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Original Article

GGDEF and EAL proteins play different roles in the control of *Sinorhizobium meliloti* growth, motility, exopolysaccharide production, and competitive nodulation on host alfalfa

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A new bacterial secondary messenger, bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), is usually synthesized or decomposed by proteins containing GGDEF or glutamate–alanine–leucine (EAL) domain. They often act as cyclase or phosphodiesterase of c-di-GMP and their genes are distributed among almost all bacteria according to known genomic DNA sequences. However, the systematic identification of GGDEF and EAL genes remains unclear in rhizobia, soil bacteria that interact with compatible legumes to form nitrogen-fixing nodules. In this study, 19 putative GGDEF and EAL genes were identified in a model rhizobium, *Sinorhizobium meliloti*, by bioinformatic analysis (encoding 5 GGDEF proteins, 4 EAL proteins, and 10 GGDEF and EAL double-domain proteins). Null mutants of 14 genes were constructed through systematic plasmid insertion. All 14 gene mutants showed deficient growth in minimal medium and defective motility, and 11 gene mutants produced a lot more exopolysaccharide and displayed less competitive nodulation on the host plant, alfalfa. Our results suggested that GGDEF and EAL proteins may play different roles in the control of *S. meliloti* physiology, although they contain conserved catalytic (GGDEF or EAL) domains. Our finding also implied that c-di-GMP may play an important role in the interactions between this rhizobium and its host plants to establish efficient symbiosis.

Keywords GGDEF domain; EAL domain; c-di-GMP; competitive nodulation

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Introduction

In the absence of combined nitrogen, Gram-negative soil bacteria called rhizobia are able to interact with compatible legumes through a complex progress to form root or stem nodules, in which differentiated rhizobial cells called bacteriods convert atmospheric dinitrogen into ammonia in exchange for carbon sources from host plants [1]. The efficiency of symbiotic nitrogen fixation is influenced by many environmental factors, including levels of oxygen, ammonia, nitrate, and phosphate [2]. In the absence of combined nitrogen in soil, a histidine kinase, NtrB, is autophosphorylated in rhizobia, and then delivers phosphate groups to its partner, NtrC, which activates the expression of nitrogen-fixing genes (*nif*/*fix*) to fix dinitrogen. In contrast, excessive ammonia or nitrate suppresses the phosphorylation of this two-component system. Similarly, under limited oxygen or phosphate, the phosphorylation system, FixL/FixJ or PhoR/PhoB, is rapidly activated to regulate expression of a large number of target genes and adjust rhizobium adaptation to stress. The response regulator of these two-component systems is often composed of one phosphate group receipt domain (such as Rec) and one DNA-binding domain. The Rec domain is sometimes organized with a GGDEF or glutamate–alanine–leucine (EAL) domain in a few response regulators that play signaling roles in the control of bacterial physiology [3].

Bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is an important secondary messenger of bacteria, and is usually synthesized and decomposed by proteins containing GGDEF and EAL domains [4,5]. This signal molecule was first discovered in *Gluconacetobacter xylinus* as a factor that activates membrane-bound cellulose synthase [6]. A few GGDEF or EAL proteins are known to contribute to the catabolism of c-di-GMP and control bacterial motility, virulence, exopolysaccharide production, sessility, biofilm formation, and cell cycle progression [4]. In *Caulobacter crescentus*, the PleD protein consists of two Rec domains and one GGDEF domain, which

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positively regulates bacterial holdfast production, stalk formation, and flagellum ejection; its homologous protein, PopA, functions in S phase entry and CtrA degradation. In contrast with PleD, an EAL protein, TipF, suppresses the assembly of bacterial flagella [5]. In *Vibrio cholerae*, an EAL protein, VieA, functions as a partner of a histidine kinase, VieS, which causes biofilm formation and virulence to its host by changing c-di-GMP content; a similar system (SadR/RocR) exists in *Pseudomonas aeruginosa* [7]. In *Salmonella typhimurium*, all 12 GGDEF genes have been removed and reintroduced using a mutant; their proteins were found to work together as a signaling network to control bacterial physiology [8].

