A Cyclic di-GMP Network Is Present in Gram-Positive Streptococcus and Gram-Negative Proteus Species

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ABSTRACT: The ubiquitous cyclic di-GMP (c-di-GMP) network is highly redundant with numerous GGDEF domain proteins as diguanylate cyclases and EAL domain proteins as c-di-GMP specific phosphodiesterases comprising those domains as two of the most abundant bacterial domain superfamilies. One hallmark of the c-di-GMP network is its exalted plasticity as c-di-GMP turnover proteins can rapidly vanish from species within a genus and possess an above average transmissibility. To address the evolutionary forces of c-di-GMP turnover protein maintenance, conservation, and diversity, we investigated a Gram-positive and a Gram-negative species, which preserved only one single clearly identifiable GGDEF domain protein. Species of the family Morganellaceae of the order Enterobacterales exceptionally show disappearance of the c-di-GMP signaling network, but Proteus spp. still retained one diguanylate cyclase. As another example, in species of the bovis, pyogenes, and salivarius subgroups as well as Streptococcus suis and Streptococcus henryi of the genus Streptococcus, one candidate diguanylate cyclase was frequently identified. We demonstrate that both proteins encompass PAS (Per-ARNT-Sim)-GGDEF domains, possess diguanylate cyclase catalytic activity, and are suggested to signal via a PilZ receptor domain at the C-terminus of type 2 glycosyltransferase constituting BcsA cellulose synthases and a cellulose synthase-like protein Cea, respectively. Preservation of the ancient link between production of cellulose-like exopolysaccharides and c-di-GMP signaling indicates that this functionality is even of high ecological importance upon maintenance of the last remnants of a c-di-GMP signaling network in some of today’s free-living bacteria.

KEYWORDS: cellulose biosynthesis, cyclic di-GMP signaling, EAL domain, GGDEF domain, Proteus mirabilis, Streptococcus galgallicus subsp. galgallicus

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ignaling systems couple sensing and information trans-
mission and amplification in order to adapt physiology and metabolism to changing external and internal stimuli. Thus, those modules are highly prone to mutation and/or horizontal gene transfer. The cyclic dinucleotide (CDN) molecule bis(3′,5′)-cyclic diguanosine monophosphate (c-di-GMP), identified in 1987 as an allosteric activator of the cellulose synthase in the bacterium Komagataeibacter xylinus (previously Gluconacetobacter (Acetobacter) xylinus (G. xylinus)) is the most abundant CDN-based second messenger signaling system in bacteria. Cyclic-di-GMP regulates a multitude of fundamental physiological and metabolic processes, such as single cell motility-to-sessility transition with the promotion of biofilm formation, chronic versus acute virulence, antimicrobial and detergent tolerance, cell cycle progression, nutrient acquisition, electron transfer, and cell morphology. Essential signaling modules of this pathway comprise the GG(D/E)EF domain with diguanylate cyclase (DGC) activity and the EAL and HD-GYP domain with phosphodiesterases (PDE) activity. The GG(D/E)EF domain synthesizes c-di-GMP in a two-step reaction with S′-pppGpG as an intermediate and two molecules of pyrophosphate as byproducts. The EAL- and HD-GYP domains hydrolyze c-di-GMP into linear 5′-pGpG and GMP, respectively. Numerous proteins are bifunctional through a combination of GGDEF with EAL/HD-GYP domains.

In these three superfamilies, catalytic domains have evolved into receptors or act though protein–protein interactions. Although an intact GG(D/E)EF motif is usually an indicator for catalytic activity, due to the requirement of extended consensus motifs, the presence of such a motif and even the presence of extended consensus signature motif(s), including ligands binding divalent ion required for catalytic activity, is not a guarantee for catalytic activity or substrate specificity and vice versa.

The activity of DGCs and PDEs is controlled by a diversity of N-terminal sensory domains that receive and respond to...
various signals, such as oxygen, nucleotide-based small molecules, and light.\textsuperscript{13–15} Thereby, the most frequently associated N-terminal signaling domain in this context is the versatile PAS (Per-ARNT-Sim) domain.\textsuperscript{16,17} With diverse primary sequences of less than 150 amino acids in size, compact PAS domains possess an interior pocket built characteristically by five antiparallel $\beta$-strands and flanked by a few $\alpha$-helices to host a variety of prosthetic groups and ligands, with few functional amino acids to determine the binding specificity.\textsuperscript{16,18}

Furthermore, signaling of c-di-GMP is translated through protein and RNA-based receptors such as the PilZ domain, MshEN domain, the inhibitory I-site of GGDEF domains, inactive EAL/HD-GYP domains, various classes of transcription regulators, and distinct RNA aptamers.\textsuperscript{5,19–21} PilZ domains, the first c-di-GMP receptors discovered, are widespread among bacteria.\textsuperscript{22,23} As a fundamental mechanism, the catalytic activity of the cellulose synthase BcsA and other exopolysaccharide synthases is regulated by PilZ domains.\textsuperscript{24} Riboswitches, consisting of a high affinity CDN binding RNA aptamer and an expression platform located within the 3$'$-untranslated region (3$'$-UTR), respond to c-di-GMP binding with conformational changes that alter downstream transcriptional termination, translation, and ribozyme activity.\textsuperscript{19,20} Two classes of c-di-GMP responsive riboswitches, type I and type II, with Genes for the Environment, for Membranes and for Motility (GEMM) motifs have been identified in Gram-negative and Gram-positive species, such as \textit{Vibrio cholerae}, \textit{Geobacter metallireducens}, and \textit{Clostridium difficile}. Compared with protein receptors, which have dissociation constants ($K_d$) in the low $\mu$M range for c-di-GMP, RNA aptamers have dissociation constants in the nanomolar range.

