aliquot was used per transformation. Cells were thawed on ice and 10 µl of ligation mixture was added to the cells and mixed carefully. After incubation on ice for 30 min, cells were heat-shocked in water bath at 42 °C for 1 min 20 sec. After 5 min incubation on ice, 1 ml LB-medium was added and cells were incubated for 60 min shaking at 37 °C. Then, cells were pelleted for 30 sec, the supernatant was discarded and cells were resuspended in 50 µl LB medium and plated on LB plates with appropriate antibiotics. Plates were incubated at 37 °C over night. Grown colonies were transferred to the fresh agar plates and checked for the presence of the plasmid containing the insert by restriction digestion.

4.4.11. Preparation and transformation of electrocompetent *M. xanthus* cells

For transformation of *M. xanthus* cells, 2 ml of an overnight culture OD_{550} 0.6-0.9 were harvested at 13 000 rpm for 1 min and the pellet was washed twice in 1 ml sterile ddH_2O and resuspended in 40 µl H_2O. The cell suspension was immediately used for electroporation.

0.1 µg DNA for plasmids integrating at the Mx8 site and 1 µg of DNA for plasmids integrating at the endogenous site was added to 40 µl cells and the mixture was transferred into an electroporation cuvette (Bio-Rad, Munchen) and pulsed with 0.65 kV, 25 µF and 400 Ω. 1 ml of CTT media was added and mixed with cells, the cell suspension was transferred to a 25 ml Erlenmyer flask containing 1 ml of CTT media and incubated with shaking at 230 rpm at 32 °C for 6 h. Then, 1 ml (for plasmids integrating at the endogenous site) and 100 µl (for plasmids integrating at the Mx8 site) of the culture were mixed with 2 ml of soft agar and plated on CTT agar plates supplemented with appropriate antibiotics. The plates were incubated for 5-10 days at 32 °C in the dark. Grown colonies were transferred to fresh agar plates. Plasmid integration was verified by colony PCR.

4.5. Biochemical methods

4.5.1. Purification of proteins

To purify the proteins, the appropriate plasmids were introduced into *E. coli* Rosseta 2 (DE3)/pLysS strain (Novagen). The cultures were grown in 0.5l or 1l LB or 2xTY medium containing appropriate antibiotics at 37 °C to an OD_{600} of 0.5-0.7. The protein expression was induced by addition of IPTG to a final concentration of 0.3 mM and cells were grown overnight at 18 °C. Alternatively, the autoinduction medium was used (Table 6).

The cells were harvested by centrifugation at 10 000 x rpm for 10 min at 4 °C and resuspended in 25 ml lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 10 mM imidazole, 10% glycerol) with added protease inhibitors (Complete Protease Inhibitor Cocktail Tablets from Roche). Cells were lysed using a French pressure cell and centrifuged at 20 000 rpm for 1h at 4 °C to collect cell debris. The supernatant containing soluble proteins was used for the protein purification. His6-tagged proteins were purified using Ni^{2+}-NTA-agarose columns (Qiagen) as recommended by the manufacturers. The supernatant was mixed with appropriate amounts of the beads and was loaded on a Pierce centrifuge column. After collecting the flow through, the column

was washed twice with 20 ml lysis buffer with 20 mM imidazole. Bound protein was eluted with imidazol gradient from 50 mM to 500 mM.

For antibody production 1mg of purified DmxB was to Eurogentec (Seraing, Belgium).

4.5.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

To separate proteins under denaturing conditions SDS-PAGE with 12% or 15% polyacrylamide gels was performed. To denature proteins, the protein samples were mixed with loading buffer (10% (v/v) glycerol, 60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 100 mM DTT, 3 mM EDTA, 0.005% (w/v) bromophenol blue) and heated for 10 min. at 98 °C before loading on the gel. Gel electrophoresis was performed in Bio-Rad electrophoresis chambers (Bio-Rad, München) at 120-150 V in 1x Tris/Glycine SDS (TGS) running buffer (Bio-Rad). Size of proteins was determined by comparison to the protein marker, the PageRuler Prestained Protein Ladder (Fermentas). Proteins were visualized by staining with Instant Blue protein staining solution (Expedeon).

4.5.3. Determination of protein concentration by Bradford

To determine protein concentrations the Bradford reagent (Bio-Rad) was used in 1:5 dilution. The protein standard curve was generated using bovine serum albumin (BSA). The reaction samples were prepared in duplicates 1 ml reaction volume. After 10 min incubation at room temperature in the dark, the absorbance was measured at 595 nm with the Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, München). Protein concentrations were determined based on the linear slope of the standard curve.