Today, with an explosive increase in bacterial genomic DNA data, a large number of GGDEF/EAL protein genes have been predicted in almost all bacteria. However, for most GGDEF/EAL protein genes in bacteria, especially in plant-associated bacteria, their functions have not been investigated in detail through genetic or biochemical approaches. In *Rhizobium leguminosarum* bv. *trifolii*, *celR2* mutant (*pleD* homologous gene) decreases cellulose production, although the overexpression of three other GGDEF protein genes, *rge1*, *dgcAS*, and *yhcK*, can restore cellulose synthesis deficiency in this mutant [9]. It remains an open question whether GGDEF/EAL protein genes play important roles during nodulation and nitrogen fixation, although several rhizobial genomes have been sequenced and published, including a model rhizobium species, *Sinorhizobium meliloti*. Through genomic DNA sequence analysis, 19 new GGDEF/EAL protein genes have been annotated for the *S. meliloti* genome, excluding one PleD homolog [10]. In this study, we determined the roles of these new genes in the control of bacterial physiology and symbiotic interactions with its host plant, alfalfa, by systematically constructing plasmid-insertion mutants.

Materials and Methods

Strains and bacterial media

The *S. meliloti* strains used in this study are shown in **Table 1**. *Sinorhizobium meliloti* Rm1021 (Str^r) was used as the wild-type strain and MT616 (pRK600) was used as a helper for conjugation [11]. The *S. meliloti* phage ΦM12 was used for general transduction [12]. Luria–Bertani (LB) medium was used for the growth of *Escherichia coli* strains and LB supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC) was used for the growth of all *S. meliloti* strains. Agar (1.5%) was added to make solid medium. The antibiotics used included 10 μg/ml chloramphenicol (Cm), 25 μg/ml kanamycin (Km), 200 μg/ml neomycin (Nm), and 200 μg/ml streptomycin (Sm). All reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Construction of gene mutations by plasmid insertion

Plasmid insertion mutagenesis was conducted as described by Luo *et al*. [12]. Briefly, oligo primers (**Supplementary Table S1**; Invitrogen, Carlsbad, USA) were used to amplify ~300 bp DNA fragments of GGDEF/EAL protein genes by PCR and were then cloned into pK19mob2ΩHMB (Transgene, Beijing, China). The resultant recombinant plasmids (**Table 1**) were used for conjunction. The recombinant rhizobium was lysed using ΦM12 phage and the lysate was used for transduction of Rm1021 to harvest a single insertion mutant (**Table 1**). The null mutants were confirmed by PCR with specific oligo primers (**Supplementary Table S1**).

Rhizobial growth kinetic analysis

Each rhizobium strain was inoculated into 3 ml LB/MC broth in the presence of the appropriate antibiotics and grown at 28°C overnight. The rhizobium culture was diluted to 20 ml M9/glucose broth at an initial cell density (OD_{600}) of 0.02 and incubated at 28°C. The rhizobium cell density was monitored by spectrophotometer (Techcomp, Shanghai, China) every 12 h. Three replicates were used and the experiment was repeated three times.

Motility test

Cell motility was examined as described by Luo and colleagues [13]. Briefly, each rhizobium inoculation from fresh LB/MC plates with appropriate antibiotics was dipped into LB/MC soft agar medium (0.25%) with a toothpick and incubated at 28°C for 3 days. Then, the diameter of each rhizobium colony (φ) was measured and the dimension was calculated by the formula:

$$A = \pi(\varphi/2)(\varphi/2).$$

Photographs were taken using a Coolpix 4500 digital camera (Nikon, Tokyo, Japan). Three replicates were used and the experiment was repeated at least three times.

Exopolysaccharides assay

Rhizobium overnight cultures in 3 ml LB/MC broth were washed with sterile saline and diluted to a cell density of 0.2. Then 5 μl of this dilution was dropped onto LB/MC solid medium containing 0.02% calcofluor white M2R (Sigma, St. Louis, USA) and 0.01 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. After incubation of 3 days, exopolysaccharides (EPS) production was visualized under optical and long-wave UV light. The 0.25 ml supernatant was collected from the overnight subculture of rhizobium in LB/MC broth and 0.75 ml isopropanol was used to precipitate EPS. The pellet was washed once with 70% ethanol, air dried, and then resolved in 0.25 ml distilled water, which was used for total sugar,

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Table 1 Bacterial strains and plasmids

