

A Role for the EAL-Like Protein STM1344 in Regulation of CsgD Expression and Motility in *Salmonella enterica* Serovar Typhimurium[∇]

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Received 4 March 2009/Accepted 8 April 2009

The bacterial second messenger cyclic di-GMP (c-di-GMP) regulates the transition between sessility and motility. In *Salmonella enterica* serovar Typhimurium, the expression of CsgD, the regulator of multicellular rdar morphotype behavior, is a major target of c-di-GMP signaling. CsgD expression is positively regulated by at least two diguanylate cyclases, GGDEF domain proteins, and negatively regulated by at least four phosphodiesterases, EAL domain proteins. Here, we show that in contrast to EAL domain proteins acting as phosphodiesterases, the EAL-like protein STM1344 regulated CsgD expression positively and motility negatively. STM1344, however, did not have a role in c-di-GMP turnover and also did not bind the nucleotide. STM1344 acted upstream of the phosphodiesterases STM1703 and STM3611, previously identified to participate in CsgD downregulation, where it repressed their expression. Consequently, although STM1344 has not retained a direct role in c-di-GMP metabolism, it still participates in the regulation of c-di-GMP turnover and has a role in the transition between sessility and motility.

Cyclic di-GMP (c-di-GMP) is a recently recognized secondary messenger which regulates the transition between sessility and motility in many bacteria (4, 27). The output of c-di-GMP signaling is determined by the temporally and spatially regulated delicate balance between the synthesis and degradation of the secondary messenger. The synthesis of c-di-GMP occurs through diguanylate cyclases which have a catalytic GGDEF domain in common (23, 35). The degradation of c-di-GMP by c-di-GMP-specific phosphodiesterases, on the other hand, is an enzymatic activity carried out by nonhomologous EAL and HD-GYP domains (32, 36). GGDEF, EAL, and HD-GYP domain proteins constitute superfamilies (7), in which subgroups of proteins can be identified (24). Although not applicable to all individual c-di-GMP-metabolizing enzymes, some general principles underlie the phenotypic output of c-di-GMP-synthesizing and -degrading activities. The expression of diguanylate cyclases leads to enhanced (local or general) intracellular c-di-GMP concentrations, which consequently promote sessility and inhibit motility. In contrast, low c-di-GMP levels promote motility and inhibit sessility. Signature motifs composed of highly conserved residues which are crucial for the c-di-GMP-metabolizing activities of GGDEF and EAL domains have been identified previously (25, 36), although the core residues required for catalytic activity are not entirely defined. Alternative functions for GGDEF and EAL domain proteins with motifs deviating from the consensus sequence have been identified (3, 6, 22, 40, 43). For example, the GGDEF-EAL domain protein LapD has been shown to bind c-di-GMP via its EAL domain (22), while CsrD has been shown to target small RNAs for degradation (40).

The transcriptional regulator CsgD is a master regulator of a multicellular/biofilm behavior in *Salmonella enterica* serovar Typhimurium and other *Enterobacteriaceae* which produces a distinct colony morphology on agar plates, the rdar (red, dry, and rough) morphotype (8). Characteristic of the rdar morphotype is the expression of the extracellular matrix components curli fimbriae and cellulose. Not only the biosynthesis of proteinaceous and polysaccharide extracellular matrix components (10, 19, 28, 47), but also the physiological adaptation to the sessile mode of growth (2) is regulated by CsgD.

CsgD and the rdar morphotype are major targets for regulation by c-di-GMP signaling (27, 33). Screens for proteins in *S. Typhimurium* that contribute to rdar morphotype formation revealed that at least two diguanylate cyclases (GGDEF/GGDEF-EAL domain proteins STM2123 and STM3388) and four phosphodiesterases (EAL/GGDEF-EAL domain proteins STM1703, STM1827, STM3611 [YhjH], and STM4264) are involved in the expression of CsgD in a temporally and hierarchically organized fashion (16, 37). Knockouts of STM1703 and STM4264 have more significant effects than knockouts of the other proteins, enhancing CsgD expression at least fourfold (37). STM4264 acts upstream of STM1703, as the overexpression of STM1703 downregulates CsgD expression in an STM4264 mutant while the overexpression of STM4264 in an STM1703 mutant fails to do so. Consistently, STM4264 affects a much greater fraction of the cellular c-di-GMP than STM1703. On the other hand, only one of the phosphodiesterases, STM3611, has an effect on swimming motility in the wild-type background (37), thus inversely regulating sessility and motility.