In many instances, cyclic di-GMP activates biofilm formation, a ubiquitous multicellular sessile lifestyle of bacteria and represses motility.\textsuperscript{25} This physiological regulation by c-di-GMP is evolutionarily conserved from ancient thermophiles to human pathogens where biofilm formation contributes to chronic infections.\textsuperscript{26,27}

In this study, we identified novel DGCs in two pathogens, the Gram-negative \textit{Proteus mirabilis} UEB50 and Gram-positive \textit{Streptococcus gallolyticus} subsp. \textit{gallolyticus} UCN34.\textsuperscript{28} (\textit{S. gallolyticus}). Previously, to our knowledge, a functional c-di-GMP signaling network has not been reported for those two species. Moreover, we verified the catalytic activity of the DGCs in \textit{vivo} by diverse experimental approaches, for instance, regulation of downstream protein production by chromosomally integrated c-di-GMP specific Vc1 and Vc2 translational riboswitches, a rapid cell-lysate-based matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS)-based screening approach, and detection of c-di-GMP by standard liquid chromatography-mass spectrometry (LC-MS/MS) of cell extracts. Combined with phenotypic analyses to promote rdar (red, dry, and rough) biofilm formation and motility in the heterologous host \textit{Salmonella typhimurium}, our results demonstrate PMI3101\textsubscript{v} and GGDEFUCN34 to be active DGCs. Bioinformatic and gene synteny analyses predict that c-di-GMP produced by PMI3101\textsubscript{v} and GGDEFUCN34 activates exopolysaccharide biosynthesis in their native hosts by binding to an C-terminal PilZ domain of a type 2 glycosyltransferase. Of note, the c-di-GMP modules in streptococcal species were found to be highly variable with respect to the location and genetic context with some modules even containing predicted EAL phosphodiesterases. Collectively, our results demonstrate the presence of a functional c-di-GMP signaling network predominantly in...
species of a phylogenetically distinct branch of the genus \textit{Streptococcus} and maintenance of c-di-GMP regulated cellulose biosynthesis in \textit{Proteus} spp., which poses the question of the ecological importance of the conservation of those signaling pathways.

\section*{RESULTS}

\textbf{Phylogeny of the c-di-GMP Signaling Network in Selected Gram-Negative and Gram-Positive Genera.}

The c-di-GMP signaling network can rapidly alter even on a short evolutionary scale. Within a genus, species can possess distinct c-di-GMP networks, highly variable in numbers and types of GGDEF and EAL domain proteins (https://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html). Disappearance of signaling systems including the c-di-GMP signaling network can be triggered by a substantial lifestyle change toward a parasitic invasive intracellular lifestyle. An extreme lifestyle adaptation is even manifested as a dramatic reduction of genome size with a concomitant reduction of all signaling networks. Additional reasons for a dramatic deterioration of bacterial signaling systems are unknown. In addition, c-di-GMP turnover proteins, as preferentially encoded on transmissible plasmids and enhancing conjugative transfer, have a statistically significant higher likelihood to be horizontally transferred. We are interested to examine the evolutionary forces, which cause dramatic consistent alterations in the c-di-GMP signaling network. Within the class of \textit{γ}-proteobacteria, the Enterobacteriales order consists of the families Enterobacteriaceae, Erwiniaceae, Yersiniaceae, Pectobacteriaceae, Hafniaceae, Budviciiaceae, and Morganellaceae. Cyclic di-GMP signaling systems of species of the type genera \textit{Escherichia}, \textit{Erwinia}, \textit{Yersinia}, and \textit{Dicyea} of the four first families, which usually possess a high density of c-di-GMP turnover proteins, have been well investigated. Examples are \textit{Escherichia coli} K-12 MG1655 (6.3 c-di-GMP turnover proteins per Mbp (6.3/Mbp) at a genome size of 4.64 Mbp), \textit{Salmonella typhimurium} ATCC14028 (4.4/Mbp; genome size: 4.96 Mbp), \textit{Klebsiella pneumoniae} subsp. pneumonia (5.3/Mbp; genome size: 5.33 Mbp), \textit{Erwinia amylovora} ATCC49946 (3.2/Mbp; genome size: 3.8 Mbp), \textit{Yersinia enterocolitica} subsp. enterocolitica 8081 (4.6/Mbp; genome size: 4.55 Mbp), \textit{Serratia marcescens} subsap. marcescens Db11 (4.0/Mbp; genome size: 5.11 Mbp), and \textit{Dickeya dianensis} DSM18020 (5.6/Mbp; genome size: 4.82 Mbp). Equally, the less investigated genera of the families Hafniaceae and Budviciiaceae possess a significant number of c-di-GMP turnover proteins (Figure 1A). In contrast, we noticed that, within the family Morganellaceae, which consists presently of the eight genera \textit{Arsenophonus}, \textit{Cosenzaea}, \textit{Moellerella}, \textit{Morganella}, \textit{Photobadus}, \textit{Proteus}, \textit{Providencia}, and \textit{Xenorhabdus}, sequenced genomes from representative species of all genera, with the exception of \textit{Xenorhabdus nematophila} ATCC19061, are consistently missing functional c-di-GMP turnover proteins (Figure 1A, Supporting Table S1). Species of the genera \textit{Proteus} and \textit{Cosenzaea} represented by, for example, \textit{P. mirabilis} HI4320, \textit{Proteus hauseri} ATCC700826, \textit{Proteus vulgaris} ATCC49132, and \textit{Cosenzaea myxofaciens} ATCC19692 (WP_006749622.1) have, however, still retained a single GGDEF domain protein. The family of Morganellaceae encompasses predominantly environmental bacteria with...
a genome size of 3.8 Mbp or higher, although symbionts with highly reduced genome size are present, e.g., within the genus *Arsenophonus*. The reason for the disappearance of the c-di-GMP network in most species of the family Morganellaceae, equally as its reduced retention in genus *Proteus* spp. is not obvious.

Upon the reduction of genome size due to habitat restriction, signaling networks are not only reduced but can become (partially) impaired in functionality. For example, in the human-adapted species *Staphylococcus aureus* and *Staphylococcus epidermidis*, the remaining GGDEF domain proteins have lost their catalytic activity, although they provide physiological functionalities, which manifest, for instance, through protein–protein interactions.7,8 The family of Streptococci is composed of three different genera, *Streptococcus*, *Lactococcus*, and *Lactovum*. Performing a BLAST search,33 we discovered that strains of distinct streptococcal species, among them *S. gallyoticus*, *Streptococcus infantarius*, *Streptococcus equinus*, *Streptococcus lutetensis*, *Streptococcus salivarius*, *Streptococcus vestibularis*, and *Streptococcus agalactolyticus* as well as the unassigned species *Streptococcus suis* and *Streptococcus henryi* (Figure 2A and data not shown), encode one GGDEF domain DGC candidate with a similar domain structure (see below). Those streptococcal species mainly belong to one of two distinct phylogenetic lineages of the genus *Streptococcus*, which includes the Bovis, Pyogenic, and Salivarius subgroups (Figure 2A34), suggesting particular evolutionary forces for the maintenance of a DGC.