Strains/plasmids	Relevant properties	Reference
Sinorhizobium meliloti		
Rm1021	Wild-type, Str^r	[11]
Sm414	SMa0137:: pK19mob2ΩHMB, Neo^r	This work
Sm416	SMa1548:: pK19mob2ΩHMB, Neo^r	This work
Sm418	SMb20389:: pK19mob2ΩHMB, Neo^r	This work
Sm419	SMb20447:: pK19mob2ΩHMB, Neo^r	This work
Sm420	SMb20523:: pK19mob2ΩHMB, Neo^r	This work
Sm421	SMb20900:: pK19mob2ΩHMB, Neo^r	This work
Sm422	SMc00033:: pK19mob2ΩHMB, Neo^r	This work
Sm423	SMc00038:: pK19mob2ΩHMB, Neo^r	This work
Sm424	SMc00992:: pK19mob2ΩHMB, Neo^r	This work
Sm426	SMc03942:: pK19mob2ΩHMB, Neo^r	This work
Sm427	SMc04015:: pK19mob2ΩHMB, Neo^r	This work
Sm428	SMa2301:: pK19mob2ΩHMB, Neo^r	This work
Sm430	SMc00887:: pK19mob2ΩHMB, Neo^r	This work
Sm432	SMc03178:: pK19mob2ΩHMB, Neo^r	This work
Escherichia coli		
DH5α	*sup E44Δ lac U169 (φ80 lacZΔM15) hsdR 17 rec A1 end A1 gyr A 96 thi21 rel A1*	Lab stock
MT616	*pro282 thi21 endA1supE44 hsdR17 - hsdM + recA56*, *Chlr*, carrying pRK600, Cm^r	[11]
Plasmids		
pK19mob2ΩHMB	Km^r	[12]
plws1	SMa0137:: pK19mob2ΩHMB, Km^r	This work
plws2	SMa1548:: pK19mob2ΩHMB, Km^r	This work
plws3	SMb20389:: pK19mob2ΩHMB, Km^r	This work
plws4	SMb20447:: pK19mob2ΩHMB, Km^r	This work
plws5	SMb20523:: pK19mob2ΩHMB, Km^r	This work
plws6	SMb20900:: pK19mob2ΩHMB, Km^r	This work
plws7	SMc00033:: pK19mob2ΩHMB, Km^r	This work
plws8	SMc00038:: pK19mob2ΩHMB, Km^r	This work
plws9	SMc00992:: pK19mob2ΩHMB, Km^r	This work
plws11	SMc03942:: pK19mob2ΩHMB, Km^r	This work
plws12	SMc04015:: pK19mob2ΩHMB, Km^r	This work
plws13	SMa2301:: pK19mob2ΩHMB, Km^r	This work
plws14	SMc00074:: pK19mob2ΩHMB, Km^r	This work
plws17	SMc03178:: pK19mob2ΩHMB, Km^r	This work

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assayed by the sulfate−anthrone method [14]. Three replicates were used and this experiment was repeated at least three times.

Nodulation and competition test

Alfalfa (*Medicago sativa* cv. Iroquois) seeds were surface sterilized as described by Leigh *et al*. [11]. Then the seeds were soaked in a rhizobium suspension for 30 min and sown on the top of a vermiculite−perlite (3:1) mixture autoclaved and pre-watered with 100 ml Fahraeus liquid medium in three pots. All seeds were covered with a thin layer of autoclaved vermiculite−perlite mixture (depth, 0.5 cm). Each pot was wrapped in a piece of plastic film, covered with a piece of paper, and left for the seeds to germinate at room temperature for 48 h. All seedlings were then incubated in a greenhouse (23 ± 1°C, 16 h daylight per day and 60% humidity). Pots were watered with 100 ml autoclaved Fahraeus liquid medium every 3−4 days. Four weeks later, the fresh weight of alfalfa seedlings was measured, root nodules were counted, and nitrogenase activity was determined by gas chromatography using a C_2H_2 reduction method. The nodulation competition test has been described by Yu *et al*. [15]. Briefly, rhizobium cells were pelleted from overnight LB/MC cultures,

washed with saline, and resuspended in saline to OD_{600} 0.02. A 1:1 mixture of wild-type Sm1021 and each mutant of GGDEF/EAL protein genes were used to soak surface-sterilized alfalfa seeds, which was then sown as described above. Four weeks later, more than 30 root nodules were collected, surface sterilized, crushed, and plated on LB/MC/Sm agar, followed by transfer to LB/MC/Nm agar. The mutant rhizobium isolated from root nodules was able to grow on plates containing neomycin, but the wild-type strain did not. Therefore, the nodule occupation ratio of each mutant was calculated as the number of colonies on LB/MC/Sm plates divided by the number of colonies on LB/MC/Nm plates.