In *S. Typhimurium*, bioinformatic analyses previously detected the presence of five GGDEF, seven EAL, and seven GGDEF-EAL domain proteins. Among them, the EAL-like protein STM1344, highly similar to the phosphodiesterase STM3611, was identified (26). STM1344 behaves unconventionally, as it is required for rdar morphotype and CsgD expression but downregulates motility. Although STM1344 af-

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[∇] Published ahead of print on 17 April 2009.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description ^a	Reference or source
S. Typhimurium strains		
ATCC 14028	Wild type	
UMR1	ATCC 14028-1s Nal ^r	28
MAE46	UMR1 $\Delta ompR101::Amp^r$	28
MAE52	UMR1 <i>P_{csgD1}</i>	31
MAE420	UMR1 STM3611::Cm ^r	37
MAE424	UMR1 STM1344::Cm ^r	37
MAE282	UMR1 STM1703::Cm ^r	16
MAE425	UMR1 STM4264::Cm ^r	37
MAE422	UMR1 STM1827::Cm ^r	37
MAE130	UMR1 STM3611-SPA Km ^r	This study
MAE131	UMR1 STM1703-SPA Km ^r	This study
MAE132	UMR1 STM1344-SPA Km ^r	This study
MAE440	UMR1 STM1344::Cm Δ STM1703::101	This study
MAE1251	UMR1 STM1344::Cm Δ STM1827::101	This study
MAE451	UMR1 STM1344::Cm Δ STM3611::101	This study
MAE439	UMR1 STM1344::Cm Δ STM4264::101	This study
MAE1253	UMR1 STM1344::Cm STM1703-SPA Km ^r	This study
MAE1252	UMR1 STM1344::Cm STM3611-SPA Km ^r	This study
LT2	Wild type	
LB5010	<i>metA22 metE551 ilv-452 leu-3121 trpO2 xyl-404 galE856 hsdLT6 hsdSA29 hsdSB121 rpsL120</i>	31
E. coli strains		
K-12 DH5 α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 (ϕ80lacZΔM15)</i>	Laboratory collection
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3)	Novagen
BL21(DE3)(pLysS)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3) pLysS	Novagen
Plasmids		
pBAD30	Arabinose-regulated promoter; Amp ^r	11
pKD46	Expresses λ Red recombinase system; Amp ^r ; temp-sensitive replicon	5
pET23a(+)	Expression vector with T7 promoter; Amp ^r	Novagen
pRGS40 (pSTM1344)	pBAD30::STM1344-His ₆ Amp ^r	This study
pRGS42	pBAD30::STM1344(N85A)-His ₆ Amp ^r	This study
pRGS44	pBAD30::STM1344(F144A)-His ₆ Amp ^r	This study
pRGS45	pBAD30::STM1344(D165A)-His ₆ Amp ^r	This study
pRGS46	pBAD30::STM1344(F168A)-His ₆ Amp ^r	This study
pRGS47	pET23a::STM1344; expresses C-terminal His ₆ tag; Amp ^r	This study
pRGS48	pET23a::STM3611; expresses N-terminal FLAG tag; Amp ^r	This study
pMMB-PA3702	pMMB::PA3702 Amp ^r	18
pEZ-YcgR	pET23a::K-12ycgR; expresses C-terminal His ₆ tag	34
pJL148	Expresses SPA tag (calmodulin binding peptide, tobacco etch virus protease cleavage site, and three FLAG tags); Km ^r	46

^a Nal^r, nalidixic acid resistant; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant.