**Basic Characteristics of GGDEF Domain Proteins from *P. mirabilis* and *S. gallyoticus Subspecies gallolyticus***

As the human pathogen *P. mirabilis* is the most well investigated *Proteus* species, we decided to assess the GGDEF domain protein of *P. mirabilis* UEB50,35 an isolate from a urinary catheter (Figure 1B). PMI3101_v, a homologue of PMI3101 from *P. mirabilis* HI4320, differs from PMI3101 by the N133T exchange. However, proteins identical to PMI3101_v are present in *P. mirabilis* strains deposited in the NCBI database.
Alignment of PMI3101 with previously functionally characterized GGDEF domain proteins indicated that the GGDEF domain of PMI3101 possesses a conserved RxGGDEF motif, the lysine that stabilizes the transition state and all amino acids involved in substrate binding as identified in the DGC PleD besides the homologue of arginine446 (Figure 1C and Figure 3A, B). No c-di-GMP binding RxxD inhibitory (I)-site motif is present.38 A class 9 PAS domain (residues 25–123) is N-terminal linked to the GGDEF domain (residues 135–293). The same GGDEF protein domain architecture is found in P. vulgaris, Proteus columbae, Proteus alimen torum, P. hauseri, and Proteus genomosp. 6 (Figure 1B and Figure 3). Outside of the genus Proteus, homologous proteins are present in C. myxofaciens (Supporting Table S1). A structural model of the PAS-GGDEF domain showed the closest structural homology to the PAS domain of PA0861 (PDB: 5XGD chain A), a P. aeruginosa PAS-GGDEF-EAL domain protein. A S-helix-like linker region connects the PAS and GGDEF domain (Supporting Figure S1). Of note, some close homologues of the protein in species outside the Proteus genus are more complex, implying that a modulation of signaling with the catalytic GGDEF domain. Close GGDEF-U CN34 homologues (>80–90% amino acid sequence identity) are present in isolates of other species of the bovis subgroup such as S. equinus, S. lutetiens, S. infantarius, and Streptococcus macedonicus (>60% identity), in the salivarius subgroup such as in the probiotic species S. salivarius and Streptococcus thermophilus and the commensal Streptococcus vestibularis (>90% identity), and unassigned Streptococcus spp. from those two subgroups (Figure 2A). The presence of a GGDEF-U CN34 homologue in the mitis subgroup member Streptococcus rubneri (data not shown) suggests that lifestyle rather than taxonomic relationship might determine the
presence of a GGDEF domain protein, however, more detailed investigation of the prevalence of GGDEF domain proteins in species of the mitis/mutans/anginosus subgroups requires additional analysis. Furthermore, homologous proteins with the same domain structure, partially lacking one or both transmembrane helices and with a low sequence identity, are harbored in pyogenes subgroup members such as *S. parauberis* (approximately 25% identity compared to GGDEFUCN34), *S. uberis* (25% identity), and *Streptococcus dysgalactiae* (18% identity) but also *S. henryi* (44% identity) and *S. suis* (25% identity), which are not assigned to a subgroup (Figure 2A). Outside of the *Streptococcus* genus proteins, homologues over the entire length are found in *Weissella soli* (WP_070230170.1) and *Lactococcus* spp. (WP_096819183) (data not shown). Of note, other close homologues of the protein in species outside the *Streptococcus* genus proteins, homologues over the entire length are found in *Weissella soli* (WP_070230170.1) and *Lactococcus* spp. (WP_096819183) (data not shown). Of note, other close homologues of the protein in species outside the *Streptococcus* genus are more complex, implying that a modulation of domain composition through chromosomal recombination or convergent evolution of GGDEF domain proteins has readily occurred (Supporting Figure S3A and B). These observations hint to the still largely unexplored evolutionary plasticity of the c-di-GMP signaling system.

**Assessment of DGC Catalytic Activity by a Riboswitch-Based Screening System.** In order to assess the catalytic functionality of the two candidate DGCs, we used a previously developed riboswitch-based system to detect alterations in c-di-GMP concentrations in vivo (Figure 4A). The genome of the pathogenic bacterium *V. cholerae* encodes two c-di-GMP specific riboswitches, Vc1 and Vc2, located upstream of the *gbpA* and *VC1722* gene, respectively. The Vc1 and Vc2 riboswitches have been characterized as “off” and “on” riboswitches in *V. cholerae* with c-di-GMP to promote and repress the expression of the downstream genes, respectively. Nevertheless, both riboswitches consistently functioned as “off” riboswitches in *E. coli* TOP10 (Supporting Figure S4), probably due to a remodeled conformation of the riboswitch upon binding to cellular components in *E. coli*. Upon overexpression of PMI3101_v, we observed downregulation of Vc2 riboswitch mediated β-galactosidase expression, and upon overexpression of GGDEFUCN34, Vc1 and Vc2 riboswitch dependent β-galactosidase expression was downregulated (Figure 4). In contrast, there was no effect upon overexpression of the catalytic mutants, PMI3101_v_D215A and GGDEFUCN34_D273A. These results suggest that the candidate DGCs produce c-di-GMP when expressed heterologously in *E. coli*.

Furthermore, we observed that induction of GGDEFUCN34 with >0.01% L-arabinose had a substantial cytotoxic effect on *E. coli* TOP10, while cytotoxicity was not observed upon expression of the GGDEFUCN34_D273A catalytic mutant. More-
over, the wild type protein was expressed at a lower level than the inactive mutant (Supporting Figure S5). Cytotoxicity upon overexpression of DGCs has been observed previously in the E. coli BL21 background. The selective cytotoxicity upon overexpression of GGDEFUCN34 but not PMI3101_v in E. coli TOP10 implies a distinct mechanism of action by GGDEFUCN34 due to elevated c-di-GMP synthesis, catalytic activity of GGDEFUCN34, or subsequent c-di-GMP binding.

