RsmA Regulates Biofilm Formation in Xanthomonas campestris through a Regulatory Network Involving Cyclic di-GMP and the Clp Transcription Factor

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Abstract

Biofilm formation and dispersal in the black rot pathogen Xanthomonas campestris pathovar campestris (Xcc) is influenced by a number of factors. The extracellular mannanase ManA has been implicated in biofilm dispersal whereas biofilm formation requires a putative glycosyl transferase encoded by the xag gene cluster. Previously we demonstrated that the post-transcriptional regulator RsmA exerts a negative regulatory influence on biofilm formation in Xcc. Here we address the mechanisms by which RsmA exerts this action. We show that RsmA binds to the transcripts of three genes encoding GGDEF domain diguanylate cyclases to influence their expression. Accordingly, mutation of rsmA leads to an increase in cellular levels of cyclic di-GMP. This effect is associated with a down-regulation of transcription of manA, but an upregulation of xag gene transcription. Mutation of clp, which encodes a cyclic di-GMP-responsive transcriptional regulator of the CRP-FNR family, has similar divergent effects on the expression of manA and xag. Nevertheless Clp binding to manA and xag promoters is inhibited by cyclic di-GMP. The data support the contention that, in common with other CRP-FNR family members, Clp can act as both an activator and repressor of transcription of different genes to influence biofilm formation as a response to cyclic di-GMP.

Introduction

The formation, maturation and dispersal of biofilms are key processes within the lifestyle of many bacteria including animal and plant pathogens [1,2]. Biofilm formation is important for many pathogenic organisms, as bacteria within these multicellular structures are often considerably more resistant to diverse stresses than can include the action of host defences [1,2]. Equally the release of bacteria from biofilms is significant for the progression of disease into uninfected tissue and for completion of the disease cycle [3]. The extracellular environment undoubtedly influences many aspects of bacterial behaviour including the dynamics of biofilm formation and dispersal. An array of signal transduction systems link the sensing of specific environmental cues to appropriate alterations in bacterial physiology and/or gene expression. An understanding of these different regulatory elements or pathways, how they are integrated and how they act to influence biofilm dynamics may have important implications for the control of bacterial disease.

Xanthomonas campestris pathovar campestris (hereafter Xcc) is the causative agent of black rot disease of cruciferous plants, which is an important disease globally [4]. A number of factors and several regulatory pathways have been implicated in the formation or dispersal of biofilms by Xcc [5]. Biofilm formation requires the synthesis of the extracellular polysaccharide xanthan and an uncharacterised polysaccharide whose synthesis is directed by the products of the xag gene cluster [6]. Conversely, the extracellular enzyme beta (1,4)-mannanase has been implicated in biofilm dispersal [5,6]. Cell-to-cell signalling mediated by the diffusible signal molecule DSF (for Diffusible Signal Factor; cis-11-methyl-dodecenoic acid) regulates biofilm dispersal [5]. Both the synthesis and perception of the DSF signal require products of the rpf gene cluster [7,8]. The synthesis of DSF is dependent on RpfF, whereas the two-component system comprising the sensor kinase RpfC and regulator RpfG is implicated in DSF perception and signal transduction [7,8]. RpfG is a regulator with a CheY-like receiver (REC) domain attached to an HD-GYP domain, which acts to degrade the second messenger bis (3’,5’) cyclic diguanosine monophosphate (cyclic di-GMP) [9,10]. Mutation of rpfF, rpfG, or rpfC leads to an elevated level of cyclic di-GMP and the mutants exhibit an aggregative behaviour when grown in certain media, unlike the wild-type, which grows in a dispersed...
Figure 1. The rsmA mutant has an increased biofilm phenotype and is associated with an elevated intracellular level of cyclic di-GMP. (A) The rsmA mutant grows in an aggregated fashion in L medium, whereas the wild-type strain (8004) grows in a dispersed fashion. In trans expression of the gene encoding rsmA in the mutant restores the phenotype to wild-type. (B) The rsmA mutant also has increased biofilm and cell adhesion on a glass surface when assessed by crystal violet staining (see Methods for details). Values given are the mean and standard deviation of triplicate measurements. (C) Effects of mutation of rsmA on cyclic di-GMP level in Xanthomonas. Elevated levels of extractable nucleotide were seen after mutation of rsmA. Introduction of the cloned rsmA gene (pRSMA) into the rsmA mutant reduced cyclic di-GMP levels to wild-type. Values given are the mean and standard deviation of triplicate measurements (three biological and three technical replicates).
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fashion [4,5,9,11]. This is consistent with a body of work in a number of bacteria in which biofilm formation has been associated with elevated levels of cyclic di-GMP (reviewed recently by [12–16]).