Bioinformatic analysis

The amino acid or DNA sequences of GGDEF and EAL proteins or genes were retrieved from the *S. meliloti* genome server (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi). Protein domains were predicted using SMART (http://smart.embl-heidelberg.de/help/smart_glossary.shtml) or Pfam (http://pfam.janelia.org/). Nucleotide or amino acid sequences were aligned using the Blast server from NCBI (http://www.ncbi.nlm.nih.gov/guide/sequence-analysis/).

Results

Identification of 14 putative genes encoding new GGDEF/EAL proteins in *S. meliloti*

The amino acid sequence of PleD was downloaded from the *S. meliloti* Rm1021 genome server and its conserved domain was predicted using the SMART program from the European Bioinformatics Institute (EBI). One conserved GGDEF with two Rec (CheY-like received) motifs was found as a homolog for *C. crescentus* and *R. leguminosarum* bv. *trifolii* [9]. We used this as a query sequence to search for homologous protein genes in *S. meliloti* (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Fifteen novel genes were presented with high scores and identities: SMa0137, SMa1548, SMa2301, SMb20389, SMb20447, SMb20523, SMb20900, SMc00038, SMc00992, SMc0 1464, SMc03942, SMc04015, SMc00074, SMc00887, and SMc03178 (**Table 2**). The conserved GGDFE domains from these protein genes were again confirmed by SMART. Ten had additional EAL domains encoded by SMa0137, SMa1548, SMb20447, SMb20900, SMc00038, SMc00992, SMc03942, SMc00074, SMc00887, and SMc03178, and five (SMa2301, SMb20389, SMb20523, SMc01464, and SMc04015) were single GGDEF domain

Table 2 GGDEF or EAL genes in *S. meliloti*

Genes	Length (aa)	GGDEF/EAL (position)	*E*-value	Other domains
pleD	455	GGDEF(279–453)	7.20e−67	2Rec
SMa0137	733	GGDEF(293–462)/EAL(472–714)	48e−54/1.28e−90	2TM
SMa1548	1071	GGDEF(639–809)/EAL(819–1061)	2.42e−59/2.00e−115	5PAS/4PAC
SMa2301	448	GGDEF(264–441)	5.13e−63	5TM
SMa0369[a]	88	EAL(10–70)	3.5e−15	–
SMb20389	341	GGDEF(167–339)	2.98e−50	GAF
SMb20447	564	GGDEF(124–294)/EAL(304–545)	3.55e−57/1.36e−108	–
SMb20523	402	GGDEF(212–381)	1.41e−42	7TM
SMb20900	644	GGDEF(279–451)/EAL(461–628)	2.10e−59/3.68e−32	5TM/PAS
SMb21517	271	EAL(6–248)	1.70e−11	–
SMc00033	599	EAL(15–256)	1.55e−39	–
SMc00038	772	GGDEF(333–506)/EAL(516–762)	7.15e−62/6.38e−132	PAS/PAC/GAF
SMc00992	790	GGDEF(327–507)/EAL(516–761)	1.08e−47/3.88e−72	2TM
SMc01464	426	GGDEF(232–393)	2.28e−46	5TM
SMc03942	773	GGDEF(330–502)/EAL(512–755)	2.59e−27/1.07e−92	5TM/PAS
SMc04015	773	GGDEF(120–292)	1.08e−47	–
SMc00074	970	GGDEF(527–700)/EAL(710–955)	1.21e−41/1.24e−97	8TM/PAC
SMc00887	448	GGDEF(9–181)/EAL(191–433)	1.74e−33/5.35e−84	–
SMc03141	349	EAL(91–332)	6.07e−74	–
SMc03178	772	GGDEF(446–616)/EAL(626–869)	2.59e−50/5.30e−97	2TM/PAS/PAC

[a]The EAL domain of this gene was predicted by Pfam program, others were predicted by SMART. GGDEF, diguanylate cyclase domain; EAL, glutamate–alanine–leucine. Rec, CheY-homologous receiver domain; TM, transmembrane region; PAS, domain of Per-period circadian; Arnt-, Ah receptor nuclear translocator; Sim-, single-minded proteins; PAC, C-terminal of PAS domain; GAF, domain present in phytochromes and cGMP-specific phosphodiesterases.

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proteins (**Table 2**). The amino acid sequence of the SMa0137 EAL domain was used to search for other EAL protein genes. Four new EAL genes, SMa0369, SMb21517, SMc00033, and SMc03141, were found and confirmed by SMART or Pfam (**Table 2**). Taken together, 19 putative GGDEF and EAL genes were found in the *S. meliloti* Rm1021 genome: 10 genes that encode proteins with both GGDEF and EAL domains, five that encode single GGDEF proteins, and four that encode single EAL proteins. It is notable that other conserved domains also appear in these proteins, such as Rec, PAS, PAC, GAF, and transmembrane (TM) regions.