4.5.4. Immunoblot analysis

Protein solutions or proteins from cell extracts were separated in the gel by SDS-PAGE and transferred to a nitrocellulose membrane using „TransBlot® Turbo™ Transfer System“ from Bio-Rad at 1.3 A, 25 V for 7 min with transfer buffer (300 mM Tris and 300 mM Glycin, and 0.05% SDS, pH 9.0). After transfer the membrane was blocked in 5% non-fat milk powder (w/v) in 1 x TTBS buffer (0.05% (v/v) Tween 20, 20 mM Tris-HCl, 137 mM NaCl pH 7.0) for 1 h or over night at 4 °C. After washing with 1 x TTBS buffer, the primary antibody (rabbit) was added in proper dilutions (Table 16) in 1

x TTBS supplemented with 2% non-fat milk powder over night at 4 °C. Next, membranes were washed again with 1 x TTBS buffer and incubated with secondary anti-rabbit immunoglobulin G peroxidase conjugate (Sigma) in a dilution of 1:15 000 for 1h at 4 °C. After washing with 1 x TTBS buffer the blot was developed with the Luminata Western HRP Substrate (Merck Millipore) and visualized with the luminescent image analyzer LAS-4000 (Fujifilm).

Table 16. Dilutions of primary antibodies used for immunoblot anaylsis

antibody	α-DmxB	α-PilA	α-PilC	Strep-Tactin-HRP
dilution	1:1000	1:5000	1:5000	1:10 000

4.5.5. Preparation of [α-^{32}P]-labeled c-di-GMP

[α-^{32}P]-labeled c-di-GMP was prepared in house by incubating 10 µM His6-tagged DgcA^{WT} (final concentration) with 1 mM GTP/[α-^{32}P]-GTP (0.1 µCi/µl) (Hartmann Analytic) in reaction buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM $MgCl_2$) in total volume of 200 µl, overnight at 30 °C. The reaction mixture was then incubated with 5 units of calf intestine alkaline phosphatase (Fermentas) for 1 h at 22 °C to hydrolyze unreacted GTP. The reaction was stopped by incubation for 10 min at 95 °C in order to precipitate the proteins. The reaction was centrifuged (10 min, 15 000× g, 20 °C) and the supernatant containing [α-^{32}P]-c-di-GMP was used directly for the assays or stored at 4 °C.

4.5.6. *In vitro* DGC and PDE assays

DGC and PDE activities were determined using [α-^{32}P]-GTP and [α-^{32}P]-c-di-GMP respectively. Briefly, assays were performed with 10 µM of purified proteins (final concentration) in a final volume of 40 µl. Reaction mixtures were pre-incubated for 5 min at 30 °C in reaction buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM $MgCl_2$). DGC reactions were initiated by adding 1 mM GTP/[α-^{32}P]-GTP (0.1 µCi/µl) and incubated at 30 °C for the indicated periods of time. PDE reactions were initiated by adding ^{32}P-labeled c-di-GMP. Reactions were stopped by addition of one volume 0.5 M EDTA. Reaction products were analyzed by polyethyleneimine-cellulose TLC chromatography as described (Christen *et al*., 2005). Briefly, 2 µl aliquots were spotted on TLC plates (Millipore), dried, and developed in 2:3 (v/v) 4 M $(NH_4)_2SO_4$/1.5 M

KH_2PO_4 (pH 3.65). Plates were dried prior to exposing a phosphor-imaging screen (Molecular Dynamics). Data were collected and analyzed using a STORM 840 scanner (Amersham Biosciences) and Image Quant 5.2 software.

4.5.7. *In vitro* c-di-GMP binding assay

c-di-GMP binding was determined using a DRaCALA assay (Differential Radial Capillary Action of Ligand Assay) with ^{32}P-labeled c-di-GMP. Briefly, ^{32}P-labeled c-di-GMP was prepared as described above, mixed with 20 µM protein and incubated for 10 min. at room temperature in binding buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$). 10 µl of this reaction mixture was transferred to a nitrocellulose filter (GE Healthcare), allowed to dry and imaged as described above. For competition experiments, 0.4 mM unlabelled c-di-GMP (Biolog) or GTP (Sigma) was added to the protein and incubated for 10 min before addition of ^{32}P-labeled c-di-GMP.

4.5.8. Capture compound mass spectrometry (CCMS)

M. xanthus cells were grown to exponential phase or alternatively starved in the MC7 shaking suspension for 24h and pelleted by centrifugation for 20 minutes at 4700 rpm. The pellet was resuspended in lysis buffer (6.7 mM MES, 6.7 mM HEPES, 200 mM NaCl, 6.7 mM KAc, pH 7.5) and protease inhibitor (complete mini, EDTA-free, Roche) was added. Cells were lysed by passing it 3 x through a French pressure cell. After centrifugation at 40 000 rpm for 1 h the supernatant was used for CCMS experiments of soluble proteins.