**PMI3101_v and GGDEFUCN34 Elevate the Intracellular c-di-GMP Concentration.** The riboswitch assay is an indirect approach to assess c-di-GMP levels in vivo. To confirm that PMI3101_v and GGDEFUCN34 can produce c-di-GMP in vivo, we developed a MALDI-FTMS-based screen as a first-line evaluation. We overexpressed PMI3101_v, GGDEFUCN34, and their catalytic mutants in E. coli TOP10 and subsequently used the crude lysates to assess cyclic dinucleotide production by MALDI-FTMS mass spectrometry. MALDI-FTMS determines the weight-to-charge ratio by measuring the flying time of the ionized molecules in the electric field. Because major molecules have a single positive charge, ions are actually separated by their mass. As no extraction of the molecule or isolation of the protein is necessary, this experimental approach can be applied as a rapid screen for DGC activity.

The ionized c-di-GMP, c-di-AMP, and c-GMP-AMP generally have a mass/charge (m/z) ratio [M + H+] of 691.104 (Figure 5), 659.114 and 675.107, respectively. A signal corresponding to c-di-GMP was detectable in the samples from cells expressing PMI3101_v and GGDEFUCN34, whereas no signal was measurable when the catalytic mutants and the vector control were expressed (Figure 5A, Supporting Figure S6), which suggested that c-di-GMP could be synthesized by PMI3101_v and GGDEFUCN34 in E. coli TOP10. Nonetheless, a mass-to-charge of 691.1040 could be created by other molecules with the same molecular weight.

To this end, we monitored the cyclic dinucleotide activity of PMI3101_v and GGDEFUCN34 conventionally by LC-MS/MS after extraction of the molecules. Indeed, again, we observed a high concentration of c-di-GMP upon expression of PMI3101_v and GGDEFUCN34, but concentrations remained unaltered upon expression of the catalytic mutants (Figure 5B). Proteins were expressed in the S. typhimurium background (see below) due to the elevated cytotoxic effect of GGDEFUCN34 in E. coli TOP1.

We also purified PMI3101_v and assessed its enzymatic activity by an in vitro assay analyzing the product by thin-layer chromatography (TLC); however, purified PMI3101_v did not show the expected catalytic activity, as c-di-GMP synthesis was not observed (data not shown). We conclude that either a cofactor(s) and/or signals to stimulate the catalytic activity of the DGC are missing in vitro.

**The DGCs PMI3101_v and GGDEFUCN34 Promote rdar Biofilm Morphotype Expression of S. typhimurium.** Cyclic di-GMP activates a multicellular behavior of S. typhimurium, rdar biofilm morphotype formation with a characteristic red, dry, and rough colony morphology on a Congo Red (CR) agar plate due to expression of extracellular matrix components cellulose and curli fimbria. This behavior serves as a biologically relevant read-out for elevated c-di-GMP levels. To investigate whether the DGCs PMI3101_v and GGDEFUCN34 affect rdar biofilm formation,
we expressed the proteins in *S. typhimurium* UMR1 grown on CR agar plates at 28 °C. The native PMI3101_v and GGDEF<sup>UCN34</sup> but not their catalytic mutants up-regulated the rdar biofilm morphotype (Figure 6A), which added supporting evidence for being catalytically active in *vivo*.

**The DGCs PMI3101_v and GGDEF<sup>UCN34</sup> Suppress Motility of *S. typhimurium*.** The transition from motility to sessility contributes to the development of multicellular behavior with motility to be inhibited by c-di-GMP. Thus, we assessed the effect of PMI3101_v and GGDEF<sup>UCN34</sup> on flagellar-based motility of *S. typhimurium* UMR1, as observed by the apparent motility in a semisolid LB agar plate at 37 °C (similar results were obtained at 28 °C). Wild type PMI3101_v (Figure 6B) and GGDEF<sup>UCN34</sup> (Figure 6C) consistently down-regulated the swimming ability compared to the positive control *S. typhimurium* UMR1, while the corresponding mutants had only a minor effect, which suggested that the two novel GGDEF domain proteins affect flagella-mediated motility by producing c-di-GMP. Noteworthy, the catalytic mutant of GGDEF<sup>UCN34</sup> slightly promoted apparent swimming. This marginal up-regulation of swimming motility by GGDEF<sup>UCN34</sup> D<sup>2673A</sup> is probably caused by an alternative binding site for c-di-GMP, as an I-site is lacking. The mutant of PMI3101_v still suppressed swimming of UMR1 to a small extent compared with the vector control, which can be explained by residual catalytic activity of the mutated GGDEF domain. In summary, suppression of motility by PMI3101_v and GGDEF<sup>UCN34</sup> but not their catalytic mutants again added supporting evidence for the two proteins being catalytically active as DGC *in vivo*.

**Genomic Context of the GGDEF Domain Protein in *Proteus mirabilis*.** Our experiments showed that PMI3101_v of *P. mirabilis* and GGDEF<sup>UCN34</sup> of *S. gallolyticus* are bona fide DGCs. As the sole DGC encoded by the respective chromosome, we were wondering about the genomic context of the gene products and their physiological targets. Investigating the gene synten in *P. mirabilis* HI4320 (as *P. mirabilis* UEB50 has not been sequenced), we found that PMI3101 is embedded into a type-IB-like hybrid cellulose biosynthesis gene cluster consisting of a bcsOABCD-dgcPMI3101-galU-bcsZ structure (Figure 7A<sup>47</sup>). Such a gene cluster is invariably present in representative isolates of *Proteus* species such as *P. vulgaris*, *P. hauseri*, *P. cibarium*, and *P. vulgaris* species such as *P. vulgaris* UEB50 has not been sequenced), we found that PMI3101 is embedded into a type-IB-like hybrid cellulose biosynthesis gene cluster consisting of a bcsOABCD-dgcPMI3101-galU-bcsZ structure (Figure 7A<sup>47</sup>). The catalytic subunit BcsA, which is identical to BcsA of *S. typhimurium* UMR1 grown on LB agar plates at 37 °C (similar results were obtained at 28 °C). Wild type PMI3101_v (Figure 6B) and GGDEF<sup>UCN34</sup> (Figure 6C) consistently down-regulated the swimming ability compared to the positive control *S. typhimurium* UMR1, while the corresponding mutants had only a minor effect, which suggested that the two novel GGDEF domain proteins affect flagella-mediated motility by producing c-di-GMP. Noteworthy, the catalytic mutant of GGDEF<sup>UCN34</sup> slightly promoted apparent swimming. This marginal up-regulation of swimming motility by GGDEF<sup>UCN34</sup> D<sup>2673A</sup> is probably caused by an alternative binding site for c-di-GMP, as an I-site is lacking. The mutant of PMI3101_v still suppressed swimming of UMR1 to a small extent compared with the vector control, which can be explained by residual catalytic activity of the mutated GGDEF domain. In summary, suppression of motility by PMI3101_v and GGDEF<sup>UCN34</sup> but not their catalytic mutants again added supporting evidence for the two proteins being catalytically active as DGC *in vivo*.