To determine the roles of these putative GGDEF and EAL genes in the control of bacterial physiology, a suicide plasmid, pK19mob2ΩHMB, was used to construct insertion mutants (see 'Materials and Methods'). Fourteen genes were successfully mutated and their transducers of ΦM12 were harvested in Rm1021 background (**Table 1**). These 14 null mutants were confirmed by PCR (**Supplementary Fig. S1**).

Growth deficiency of GGDEF and EAL protein gene mutants

One of the GGDEF proteins, MSDGC1, is required for the growth of *Mycobacterium smegmatis* [16]. It is possible that GGDEF and EAL proteins may function like MSDGC1 in *S. meliloti*. To confirm this possibility and to determine which GGDEF/EAL proteins are needed for the growth of *S. meliloti*, growth was monitored in LB/MC rich medium and in M9 minimal broth with glucose as a sole carbon source. In LB/MC broth, three mutants (Sm428, Sm430, and Sm432) grew faster than the wild-type (Rm1021), although the other 11 mutants showed no difference from the wild-type over 30 h incubation [**Fig. 1(A)**]. In M9/glucose broth, all 14 mutants grew slower than the wild-type during 60 h incubation [**Fig. 1(B)**]. These results suggested that the 14 GGDEF and EAL proteins are required for the growth of *S. meliloti* in limited nutrients, and three of them switch their roles depending on nutrient abundance.

Motility deficiency of GGDEF and EAL gene mutants

Several GGDEF proteins in one *S. typhimurium* are essential for bacterial motility [8], so we checked the motile phenotype of these *S. meliloti* GGDEF and EAL gene mutants on LB/MC swarming plates. Eleven gene mutants (Sm414, Sm416, Sm418, Sm419, Sm420, Sm421, Sm422, Sm423, Sm424, Sm426, and Sm427) swarmed slower than the wild-type, whereas the other three mutants (Sm428, Sm430, and Sm432) swarmed faster than Rm1021 [**Fig. 2(A)**]. We further measured the diameter of each swarming colony and calculated its dimension. Among the 11 slower-swarming mutants, Sm420 formed the smallest colony, 55.6% smaller than that of the wild-type; the dimensions of the other 10 mutant colonies were 35.2–48.1% smaller than that of wild-type [**Fig. 2(B)**]. In contrast, the colony dimensions of mutants Sm428, Sm430, and Sm432 were 30.9, 15.8, and 37.4% greater than that of Rm1021, respectively [**Fig. 2(B)**]. These results suggested that 11 of the GGDEF and EAL proteins are in positive control of *S. meliloti* motility, but the other three proteins suppress it.

EPS production deficiency of GGDEF and EAL protein gene mutants

To determine the roles of GGDEF and EAL proteins in the production of EPS, we assayed the EPS secreted by all 14 GGDEF and EAL protein gene mutants on LB/MC/calcofluor plates. As shown in [**Fig. 3(A)**], all mutants grew almost as well as the wild-type. Under long-wave UV, 11 mutants (Sm414, Sm416, Sm418, Sm419, Sm420, Sm421, Sm422, Sm423, Sm424, Sm426, and Sm427) exhibited stronger fluorescence than Rm1021, and the other three mutants (Sm428, Sm430, and Sm432) were similar to the wild-type [**Fig. 3(B)**]. Quantitative analysis of sulfate–anthrone indicated that the same 11 mutants had 4–5-fold increases in EPS production compared with that

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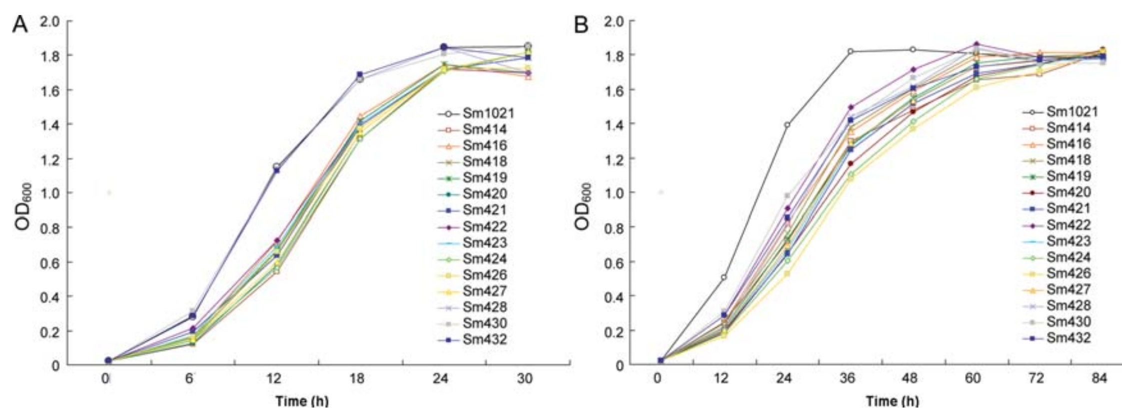