The Rpf/DSF system positively influences the expression of the manA gene, encoding endo-mannanase, but negatively influences the expression of the xag gene cluster [6]. The regulatory effect of the DSF/rpf system on the expression of manA is believed to involve the transcriptional regulator Clp [17]. Elevated levels of cyclic di-GMP negatively influence the ability of Clp to bind to DNA [18,19].
In previous work we have shown that the post-transcriptional regulator RsmA also influences biofilm formation in Xcc. RsmA is an RNA-binding protein functioning as a global regulator of various cellular processes in bacteria [20]. Deletion of rsmA in Xcc results in complete loss of virulence, a significant reduction in the production of the extracellular polysaccharide xanthan and extracellular enzymes including mannanase but an enhanced bacterial aggregation and cell adhesion. Mutation of rsmA does not alter the expression of rpf genes or the level of DSF in Xcc however, suggesting that the regulatory action of RsmA on biofilm formation is independent of cell-to-cell signalling under the conditions tested [21].

In this paper we address the mechanisms by which RsmA exerts a regulatory effect on biofilm formation in Xcc. In Escherichia coli and Salmonella, the RsmA homolog CsrA controls cyclic di-GMP metabolism [20,22,23]. Our initial finding that mutation of rsmA in Xcc also leads to an increase in levels of cyclic di-GMP prompted us to study the influence of RsmA on expression of genes in Xcc encoding GGDEF, EAL or HD-GYP domain proteins that are involved in cyclic di-GMP synthesis or degradation [12]. This allowed us to define a subset of these proteins whose expression is regulated by RsmA. Further to this we demonstrate by mutational analysis that Clp has opposite effects on the expression of rsmA and sggA genes. The work defines a regulatory network controlling the formation of biofilms in Xcc that comprises elements that are likely to be responsive to diverse environmental cues.

Results

Mutation of rsmA Leads to an Alteration in Biofilm Formation and Increase in Cellular Level of Cyclic di-GMP

We have previously shown that mutation of rsmA in Xcc leads to an aggregative behaviour in liquid media and enhanced adhesion to glass surfaces [21]. This biofilm phenotype was assayed quantitatively using crystal violet staining to measure adherence to glass (see Methods). As expected, the rsmA mutant showed aggregative behaviour in liquid medium (Fig. 1A) and had higher levels of biofilm formation on glass than the wild-type (Fig. 1B). Complementation with rsmA expressed in trans restored the phenotypes to wild-type (Fig. 1A,B). In parallel, we measured the level of cyclic di-GMP in wild-type, rsmA mutant and complemented strains (see Methods). The cellular level of cyclic di-GMP in the rsmA mutant was elevated over that seen in the wild-type. Introduction of the cloned rsmA gene (pRSMA) into the rsmA mutant reduced the cyclic di-GMP level to wild-type (Fig. 1C).

RsmA Binds to the Transcripts of Genes Encoding GGDEF Domain Proteins to Influence Expression

The finding that mutation of rsmA led to an increase in cellular levels of cyclic di-GMP prompted us to study the influence of RsmA on expression of all 37 genes in Xcc encoding proteins involved in cyclic di-GMP turnover: diguanylate cyclases with a GGDEF domain and phosphodiesterases with an EAL or HD-GYP domain [24]. The regulatory influence of RmsA on cyclic di-GMP metabolism could be exerted directly, through binding to transcripts of genes encoding these proteins, although more...
indirect regulation is certainly possible. In order to address these issues, we examined the effect of rsmA mutation on the transcription of genes encoding proteins with GGDEF, EAL or HD-GYP domains by qRT-PCR and also assessed the physical binding of RsmA to transcripts by electrophoretic mobility shift assays (EMSA).