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of bcsG-bcsR-bcsQ-bcsA2-bcsB2-bcsC2 is present at a distant location on the *P. mirabilis* chromosome equally as in other *Proteus* species representatives with the exception of the *P. hauseri* isolate (Supporting Figure S8). The cellulose synthase BcsA2, which possesses 53% identity to BcsA of *S. typhimurium*, 36% identity to BcsA1, and 23% identity to BcsA of *R. sphaeroides* harbors a C-terminal PilZ domain for c-di-GMP binding (Supporting Figure S7). Thus, although cellulose biosynthesis has not been observed in *Proteus* spp. (we did not observe cellulose production upon plasmid-based expression of PMI3010_v in *P. mirabilis* UEB50, data not shown), these bioinformatic analyses suggest *P. mirabilis* and other *Proteus* spp. to synthesize cellulose by two different cellulose or cellulose-like biosynthesis operons stimulated by c-di-GMP signaling (Figure 7B; Supporting Figure S8).

**Genomic Context of the DGC GGDEF<sup>UCN34</sup> in *S. gallolyticus* UCN34.** GGDEF<sup>UCN34</sup> (SGGB_RS02220) is the first gene of a five-gene cluster genomic islet inserted into a tRNA<sub>ser</sub>_locus. The two downstream overlapping open reading frames encode two type 2 glycosyltransferases. SGGB_RS02225, which overlaps with SGGB_RS02230 encoding a Dpm1-GtrA hybrid protein and SGGB_RS02230 coding for a cellulose synthase-like protein CelA. The SGGB_RS02235 gene (membrane) encodes an integral membrane protein with 16 predicted transmembrane helices. Other *S. gallolyticus* strains can have a variable islet composition with *S. gallolyticus* BI02 entirely missing the islet. (B) Localization and functionality of gene products of the genomic islet of *S. gallolyticus* UCN34. Two type 2 glycosyltransferases, a Dpm1-GtrA hybrid protein and a cellulose synthase-like protein CelA, are membrane proteins involved in (exo)polysaccharide synthesis. The C-terminal PilZ domain of CelA suggests binding of c-di-GMP for regulation of the catalytic activity. The membrane protein with 16 predicted transmembrane helices and the 60 amino acid protein is of unknown function.
Dpm1 is the catalytic subunit of eukaryotic dolichol-phosphate mannose synthase to which the N-terminal part of SGGB_RS02225 is homologous (Figure 8B). The C-terminal part consists of a GtrA-like protein with four transmembrane helices. GtrA is involved in flipping of undecaprenyl-phosphate glucose over the inner membrane in Gram-negative bacteria. Downstream, SGGB_RS02230 codes for a cellulose synthase-like protein CelA. CelA has <20% identity to all four cellulose synthases and has only four predicted membrane spanning helices, but it contains relevant signature amino acids for catalysis with the crystal structure of BcsA from R. sphaeroides as the best fit (Supporting Figure S7). Intriguingly, CelA has a C-terminal PilZ domain including signature amino acids for c-di-GMP binding, which suggests that CelA binds the CDN to regulate its catalytic activity. The SGGB_RS02235 gene downstream of SGGB_RS02230 encodes an inner membrane protein with 16 predicted transmembrane helices of unknown function followed by a short coding sequence. Besides CelA, none of these proteins have an identifiable c-di-GMP binding site. In summary, CelA is a potential target for the c-di-GMP signaling pathway in S. gallolyticus UCN34 (Figure 8B).

We were wondering whether all strains of S. gallolyticus possess this c-di-GMP signaling islet. BLAST search showed that, of nine entirely sequenced S. gallolyticus isolates, five possess the islet, while four isolates have a partial islet or no insertion (Figure 9A). In other bovis subgroup species such as S. equinus, S. luteniensis, and Streptococcus sp., the islet is present, however, not all strains within a species possess this insertion (Supporting Figure S9 and data not shown). While in S. macedonicus ACA-DC198 and S. infantarius ATCC BAA-102, a part of the islet, but not the DGC, has been retained, Streptococcus pasteurianus strains do not possess the islet. However, this bioinformatics analysis might be considered preliminary for the following reasons: (1) complete genome
sequences not available for all isolates; (2) the paucity of sequenced isolates for some of the investigated species, and (3) the biased isolate collection.

As we have observed a high amino acid sequence variability among the GGDEF\textsuperscript{UNC34} protein homologues, we were wondering whether outside of bovis subgroup species (1) the DGC is always found in the same genetic context and, if so, whether (2) the islet is always integrated at the same location in the genome.

In the salivarius subgroup, the operon structure GGDEF-Dpm1/\textit{GtrA-CellA-membrane} is conserved in \textit{S. salivarius} isolates; however, the operon is integrated into a different genomic context (Supporting Figure S10). In the pyogones subgroup member \textit{S. uberis}, an EAL only domain c-di-GMP phosphodiesterase protein ORF has been integrated downstream of the GGDEF domain again in another genomic context (Supporting Figure S11A). In \textit{S. parauberis} strains, four ORFs coding for an EAL only protein, a hydrolase, the PagC glycosyltransferase, and another EAL only protein are regularly encoded downstream of the GGDEF protein ORF (Supporting Figure S11B). Furthermore, in unassigned \textit{S. suis}, the \textit{S. uberis} type operon is rearranged with the GGDEF and EAL protein located downstream of the ORF for the uncharacterized membrane protein (Supporting Figure S12A). In all species, the operon is flanked by different genes.

Moreover, in the two sequenced \textit{S. Henryi} isolates, the GGDEF\textsuperscript{UNC34} homologue is found in a different genetic context (Supporting Figure S12B). Intriguingly, downstream of the PAS-GGDEF protein open reading frame, the open reading frame for a composite PAS-GGDEF-EAL-GGDEF domain protein is located.