Figure 1 Growth curve of GGDEF and EAL protein gene mutants (A) The growth curve of rhizobium in LB/MC broth. (B) The growth curve of rhizobium in M9/glucose broth. Experiments were repeated three times and similar growth trend was observed.

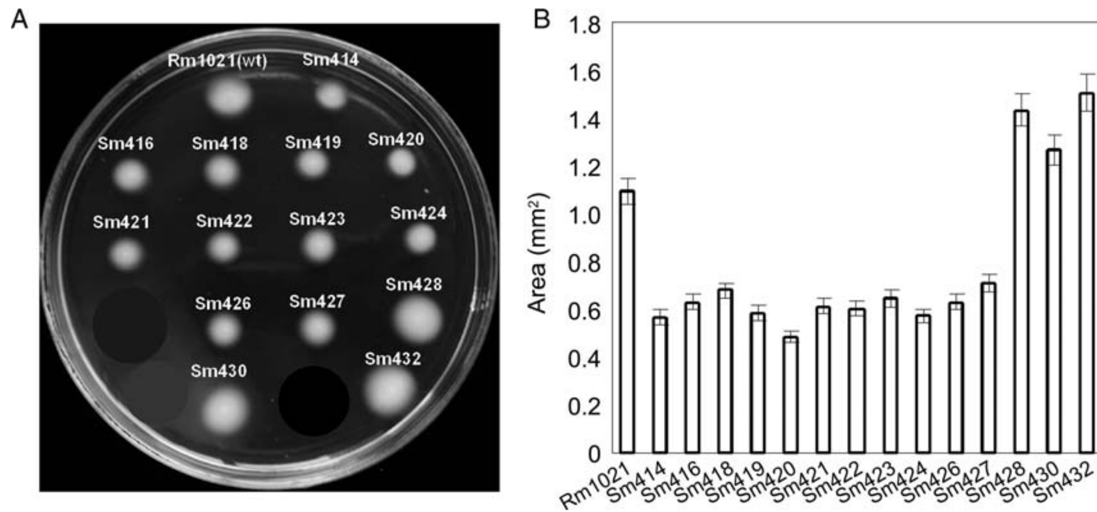

Figure 2 Motility of GGDEF and EAL protein gene mutants (A) Motility of rhizobia on LB/MC swarming plates. (B) Dimension of rhizobial colonies on LB/MC plates. Experiments were repeated at least three times and two replicates were tested.

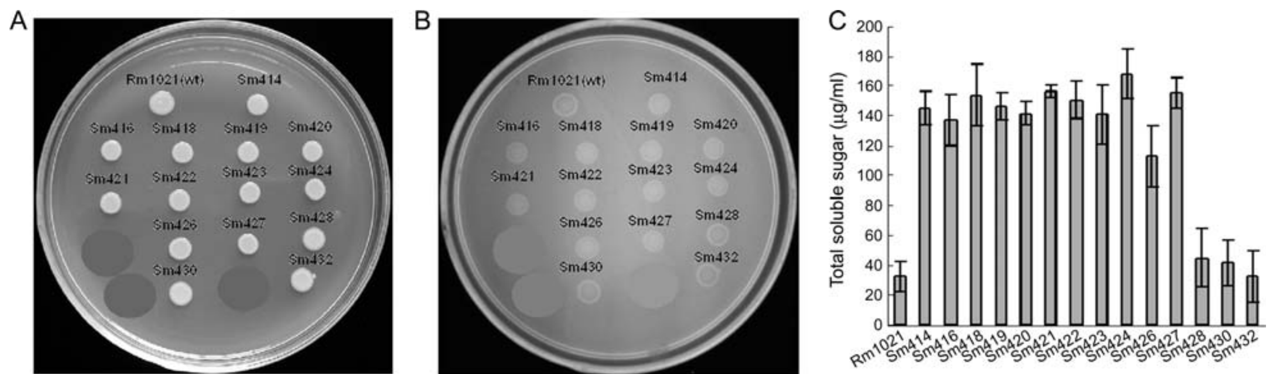

Figure 3 Exopolysaccharide production of GGDEF and EAL mutants (A) Bacterial colonies under optical light. (B) Bacterial colonies under UV light. (C) Quantitative assay of EPS produced in LB/MC broth. Experiments were repeated three times and three replicates were tested.