The capture experiments were essentially carried out as described (Nesper *et al*., 2012) and performed in 200 µl 12-tube PCR strips. Briefly, 50 µl of the soluble protein fractions were mixed with 20 µl 5 x capture buffer (100 mM HEPES, 250 mM KAc, 50 mM MgAc, 50% glycerol, pH 7.5) and with the 10 µl of c-di-GMP capture compound (cdG-CC, Caprotec Bioanalytics GmbH, Berlin). The volume was adjusted to 100 µl with H_2O and incubated for 2 h at 4 °C in the dark on a rotary wheel. The reaction was then UV irradiated for 4 minutes using a caproBox (Caprotec Bioanalytics GmbH, Berlin). 50 µl magnetic streptavidin beads (Dynabeads MyOne Streptavidin C1, Invitrogen) and 25 µl 5 x wash buffer (250 mM Tris pH 7.5, 5 M NaCl, 0.1% n-octyl-β-D-glucopyranoside) were added and the mixture was incubated for 1 h at 4 °C on a rotary wheel. The beads were then collected with a magnet (caproMag, Caprotec Bioanalytics GmbH, Berlin) and washed 6 x with 200 µl 1 x wash buffer. Beads were

analyzed by LC-MS/MS (analysis was performed by Jörg Kahnt). In first control experiment run in parallel, c-di-GMP as a competitor was added to protein extracts up to a final concentration of 1 mM and incubated for 30 minutes at 4 °C on a rotary wheel before the cdG-CC was added. In second control experiment no cdG-CC was added to the reaction.

4.5.9. c-di-GMP quantification

c-di-GMP quantification was performed in Medizinische Hochschule Hannover (Hannover) by Prof. Dr. Volkhard Kaever and Annette Garbe**.** To quantify the c-di-GMP levels in *M. xanthus* cells, the cells were grown overnight in CTT medium and harvested in exponential phase or then diluted in MC7 starvation buffer. At the indicated time points after dilution, cells were harvested at 4 °C, 2500× g for 20 min. Cells were lysed in extraction buffer (HPLC grade acetonitrile/methanol/water (2/2/1, v/v/v)), supernatants were pooled and evaporated to dryness in a vacuum centrifuge. Pellets were dissolved in HPLC grade water and analysed by LC-MS/MS. Experiments were done in three biological replicates in which at least three independent cultures were grown in parallel. For all samples, protein concentrations were determined in parallel using a Bradford assay (Bio-Rad).

4.6. Transmission electron microscopy

Transmission electron microscopy was performed in Georg-August-Universität (Göttingen) with help of Dr. Michael Hoppert. Briefly, *M. xanthus* overnight cultures were diluted 1:10 with 1% liquid CTT medium and grown on horizontal shaker at 32 °C in the dark for 1-3 h. After 1-3 h incubation, 50 µl of *M. xanthus* culture was placed on parafilm. A copper grid (PLANO) was dipped into the drop for 2 min., allowing cells to adsorb to the surface. Excess of liquid was soaked off, the film was placed briefly on a drop of distilled water, excess liquid was soaked off again, and the film was transferred on a drop of 2% uranyl acetate (wt/vol) for 2 seconds and blotted dry. Transmission electron microscopy was performed on a Philips EM 301 electron microscope at calibrated magnifications.

4.7. Bioinformatic analyses

Gene and protein sequences of *M. xanthus* were obtained from TIGR database (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) and KEGG database (http://www.genome.jp/kegg/). Functional domains were identified using SMART database (http://smart.embl-heidelberg.de/). Alignment and analysis of selected sequences was performed using Vector NTI (Invitrogen).

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Curriculum vitae

Personal data

Name: Dorota Jagoda Skotnicka
Date of birth: 09.02.1988
Place of birth: Legnica, Poland

Education

09/2003 – 06/2006	Secondary School No. 1 Legnica, Poland
10/2006 – 07/2009	Bachelor of Science University of Wroclaw, Poland Supervisor: Dr. Magdalena Wołoszyńska
10/2009 – 10/2011	Master of Science - Molecular Biology University of Wroclaw, Poland Supervisor: Prof. Dr. Maria Małgorzata Sąsiadek
from 09/2012	PhD program (Dr. rer. nat.) Philipps-Universität Marburg Max-Planck-Institute for Terrestrial Microbiology, Marburg Supervisor: Prof.Dr. Lotte Søgaard-Andersen

Internships

09/2009 – 01/2010	Erasmus Programme University of Groningen, Netherlands Supervisor: Prof. Dr. O.P. Kuipers
07/2010 – 08/2010	Praktikum Max Planck Institute of Molecular Plant Physiology, Golm Supervisor: Dr. Joost van Dongen
07/2011 – 09/2011	Praktikum Max Planck Institute of Molecular Plant Physiology, Golm Supervisor: Dr. Joost van Dongen
11/2011 – 04/2012	Internship Max-Planck-Institute for Terrestrial Microbiology, Marburg Supervisor: Prof.Dr. Lotte Søgaard-Andersen

List of publications

Eckhardt TH, **Skotnicka D**, Kok J, Kuipers OP.
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Schäper S, Krol E, **Skotnicka D**, Kaever V, Søgaard-Andersen L, Becker A.
c-di-GMP regulates multiple cellular functions in the symbiotic α-proteobacterium *Sinorhizobium meliloti.*
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