Mutation of rsmA led to an alteration in the transcript level of seven genes (XC_0831, XC_1803, XC_1824, XC_2228, XC_2715, XC_2224 and XC_2866), which were all elevated in the mutant compared to the wild-type (Fig. 2). These genes encode proteins with a GGDEF domain or with both GGDEF and EAL domains (Fig. S1). The EMSA assays showed that RsmA interacted directly with the transcripts of five of these genes (XC_1803, XC_1824, XC_2228, XC_2715 and XC_2866). However RsmA did not interact with transcripts of XC_0831 or XC_2324 or with any gene whose transcript level was unaltered (Fig. 3). The mobility shifts were reversed by competition with unlabelled RNA of the same sequence, but not by unrelated RNAs (data not shown), which indicated that the interactions were specific.

The possibility that RsmA regulates the expression of XC_1803, XC_1824, XC_2228, XC_2715 and XC_2866 was further investigated using fusions to gusA (see Methods). Differences in the level of GusA expression between the rsmA mutant and wild-type were seen with fusions to XC_1803, XC_2715 and XC_2866, which encode GGDEF domain proteins but not with fusions to XC_1824 and XC_2228, which encode GGDEF/EAL domain proteins (Fig. 4). Taken together, the findings indicate that RsmA regulates expression of three GGDEF domain proteins and are consistent with the contention that regulation occurs at the post-transcriptional level. We speculate that RsmA binding promotes enhanced degradation of transcripts, thus reducing their relative level in the wild-type compared to the rsmA mutant. The mechanism by which RsmA affects the transcript levels of XC_0831 and XC_2324 is unclear however.

**Mutation of Genes Encoding Target GGDEF Domain Proteins Reduces the Level of Cyclic di-GMP in the rsmA Background**

The elevated expression of the GGDEF domain proteins XC_1803, XC_2715 and XC_2866 in the rsmA mutant suggested that these putative diguanylate cyclases were responsible for the higher cellular level of cyclic di-GMP in this strain. In the first step towards testing this hypothesis, all three GGDEF domain proteins were purified as His6-tagged proteins and their activity in cyclic di-GMP synthesis was assessed (see Supporting Information). All three proteins were active as diguanylate cyclases (Fig. S2). Following this, the effect of mutation of XC_1803, XC_2715 and XC_2866, both singly and in combination on the level of cyclic di-GMP in the rsmA mutant of Xcc was examined. The findings (Fig. 5) showed that although single mutations had no effect, double mutants reduced the level towards wild-type, while the triple mutation reduced the level of cyclic di-GMP in the rsmA mutant to wild-type levels. The triple mutation in the wild-type background had little effect on the cyclic di-GMP levels however. These findings link alteration in level of the three GGDEF domain proteins in the rsmA mutant to alteration in cyclic di-GMP.

![Figure 5. Combinatorial mutation of three genes encoding GGDEF domain proteins restores cyclic di-GMP levels of the rsmA mutant towards wild-type.](image-url)

The relative levels of cyclic di-GMP in the rsmA mutant background upon disruption of the XC_1803, XC_2715 and XC_2866 genes singly or in combination were determined as described in Methods. Only mutation of all three genes had a significant effect on cyclic di-GMP levels of the rsmA mutant. Values given are the mean and standard deviation of triplicate measurements (three biological and three technical replicates).

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Mutation of Genes Encoding the GGDEF Domain Proteins XC_1803, XC_2715 and XC_2866 Alters Biofilm Formation by the rsmA Mutant

As described above, mutation of rsmA leads to aggregation in liquid medium and an increased biofilm formation in glass tubes without shaking [Fig. 1; [21]]. To ascertain if the GGDEF domain proteins XC_1803, XC_2715 and XC_2866 contribute to these phenotypes of the rsmA mutant, double and triple mutants that were constructed in the rsmA mutant background (see above) were assessed for biofilm formation and aggregation. The triple mutation in the rsmA background gave rise to planktonic dispersed growth in liquid medium (Fig. 6A) and a similar biofilm phenotype to that of the wild-type strain as measured by the crystal violet staining assay (see Methods for details). In contrast mutation of XC_1803, XC_2715 and XC_2866 alone or in pairwise combination has little or no effect on biofilm formation in the rsmA mutant. These findings indicate that the action of rsmA in the regulation of aggregation and biofilm formation is exerted through its influence on the expression of XC_1803, XC_2715 and XC_2866.