of the wild-type in LB/MC broth, whereas the other three mutants did not differ significantly from the wild-type [**Fig. 3(C)**], consistent with the calcofluor-staining observations on LB/MC plates. These results suggested that these 11 GGDEF and EAL proteins participate in the suppression of *S. meliloti* EPS production.

Symbiotic deficiency of GGDEF and EAL protein gene mutants

An alfalfa nodulation test was performed to analyze the symbiotic efficiency of GGDEF and EAL protein gene mutants. Alfalfa seedlings inoculated with the 14 gene mutants or wild-type rhizobia showed no significant difference in total root nodule number and the fresh weight of shoots after 4 weeks. These results suggested that GGDEF and EAL proteins are not essential to induce efficient nitrogen-fixing nodules on host alfalfa.

However, the competitive nodulation test using an equal optical density of each mutant and wild-type cells indicated that the nodule occupation ratio of the 11 mutants (Sm414, Sm416, Sm418, Sm419, Sm420, Sm421, Sm422, Sm423, Sm424, Sm426, and Sm427) was 20% lower than that of wild-type (**Fig. 4**). The other three mutants (Sm428, Sm430, and Sm432) showed similar competition as the wild-type (ratios of 55−60%). These results suggested that these 11 GGDEF and EAL proteins are required for *S. meliloti* competitive nodulation on alfalfa.

Discussion

In bacteria, c-di-GMP is considered a secondary messenger, like cAMP. Two key enzymes, GTP cyclase and c-di-GMP phosphodiesterase, synthesize and decompose c-di-GMP and modulate its content in the bacterial cell to control its physiological processes. These enzymes contain GGDEF, EAL, and HD-GYP domains that are encoded by a large number of genes in bacteria [4,5]. In this report, we first systematically studied GGDEF and EAL protein genes in

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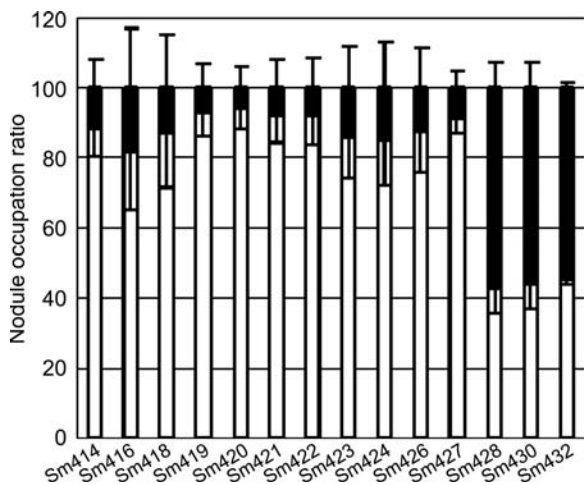

Figure 4 Nodulation competition test of each GGDEF/EAL mutant and wild-type Nodule occupation ratio of GGDEF/EAL protein gene mutants is shown as black bar, but the wild-type is indicated as blank bar. The 1:1 mixture of each mutant and the wild-type was inoculated on host alfalfa in this experiment. And the nodule occupation ratio of each mutant was calculated as the number of colonies on LB/MC/Sm plates was divided by the number of colonies on LB/MC/Nm plate.

the model rhizobium *S. meliloti* 1021. All 19 putative GGDEF and EAL protein genes were identified in this rhizobium by bioinformatic analysis. Their proteins share 30−40% identity (except SMa0369) and their GGDEF and EAL domains contain the highly conserved catalytic amino acids RxGGDEF and EALxRWxHP (**Supplementary Fig. S2**), although SMa0369 and SMb20900 only harbored incomplete EAL domains. It is an interesting question whether these proteins function in the control of physiology and symbiosis of *S. meliloti*.