In conclusion, in most of the investigated streptococcal species, a PAS-GGDEF DGC co-occurs with two type 2 glycosyltransferases followed by an inner membrane protein and variable accessory genes related to c-di-GMP signaling and exopolysaccharide biosynthesis, although the chromosomal location can vary. Of note, the gene cluster \textit{Dpm1/\textit{GtrA-CellA-membrane}} is present also outside of streptococcal species such as in \textit{Pediococcus pentosaceus} ATCC25745, with two GGDEF domain proteins to be encoded at distant locations on the chromosome (Supporting Figure S12C). Thus, those genes seem to thrive in \textit{Streptococci} and related species in various contexts.

**Streptococcal EAL Proteins Are Potentially Catalytically Active.** Sequence alignment of the EAL proteins from the different streptococcal species identified three distinct proteins, EAL1, EAL2, and PAS-GGDEF-EAL-GGDEF, within c-di-GMP signaling islets (Figure 9 and data not shown). The presence of the conserved amino acid motifs required for catalytic activity such as the homologue of the catalytic base E\textsubscript{352}GVE of the PDE RocR\textsuperscript{E53.35} indicated that at least two of the three EAL domains possess c-di-GMP phosphodiesterase activity. While conserved motifs of the EAL domain of PAS-GGDEF-EAL-GGDEF from \textit{S. Henryi} indicated catalytic functionality, both GGDEF domains are highly degenerated (Figure 9 and data not shown).

![DISCUSSION](https://dx.doi.org/10.1021/acsinfectdis.0c00314)

In this work, we have gathered experimental and bioinformatics evidence that \textit{Proteus} and \textit{Streptococcus} species possess a functional c-di-GMP signaling network and initially experimentally characterized two novel DGCs from those Gram-negative and Gram-positive species. Despite being active enzymes in the context of a cellulose or cellulose-like operon, the physiological roles of the DGCs \textit{PMI3101_y} and GGDEF\textit{UNC34} are, however, still undefined.

\textit{P. mirabilis} is well-known for its extensive flagella-mediated swimming and swarming motility.\textsuperscript{53} Swimming and swarming motility is stimulated by low c-di-GMP levels in bacteria and concurrently physically inhibited by cellulose production in \textit{S. typhimurium}.\textsuperscript{53,55} Nevertheless, \textit{Proteus} spp. possess an obviously functional cellulose biosynthesis operon with the catalytic subunit of the cellulose synthase BcsA encompassing a C-terminal PilZ domain receptor with conserved signature amino acid motifs required for c-di-GMP binding.\textsuperscript{23,24} We hypothesize that cellulose production can be involved in cell aggregation, surface adhesion, chlorine resistance, or other cellulose-mediated physiology in one of the diverse habitats where \textit{P. mirabilis} forms biofilms.\textsuperscript{50} \textit{P. mirabilis} is also a frequent cause of catheter associated urinary tract infection, where c-di-GMP mediated cellulose production might be involved in the modulation of \textit{in vivo} virulence, as observed for other bacteria.\textsuperscript{57–59}

To our knowledge, reports of a functional c-di-GMP signaling system within the family \textit{Streptococcae} had been restricted to investigate the effect of extracellular c-di-GMP on biofilm formation.\textsuperscript{60} Cyclic di-GMP mediated biofilm formation upon expression of cellulose-like exopolysaccharides could contribute to the pathogenesis of \textit{S. galolyliticus} and related bacterial species. \textit{S. galolyliticus} has been associated with colorectal cancer and causes endocarditis and bacteremia predominantly in the aged population.\textsuperscript{61,62} Further functional analysis of the DGCs and the c-di-GMP regulatory network in \textit{S. galolyliticus}, \textit{P. mirabilis}, and other streptococcal and \textit{Proteus} spp. will aid our understanding of the ecological and clinical impact of c-di-GMP signaling in biofilm formation and the pathogenesis of infection in these bacterial species.

Interestingly, in \textit{S. galolyliticus} UCN34, the DGC colocalizes with two genes coding for type 2 glycosyltransferases, one of them the cellulose synthase-like protein CellA harboring a C-terminal PilZ domain. Whether the PilZ domain binds c-di-GMP requires experimental investigation, but bioinformatic analyses indicate the conservation of signature amino acid motifs for c-di-GMP binding (Supporting Figure S7). To emphasize, the PAS-GGDEF domain proteins encoded by streptococcal species are distinguished by their remarkable low amino acid sequence similarity, which can be below 30% identity in the animal pathogens \textit{S. suis}, \textit{S. uberis}, and \textit{S. parauberis} compared to \textit{S. galolyliticus} UCN34. In comparison, the corresponding type 2 glycosyltransferases CellA and Dpm1-GtrA have evolved much slower with sequence identities of >52% and >46%, respectively, suggesting signal sensing and amplification systems particularly prone to rapid evolution in different streptococcal species.

Of note, no readily recognizable EAL or HD-GYP c-di-GMP specific PDE was identified in \textit{S. galolyliticus} and \textit{P. mirabilis}. This finding is in line with observations in other bacteria, which possess also a sole functional DGC but no identified c-di-GMP hydrolyzing enzyme.\textsuperscript{29,63} Although conventional c-di-GMP specific EAL and HD-GYP domain phosphodiesterases are not present, we cannot entirely exclude the presence of distantly related variants of those enzymes. Alternatively, other ubiquitous phosphodiesterase domains such as the Hdc domain of the bifunctional ppGpp synthase/hydrolase SpoT are candidates for such a residual functionality. On the other hand, surprisingly, the exopolysaccharide operons of the animal
pathogens *S. uberis*, *S. parauberis*, and *S. suis* had one or two EAL domain only proteins integrated into the c-di-GMP signaling islet. Equally, in *S. henryi*, a PAS-GGDEF-EAL-GGDEF protein is encoded downstream of the DGC gene. We assume that, in these cases, the EAL proteins hydrolyze c-di-GMP as signature amino acid motifs indicating catalytic activity are present.