Cyclic di-GMP Influences Expression of manA and xagA Genes in a Clp-dependent Manner

The findings outlined above raise the question of how the elevation of levels of cyclic di-GMP in the rsmA mutant is linked to the formation of biofilms in Xcc. One possible link is through the cyclic di-GMP-responsive transcriptional regulator Clp (XC_0486). Previous studies have shown that Clp binds target DNA in the absence of any effector and that DNA binding is inhibited by cyclic di-GMP [25,26]. Expression of the manA gene

Figure 6. Combinatorial mutation of three genes encoding GGDEF domain proteins in the rsmA mutant background prevents aggregation and reduces biofilm formation towards wild-type. (A) A triple disruption of XC_1803, XC_2715 and XC_2866 background prevents aggregation of the rsmA mutant, although single or double mutants (such as XC_1803/XC_2715 illustrated) have little or no effect. (B) A triple disruption of XC_1803/XC_2715/XC_2866 in an rsmA mutant background leads to reduction in biofilm to a level similar to that of the wild-type strain as measured by the crystal violet staining assay (see Methods for details). In contrast mutation of XC_1803, XC_2715 and XC_2866 alone or in pairwise combination has little or no effect on biofilm formation in the rsmA mutant. Values presented are the means and standard deviations from triplicate measurements (three biological and three technical replicates).

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encoding the biofilm dispersing enzyme endo-mannanase is dependent on Clp [17] and is decreased in the rsmA mutant [21].

These previous observations were extended to examine expression of both manA and xagA, which is required for biofilm formation, in both rsmA and clp mutants using qRT-PCR. As expected, the level of manA transcript in both rsmA and clp mutants was reduced compared to the wild-type (Fig. 7A). Complementation restored manA transcript levels towards wild-type (Fig. 7A).

In contrast, mutation of either rsmA or clp led to increased transcription of xagA (Fig. 7B). Complementation restored xagA transcript levels towards wild-type (Fig. 7B).

EMSA analysis showed that Clp bound to the promoter of both the xagA and manA genes in the absence of cyclic di-GMP (Fig. 7C,D). As expected, addition of the dinucleotide inhibited binding to both promoters (Fig. S3 and data not shown). These observations suggested that Clp has divergent action at different promoters, acting as an activator of manA but a repressor of xag.

The possible action of Clp as a repressor of xag gene expression was further investigated using a xag-gusA fusion in which the promoter of xagA (211 nt) drives expression of gusA (see Methods).

This construct was introduced into the wild-type and clp mutant. The levels of beta-glucuronidase were higher in the clp mutant compared to the wild-type background (Fig. 8). Complementation of the clp mutant led to restoration of gusA expression to levels seen in the wild-type background (Fig. S4). This experiment was repeated with a xagA promoter in which the putative binding site for Clp (TGTGA-N6-TCGAT) was altered by site directed mutagenesis to AAAAA-N6-TCGAT as described in Methods. This alteration prevented Clp binding to the promoter as measured by EMSA and led to higher levels of beta-galactosidase in the wild-type background than those seen with the native promoter (Fig. 8). In the clp mutant background however, both native and altered promoters gave similar levels of beta-galactosidase. Taken together, the findings are consistent with an action of Clp as a repressor of xag gene expression, where cyclic di-GMP binding relieves repression.

**Discussion**

The work in this manuscript provides insight into the mechanisms by which the post-transcriptional regulator RsmA...
exerts a regulatory influence on biofilm formation in Xcc. The findings support a model by which RsmA influences the expression of three genes encoding GGDEF domain proteins that leads to alteration in cellular levels of cyclic di-GMP and consequent divergent effects on the expression of manA and xag gene expression by the Clp transcriptional regulator (Fig. 9). The influence of RsmA on expression of specific GGDEF domain proteins is similar to that described previously in E. coli and Salmonella, where the RsmA homolog CsrA has been shown to directly influence expression of GGDEF domain proteins (Jonas et al., 2008; 2010). We do not exclude the possibility that RsmA has additional direct or indirect effects on manA or xag gene expression however, although RsmA does not affect transcription of the clp gene (Fig. S4).