To answer this question, we successfully constructed null mutants of 14 GGDEF and EAL protein genes in *S. meliloti*. We classified these GGDEF/EAL proteins into two groups according to the phenotypes of their gene mutants: 11 proteins (Group 1) encoded by *SMa0137*, *SMa1548*, *SMb20389*, *SMb20447*, *SMb20523*, *SMb20900*, *SMc00038*, *SMc00992*, *SMc03942*, *SMc04015*, and *SMc00033* and three proteins (Group 2) encoded by *SMa2301*, *SMc00887*, and *SMc03178*.

Growth deficiency of GGDEF and EAL mutants was observed in both minimal and rich media (**Fig. 1**). Our data indicate that the GGDEF/EAL proteins in Group 1 are required for growth of *S. meliloti* in limited nutrients. One possible explanation for this is that these GGDEF/EAL proteins take part in the uptake and utilization of specific carbon sources that are absent in minimal medium (data not shown). It is interesting that the Group 2 proteins may switch their roles depending on nutrient abundance (enhancing growth when resources are rich, limiting it when resources are scarce), which implies that the activities of these proteins could be suppressed by certain molecules but improved in their absence.

Low levels of c-di-GMP may promote bacterial motility, whereas high levels may suppress it in some bacteria [8,17]. Our data suggested that the Group 1 proteins might positively control *S. meliloti* motility, whereas the Group 2 proteins function negatively (**Fig. 2**). This implies that c-di-GMP levels are higher in Group 1 than in Group 2. A hierarchy system may regulate the motility of *S. meliloti*, which mainly consists of several regulators, including ExoR/ExoS/ChvI, VisN/VisR, ExpR, and SinR [13,18,19]. It is an intriguing question whether these regulators are associated with the effectors of c-di-GMP, because several types of c-di-GMP effectors have been identified in various bacteria [4,5].

Sinorhizobium meliloti secretes at least three EPSs, namely, succinoglycan (EPSI), galactoglycan (EPSII), and capsule polysaccharide (KPS). These are required for the successful establishment of efficient symbiosis between *S. meliloti* and alfalfa. EPS biosynthesis is regulated by multiple regulators (ExoR−ExoS−ChvI, ExpR−SinR−SinI, and so forth) in *S. meliloti*, which is stimulated by limited phosphate, sulfate, or combined nitrogen [20,21]. Our data suggest that the Group 1 proteins play negative roles in EPS biosynthesis (**Fig. 3**). The increased levels of EPS could be succinoglycan (EPSI) or cellulose in these mutants, because this type of EPS binds to the specific florescent dye calcofluor [22,23]. If cellulose, then the Group 1 proteins could function similarly to Pled, Rge1, DgcAS, and YhcK from *R. legtuminosarum* bv. *trifolii* [9]. On the other hand, if the EPS is succinoglycan, then the secondary messenger, c-di-GMP, could contribute to succinoglycan (EPSI) biosynthesis in *S. meliloti*. This would be an interesting mechanism to investigate in the future.

Several GGDEF and EAL protein gene mutants have changed the cellulase and protease activities in some phytopathogenic bacteria [23]. However, we did not observe variation in either type of enzyme activity in the 14 GGDEF/EAL protein gene mutants studied herein. We also examined biofilm formation in the *S. meliloti* wild-type and mutant strains in 96-well plates (PVC) containing LB broth [20], but the results were inconclusive.

In nature, different rhizobium strains may competitively induce the same host plant to form nitrogen-fixing nodules, which is usually called competitive nodulation [15]. It is often quantified as a nodule occupation ratio, which is one of the key features of bio-fertilizers derived from rhizobia. It has been reported that the competitive nodulation of *Bradyrhizobium japonicum* is affected by cell density [24]. In this study, we found that the proteins in Group 1 are essential for *S. meliloti* competitive nodulation on alfalfa (**Fig. 4**). These mutants exhibited slower growth in minimal medium (**Fig. 1**). We believe that they may grow more slowly in artificial soil (vermiculite−perlite) compared with the wild-type, which results in lower cell density and less competitive nodulation than the wild-type.

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In conclusion, 14 GGDEF and EAL protein genes from *S. meliloti* were identified by bioinformatics analysis and systematic insertion mutagenesis. These proteins function in two different modes to control rhizobial growth, motility, and EPS production. The 11 proteins in Group 1 are required for rhizobial competitive nodulation on host plants. More importantly, our data suggested that these proteins play different roles in the control of the same physiological behavior in *S. meliloti*. Therefore, these GGDEF/EAL proteins may modulate c-di-GMP content at different levels in a rhizobial cell.

Supplementary Data

Supplementary data are available at *ABBS* online.

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