Previously, a cyclic di-AMP signaling network had been ubiquitously identified in streptococcal species. Cyclic di-AMP signaling has been mainly investigated in the human pathogens *S. pneumoniae* and *S. pyogenes*, the dental caries causing *S. mutans*, and the animal pathogen *S. suis*, where c-di-AMP signaling controls exopolysaccharide biosynthesis and biofilm formation, antimicrobial resistance, the competence status, and regulation of host immunity, among other phenotypes. In *S. galloxyticus* subsp. *galloxyticus*, c-di-AMP signaling has been shown to promote osmoreistance, alter cell morphology, and inhibit biofilm formation and host–cell interactions. There might be a cross-talk of c-di-AMP signaling with other common nucleotide-based signaling systems such as the c-di-AMP and ppGpp signaling systems with similar pathways to be affected. Of note, the c-di-GMP signaling system is not found in all strains of the species *S. galloxyticus* and other species, but a partial or entire deletion of the c-di-GMP signaling encoding islet and rearrangements of the islet can occur (Figure 9A; Supporting Figure S10). This microheterogeneity indicates a high genomic and potentially phenotypic plasticity probably governed by the ecological niche. Remarkably, heterogeneous GGDEF proteins are present in a few strains of *S. pyogenes* and *S. pneumoniae* (data not shown). While the GGDEF proteins in *S. pyogenes* are highly degenerated (one GGDEF only could be potentially functional, though), their occurrence in *S. pneumoniae* needs to be confirmed. In the ecological niche of the human nose, *S. pneumoniae* is in an excellent position to constantly take up genes from environmental species by natural competence to test them for suitability in its genomic context.

An initial characterization of the DGC activity of *P. mirabilis* and *S. galloxyticus* proteins came from the assessment of the response of c-di-GMP responsive riboswitches. Under our experimental conditions, the systems were especially robust to detect DGCs, although, despite the nanomolar affinity, one of the aptamer-based c-di-GMP sensors did not routinely provide sensitivity. Previously, a riboswitch-based fluorescence biosensor consisting of dual aptamers, a c-di-GMP binding aptamer and the spinach aptamer that can bind to cyan fluorophores 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), was designed to visualize the intracellular CDN level to detect c-di-GMP turnover proteins. Another biosensor for monitoring changes in intracellular c-di-GMP level is based on modulation of fluorescence resonance energy transfer (FRET) upon binding to the PiiZ domain protein containing cyan CFP and yellow YFP fluorescent protein fusions. Furthermore, we applied a MALDI-FTMS-based approach to detect intracellular c-di-GMP levels from entire cell lysates without isolating the compound. To initially assess the catalytic activity of cyclic di-GMP turnover proteins by MALDI-FTMS is especially useful for difficult to purify proteins and upon undetectable in vitro catalytic activity. Our experimental approaches do not require cutting-edge facilities such as fluorescence microscopy and fluorescence activated cell sorting.

Moreover, promotion of rdar morphotype expression combined with inhibition of motility in *S. typhimurium* UMR1, as occurred overexpression of PMI3101\_v and GGDEF\_UCN34, is a sensitive in vivo assay to initially assess DGC activity. Furthermore, even though GGDEF\_UCN34 does not possess an RxxD I-site, the catalytic mutant slightly up-regulated the swimming motility of *S. typhimurium* UMR1 indicative for a depletion of the second messenger molecule as it occurs upon c-di-GMP binding. Of note, the GGDEF domain DGC XCC4471 lacking the I-site can bind a semi-intercalated c-di-GMP dimer. We therefore speculate that the GGDEF domain protein GGDEF\_UCN34 with a mutated GGAEF site has the ability to bind c-di-GMP. Thus, physiological assays proved to be relevant tools to initially validate basic functionality of candidate c-di-GMP signaling network components.

## Conclusion

In conclusion, the c-di-GMP network is more widespread than previously anticipated with the production of a c-di-GMP activated cellulose or cellulose-like macromolecule as a fundamental physiological trait in distantly related Gram-negative and Gram-positive bacteria. These readily trackable organisms with a highly reduced c-di-GMP signaling network will aid in identifying the evolutionary forces that lead to an expansion versus reduction of this ubiquitous second messenger signaling system.

## Methods

**Bacterial Strains, Plasmids, and Growth Conditions.** *E. coli* TOP10 was grown either in Luria–Bertani (LB) medium or on a LB agar plate, while *S. typhimurium* UMR1 (ATCC14028 Na\^\textcircled{L}), MAE50 (UMR1 Δ espD; biofilm negative control), and MAE108 (UMR1 Δ flaC Δ fliB; motility negative control) were grown on LB without salt agar at 30 or 37 °C. *S. galloxyticus* UCN34 was grown at 37 °C with 5% carbon dioxide in brain heart infusion (BHI) broth (Oxide) or on a BHI agar plate. *P. mirabilis* UEB50 was grown in nutrient broth or on a nutrient agar plate (Difco) at 37 °C. Supplements were ampicillin (100 μg/mL) (Sigma) for the selection for recombinant strains and L-arabinose (100 μg/mL) at the indicated concentration for induction of protein production. All strains and plasmids used in this study are listed in Supporting Table S2.

**Riboswitch Construction.** The Vc1 c-di-GMP riboswitch was amplified from the genomic DNA of *V. cholerae* strain C6706 comprising from −240 to +28 bp with respect to the ORF. The lac promoter was amplified from pUC19. Vc1 and lac promoter were ligated by overlapping PCR, the fragment digested with Smal and BamHI (New England Biolabs), and ligated into the translational reporter vector pRS414 in frame upstream from the ninth codon of the lacZ reporter gene. The genomic DNA was extracted by a GenElute Bacterial Genomic DNA kit (Sigma–Aldrich).

Subsequently, riboswitches along with lacZ were amplified from the pRS414 vector. The PCR products and pGRG25 containing the inducible highly efficient recombination system for integration into the attTn7 site were digested with PacI and NotI (New England Biolabs). Digested pGRG25 was dephosphorylated by Antarctic phosphatase (New England Biolabs) and ligated with the PCR product by T4 DNA ligase (Roche Life Science). The resulting product was transformed into chemo-competent *E. coli* TOP10 cells grown at 30 °C. Insertion of genes into the chromosomal attTn7 attachment
A 4 mL culture was harvested and resuspended in 100 μL of LB with ampicillin and grown overnight culture, which was further cultured for 4 h at 30 °C. LC-MS CHROMASOLV water (Sigma-Aldrich) of OD600 = 3 was prepared to the cell lysate for rapid c-di-GMP detection, a single reaction products are observed.