An intriguing observation in the current work is that mutation of clp leads to a down-regulation of expression of manA [5 and data not shown], that is associated with biofilm dispersal but an upregulation of xag gene expression, which is involved in biofilm formation [6]. The most parsimonious explanation for this finding is that Clp can act both as an activator and repressor of transcription of different genes. Similarly, other members of the CRP-FNR family (to which Clp belongs) have been shown to act both as activators and repressors [27,28]. The effects of mutation of the putative Clp box on expression of the xag-lacZ reporter is consistent with a direct role for Clp as a repressor but we cannot exclude the possibility that regulation of xag gene expression by Clp is more indirect, for example by positive regulation of a distinct repressor protein. It is noteworthy that mutation of clp does not lead to an aggregated phenotype in Xcc ([29]; and data not shown). This suggests that regulation of Clp is necessary, but not sufficient for biofilm formation and that other cyclic di-GMP-influenced processes are also required.

The formation of biofilms in many bacteria is responsive to diverse environmental cues or stimuli and several elements in the proposed regulatory pathway are likely to be responsive to such cues. A body of work on different bacteria has shown that small RNAs modulate the activity of RsmA (CsrA) by sequestration to prevent binding to transcripts of target genes. The small RNAs that are presumed to fulfill this role in Xcc have yet to be described. Importantly, in other bacteria, the synthesis of these regulatory RNAs is regulated by two component systems that recognize as yet unknown signals. RsmA in Xcc regulates expression of three GGDEF domain proteins, two of which contain additional domains: XC_1803 contains a GAF domain and XC_2715 has a putative periplasmic binding domain. This suggests that the enzymatic activities of these proteins in cyclic di-GMP synthesis are responsive to environmental cues.

The Rpf/DSF cell-cell signalling system also influences biofilm dispersal and inversely regulates manA and xag gene expression. The Rpf/DSF system is linked to alterations in the level of cyclic di-GMP through the action of RpfG as a cyclic di-GMP phosphodiesterase. However RsmA does not influence DSF signalling in Xcc and Rpf/DSF does not act to regulate expression of XC_1803, XC_2715 or XC_2666. This suggests that the DSF-dependent and RsmA-dependent pathways controlling biofilm dynamics intersect at the level of cyclic di-GMP, although other levels of interplay are possible.

In conclusion, the work described in this paper adds to an understanding of mechanisms by which Xcc controls biofilm formation in response to diverse environmental signals [5,6]. Our knowledge of these processes remains fragmentary however and there a large number of questions that remain to be answered. For example, what is the substrate for ManA and what is the polymer whose synthesis is directed by the Xag proteins? What other factors are required for biofilm formation? Finally (and of particular relevance to the work described here), what are the small RNAs that we presume to act to modulate RsmA action and how are they in turn regulated?

Methods

Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and mutants used in this study are listed in Table S1. Bacterial culture conditions have been previously described (Barber et al., 1997; Slater et al., 2000). L. medium contains Bactotryptone (Difco), 10 g/l; yeast extract, 5 g/l; sodium chloride, 5 g/l; and D-glucose, 1 g/l. NYGB medium contains Bacteriological Peptone (Oxoid, Basingstoke, U.K.), 5 g/l; yeast extract (Difco), 3 g/l; and glycerol, 20 g/l.

General Molecular Biology Methods

Common molecular biological methods such as isolation of plasmid and chromosomal DNA, polymerase chain reaction (PCR), plasmid transformation as well as restriction digestion were carried out using standard protocols (Sambrook et al., 1989). PCR products were cleaned using the QIAquick PCR purification kit (Qiagen) and DNA fragments were recovered from agarose gels using QIAquick mini-elute gel purification kit (Qiagen). Primer sequences are provided in Table S2.

Cellular Quantification of Cyclic di-GMP

Cyclic di-GMP was quantified as described previously [11] in bacterial strains grown to an OD at 600 nm of 0.8 in NYGB medium.

Figure 8. The transcription of xagA is negatively regulated by Clp. The action of Clp as a repressor of xagA was investigated using a xag-gusA transcriptional fusion. β-glucuronidase (GUS)-activity was determined in the wild-type and clp mutant as described in the Methods. The GUS activity was higher in the clp background than in wild-type background, consistent with role for Clp as a repressor. A clp transcriptional fusion in which the putative binding site for Clp was altered by site directed mutagenesis to AAAAA-N6-TCGAT (to generate the xag*-gusA transcriptional fusion) gave higher GUS-activity in the wild-type than seen with the native reporter, indicating a role for Clp binding in repression. In the clp mutant background, GUS activity directed by xag-gusA and xag*-gusA were similar. Bars show mean values and standard deviation from three independent experiments. doi:10.1371/journal.pone.0052646.g008
Construction of \textit{gusA} Reporter Plasmids