Cloning of GGDEF Domain Proteins. PMI3101_v and GGDEFUCN34 were PCR amplified from the genomic DNA of P. mirabilis UEB50 and S. galloyticus UCN34, respectively. A hexahistidine-tag (His6-tag) was introduced at the C-terminus of the open reading frames (ORFs) during PCR. The amplified fragments were digested by XmaI and XbaI (New England Biolabs) and subsequently cloned into the corresponding sites of the pBAD28 vector. The site-directed mutagenesis of the GGDEF domain to GGAEF was performed using a QuickChange II Site-Directed Mutagenesis Kit (Agilent). Primers are listed in Supporting Table S2.

Assessment of c-di-GMP Synthesis by Riboswitch-Regulated β-Galactosidase Activity. Vectors harboring DGCs, c-di-GMP specific PDEs, and respective mutants were transformed into chemically competent E. coli TOP10 cells containing the monitoring riboswitch plasmid on the chromosome. Individual colonies were grown overnight at 37 °C with 200 rpm shaking in LB medium supplemented with 100 μg/mL ampicillin. Cultures were diluted to an OD600 of 0.1 and subsequently grown to an OD600 around 0.6. Five μL of culture was spotted onto an LB agar plate with 100 μg/mL ampicillin, 80 μg/mL X-gal (Roche), 0–0.1% (wt/vol) L-阿拉伯宁, and 0.25 mM isopropyl β-D-1-thiogalactopyranosidase (IPTG) upon containment of the Vc1 riboswitch. The plates were incubated at 28 °C, and color development was monitored up to 72 h.

Phenotypic Assays. The swimming assay was performed in 1% tryptone, 0.5% NaCl, 0.3% (wt/vol) agar plates. Three μL of an overnight culture resuspended in water adjusted to OD600 = 5 was injected into the agar, and the plates were incubated at 37 °C for 6 h. Afterward, pictures of plates were taken with a Gel Doc XR+ system (Bio-Rad) and the diameter of the swimming zone was measured.

The rdar biofilm morphotype was assessed on CR-LB without salt agar plates. A single colony was picked from an LB agar plate incubated overnight, resuspended in water at OD600 = 5, and 3 μL spotted onto the CR plate. After 72 h of incubation at 28 °C, the morphotype of the colony was observed.

MALDI Fourier Transform Mass Spectrometry. To prepare the cell lysate for rapid c-di-GMP detection, a single colony was picked and suspended in 450 μL of LB medium with ampicillin (100 μg/mL). A 50 μL suspension was transferred into 5 mL of LB with ampicillin and grown overnight in 30 °C with shaking at 200 rpm. To induce the overexpression of proteins, 0.01% arabinose was added to the overnight culture, which was further cultured for 4 h at 30 °C. A 4 mL culture was harvested and resuspended in 100 μL of LC-MS CHROMASOLV water (Sigma-Aldrich) of OD600 = 3 supplemented with 0.5 mg/mL lysozyme. The resuspension was incubated at 24 °C for 1 h followed by two rounds of freeze and thaw (−80 °C 1 h, room temperature 1 h). The lysate was stored at −80 °C until further use.

The α-cyano-4-hydroxycinnamic acid (α-cyano, CHCA) (Sigma-Aldrich) matrix for MALDI-TOF mass spectrometry was prepared according to the manufacturer’s instruction. A 2 μL portion of lysate was mixed with 2 μL of matrix; the mixture was spotted on a metal plate of the atmospheric pressure MALDI interface (MassTech, Columbia, MD, USA) and measured by Q Exactive (Thermo Scientific) Fourier Transform mass spectrometer.

Estimation of c-di-GMP Concentration by LC-MS/MS. The extraction of c-di-GMP from bacterial cells was performed as reported. Overnight cultures from agar-grown colonies with protein expression induced by 0.1% L-arabinose were suspended in 500 μL of ice cold extraction solvent (acetonitrile/methanol/water/formic acid = 2/2/1/0.02, v/v/v/v), pelleted, and resuspended, followed by boiling for 10 min. Three subsequent extracts were combined and frozen at −20 °C overnight. The extract was centrifuged for 10 min at 20,800g, evaporated to dryness in a Speed-Vac (Savant), and analyzed by LC-MS/MS.

ASSOCIATED CONTENT
 Supporting Information
 The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00314.

Methods describing the bioinformatics analyses and protein purification; designation of proteins used in bioinformatics analyses; figures showing structural models for PMI3101_v and GGDEFUCN34, domain structure and alignments of homologous proteins outside the respective genera for PAS-GGDEF from P. mirabilis and S. galloyticus, control experiments for detection of alternating c-di-GMP concentrations by Vc1 and Vc2 riboswitches, analysis of plasmid-derived production of wild type and mutant proteins in c-di-GMP detection and motility assays by Western blot, an extended MALDI-FTMS spectrum from 600 to 700 m/z for cell extracts, bioinformatics analyses and structural modeling of the type 2 glycosyltransferases BcsA1 and BcsA2 of P. mirabilis and CeA of S. galloyticus and its c-di-GMP binding PilZ domains, visualization of the second cellulose biosynthesis gene cluster in Proteus species, comparison of the c-di-GMP signaling islet from S. galloyticus UCN34 in gene context and location with islets from bovis subgroup species, S. salivarius, S. uberis, S. parauberis, S. suis, and S. henryi, and comparison of the Dpm1/GtrA-CelA-membrane gene cluster in P. pentosa- ceus ATCC25745 to the c-di-GMP islet in S. galloyticus; tables indicating GGDEF, EAL, and HD-GYP domain proteins in species of the family of Morganellaceae, strains and plasmid used in this study, and primers used in this study (PDF)

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U.R. had the original idea and designed the study; Y.L., F.L., J.T., H.B., R.-T.G., C.-C.C., and U.R. performed the experiments; Y.L., C.L., F.L., J.T., R.-T.G., C.-C.C., A.C., R.Z., and U.R. analyzed the data; Y.L. and U.R. wrote the manuscript with input from all of the authors.

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED

C-di-GMP, bis(3′-5′) cyclic diguanylic acid; CR, Congo Red; CDN, cyclic dinucleotide; LC-MS/MS, liquid chromatography-mass spectrometry; MALDI-FTMS, matrix assisted laser desorption ionization-Fourier transformation mass spectrometry

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