Reporter plasmids \textit{pG1803}, \textit{pG1824}, \textit{pG2228}, \textit{pG2715} and \textit{pG2866} were constructed by cloning the putative promoter region and ribosome binding site (500-bp region upstream of the start codon) of \textit{XC\_1803}, \textit{XC\_1824}, \textit{XC\_2228}, \textit{XC\_2715} and \textit{XC\_2866} respectively into the broad-host-range cloning vector \textit{pL6gus} (Table S1), which harbors the coding region (without promoter and RBS) of \textit{b-glucuronidase (gusA}) gene in its MCS (multiple cloning site). The putative promoter region and RBS of \textit{XC\_1803}, \textit{XC\_1824}, \textit{XC\_2228}, \textit{XC\_2715} and \textit{XC\_2866} were amplified from the chromosomal DNA of \textit{Xcc\_8004} using the primer pairs detailed in Table S2. All constructs were sequenced to confirm the absence of mutations.

The \textit{xagA} reporter plasmid \textit{pGUSXAGA} was constructed by cloning the intergenic region 211 bp before the \textit{xagA} gene into the broad-host-range cloning vector \textit{pLAFRJ}, which harbours the promoterless \textit{b-glucuronidase (gusA}) gene in its MCS (multiple cloning site). The 211 bp region upstream of the start codon (not including GTG) was amplified by PCR using the total DNA of the wild-type strain \textit{8004} as the template (primers available upon request). The amplified DNA fragment, confirmed by sequencing, was inserted 9 bp upstream of the promoterless \textit{gusA} ATG start codon in the vector \textit{pLAFRJ} to create the recombinant plasmid \textit{pGUSXAGA} (Table S1). The recombinant plasmid obtained was further confirmed by restriction analysis and PCR. All reporter plasmids were introduced into the \textit{Xcc} strains of interest through conjugation as previously described [30].

RNA Isolation and Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) for Transcription Analysis

RNA was isolated from \textit{Xanthomonas} strains of interest as previously described (McCarthy et al., 2008). Expression of the genes \textit{XC\_1803}, \textit{XC\_1824}, \textit{XC\_2228}, \textit{XC\_2715} and \textit{XC\_2866} were monitored in both wild-type and \textit{rsmA} mutant backgrounds. Bacteria were grown to an OD\textsubscript{600} nm of 0.8 in NYGB media and RNA was sampled. Total RNA (5 \mu l per sample) was reverse transcribed to give cDNA, using primers detailed in Table S2. Quantitative RT-PCR protocols were used as described previously [30,31].
Over-expression and Purification of GST-tagged Clp

For purification of proteins that were fused to the glutathione S-transferase (GST), gel filtration was carried using a Sephadex G-75 (Sigma) as previously described [9,10]. Protein concentration was assayed using Nanodrop®. Purified proteins were stored at -20°C.

Electrophoretic Gel Mobility Shift Assays

The DNA probes used for EMSA were prepared by PCR amplification of the desired manC and sagD upstream regions, using oligonucleotides as the primers (primers available upon request). The Clp protein, the binding conditions, and detection procedures were as previously described [32]. Briefly, the purified PCR products were 3' end-labelled with digoxigenin following the manufacturer’s instructions (Roche). The EMSA was carried out using the Gel Shift Kit 2nd Generation (Roche) as recommended by the manufacturer with some modifications. Ten fmol of the DIG-labelled fragment and a range of 0–50 μM of Clp protein were added to the binding reaction. The mixture was allowed to proceed at room temperature for 45 min. The samples were separated by electrophoresis on 6% native polyacrylamide gels and transferred to Hybond-N blotting membrane (Amersham). Protein–DNA complexes were visualized using the Detector™ AP Chemiluminescent Blotting Kit (KPL, Gaithersburg, MD, USA) according to the manufacturer’s instructions (Roche).

RNA Gel Mobility Shift Assays

RNA gel mobility shift assays were carried out as previously reported by Yakhnin et al., [33]. Briefly, DNA templates for the generation of the targeted RNA transcripts were produced using wild-type strain 8004 DNA template and the specific primer pairs listed in Table S2. These DNA fragments were transcribed into RNA using the MEGashortscript kit (Ambion) following the manufacturer’s instructions. After gel purification, the 3’ ends of these transcripts were labeled with biotin using the Pierce RNA 3’ End Biotinylation Kit (Thermo Fisher Scientific). Biotin-labeled RNA was gel-purified and re-suspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), heated to 85°C and chilled on ice. Then 400 nM His6-RsmA protein and 80 pM Biotin-labeled RNA were added into a tube containing 10 μl of binding reaction systems [10 mM Tris-HCl pH 7.5, 10 mM MgCl2, 100 mM KCl, 3.25 ng total yeast RNA, 20 mM DTT, 7.5% glycerol, 4 U SUPERasin (Ambion, Austin, TX)], and mixed well. The mixtures were incubated at 28°C for 30 min to allow RsmA–RNA complex formation. Binding reaction mixtures were separated using 12% native polyacrylamide gels, and signal bands were visualized with using the Detector™ AP Chemiluminescent Blotting Kit (KPL, Inc., Gaithersburg, MD, USA) according to the manufacturer’s instructions.

Biofilm Assays

Biofilm was assessed by: (i) Aggregation in L medium as described previously [3] and (ii) Attachment to glass was determined by crystal violet staining. Where log-phase-grown bacteria were diluted to OD600 nm = 0.02 in L media broth, and 5 ml was incubated at 30°C for 24 h in 14-ml glass tubes. After gently pouring off the media, bacterial pellicles were washed twice with water and were then stained with 0.1% crystal violet. Tubes were washed and rinsed with water until all unbound dye was removed [21,24,34]. Three independent assays were carried out for each strain.

Supporting Information

Figure S1 Domain organization of the various GGDEF, EAL, GGDEF-EAL domain-containing proteins, identified by analysis of the annotated genome sequence of Xcc 8004. Domains were assigned according to Pfam and SMART [http://smart.embl-heidelberg.de/].

Figure S2 The isolated GGDEF domains of XC_1803, XC_2715 and XC_2866 possess cyclic di-GMP cyclase activity. The DNA encoding the GGDEF domain from XC_1803, XC_2228 or XC_2866 with a C-terminal His6 tag was cloned by PCR using oligonucleotide primers described in Table S2. The His-6-tagged proteins were purified by nickel affinity chromatography and analyzed by SDSPolyacrylamide gel electrophoresis with Coomassie blue staining using similar methods as described previously [Ryan et al., 2006; 2010]. Lane 1, molecular mass markers; lane 2, XC_1803 (~20 kDa); lane 3, XC_2715 (~20 kDa) and XC_2866 (~35 kDa). (B) Di-guanylate cyclase activity of the GGDEF domains from XC_1803, XC_2228 and XC_2866. GTP (top; 0 min) and products (bottom; 60 min) of the reaction, separated by reversed-phase HPLC. The standard reaction mixture (total volume, 200 μl) contained 5 μM enzyme in 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 0.5 mM EDTA, and 50 mM NaCl. The reaction was started by the addition of 50 μl of substrate (final concentration, 150 μM) to the prewarmed reaction mixture and was carried out for 60 min. The mixture was immediately placed in a boiling water bath for 3 min, followed by centrifugation at 15,000xg for 2 min. The supernatant was filtered through a 0.22 μm-pore-size filter and analyzed by high-pressure liquid chromatography (HPLC), as described previously [9]. The presence or absence of cyclic di-GMP in the HPLC fractions was confirmed by mass spectrometry. Y-axis although not shown are units of milliabsorbance.

Figure S3 EMSA assessment of the impact of cyclic di-GMP on Clp binding to the sagD promoter. DIG-labeled promoter fragments were incubated with purified Clp protein in the absence or presence of nucleotide, as indicated.

Figure S4 The expression of the clp gene is unaltered in an rsmA mutant. Transcript level of the gene that encodes the Clp protein was measured in wild-type strain (8004) and rsmA mutant backgrounds by qRT-PCR as described in the Methods. Bars show mean fold changes obtained from three independent experiments.

Table S1 Bacterial strains and plasmids used in this study.

Table S2 Primers used throughout this study.

Author Contributions

Conceived and designed the experiments: RPR, JMD DJT. Performed the experiments: XL SA YM RPR. Analyzed the data: XL SA YM RPR, JMD DJT. Contributed reagents/materials/analysis tools: XL SA YM RPR, JMD DJT JLT. Wrote the paper: SA YM RPR, JMD DJT.
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