fects the c-di-GMP pool in vivo, biochemical analysis showed that STM1344 neither degrades nor synthesizes c-di-GMP and also does not bind it. However, STM1344 is integrated into the c-di-GMP network, as it works independently of the phosphodiesterases STM4264 and STM1827 to affect the expression of the phosphodiesterases STM1703 and STM3611. Therefore, STM1344 exerts a novel function in aiding the transition between sessility and motility.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. For cloning purposes, *Escherichia coli* and *S. Typhimurium* were grown at 37°C on Luria-Bertani (LB) agar plates supplemented with the appropriate antibiotics. For analysis, bacteria were precultured on LB agar plates at 37°C overnight and directly inoculated onto LB agar plates without salt at 28 or 37°C for the times indicated below. Cells were grown on agar plates containing LB medium without salt, supplemented with Congo red (40 μ g ml⁻¹) and Coomassie brilliant blue G-250 (20 μ g ml⁻¹) or calcofluor white (fluorescence brightener 28; 50 μ g ml⁻¹) when required. For the

expression of genes, 0.1% arabinose or 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was used. The antibiotics employed were ampicillin (100 μ g ml⁻¹), chloramphenicol (20 μ g ml⁻¹), kanamycin (30 μ g ml⁻¹), and tetracycline (20 μ g ml⁻¹).

General molecular biology methods. Common molecular biological procedures such as the isolation of plasmid and chromosomal DNA, PCR analyses, and the transformation of cells with plasmids, as well as restriction digestion, were carried out using standard protocols (1). PCR amplifications were performed using GeneCraft *Taq* polymerase (Biostar). PCR products were cleaned using the QIAquick PCR purification kit (Qiagen), and DNA fragments were recovered from agarose gels by using the QIAquick elute gel purification minikit (Qiagen). Primer sequences are provided in Table 2.

Plasmid construction. For complementation, the STM1344 gene was cloned into pBAD30 with a sequence expressing a C-terminal His tag. Mutant STM1344 alleles were constructed by PCR-based mutagenesis using overlapping primers (Table 2) and subsequently cloned into pBAD30.

For the expression of STM1344 and STM3611 proteins in *E. coli* strain BL21(DE3), the corresponding genes were cloned into the expression vector pET23a(+) with sequences encoding a C-terminal His tag and an N-terminal FLAG tag, respectively.

Primers used to construct the recombinant plasmids are listed in Table 2. *E.*

TABLE 2. Primers used in this study

Purpose and name	Sequence ^a	Restriction site and/or tag
Construction of SP4-tagged proteins		
STM1344-SPA-F	TGGCCCTCCGGTGCCGGTAAGCCAGCTCATTAACCTCGTTCAGCGATCCATGGAAAAGAGAAG	
STM1344-SPA-R	AGTGTAGACGGTTAATCACCGGTTAAACACCGGCAAAACAGAAAGGCATATGAATATCCTCCTTAG	
STM3611-SPA-F	CGACCCGTGCCGCTGATATCGCTCGAAGAGGTGATTCCTGACCCCTGTCATGGAAAAGAGAAG	
STM3611-SPA-R	ATGACCGCGCCTCGTAATACCACGTATACGGGAACAGTCTGGCGCATATGAATATCCTCCTTAG	
STM1703-SPA-F	TCCTTTGAACGCTGGTACAACGTTATCAGACGAAAAAATGCGTTCATGGAAAAGAGAAG	
STM1703-SPA-R	GGCTATGCCTGTCTGCACAGGCTGTTCCCTATTATCGTCGCCACATATGAATATCCTCCTTAG	
SP4-tag control		
STM1344_SPA_control	TGCACGCTATTGTTGCCCA	
STM1344_Control_Reverse	GGCAGTAAAAGACAGGGT	
STM3611_SPA_control	TGCTGCTGCAACTGATGAAC	
STM3611_Control_Reverse	TGTGATGGCGCGGTTATT	
STM1703_SPA_Control	AGGCGCTAAATTACAAGTG	
STM1703_Control_Reverse	AAATTGATTGTTGTCGGGAGT	
Cloning		
STM1344_pET23a_Start	CGTGGCTAGCATGCTTCACTTGATGAGCTT	NheI
STM1344_pET23a_Stop	CCTAAGCTTTCGCTGAACGAGTTAATGA	HindIII
STM3611_pET23a_N-FLAG_Start	AGCCATATGACTACAAGACGATGACGACAAGGGTATAAAGCAGGTTATCCAGCA	NdeI, FLAG tag
STM3611_pET23a_N-Tag_Stop	AGCAAGCTTTTACAGGGTCAGAATCACCTCT	HindIII
Construction of variant STM1344		
E29A_forward2	TTGGTTTAGCAATTATTGCAACGT	
E29A_reverse2	GTTGCAATAAATTGCTAAACCAACAA	
N85A_forward	CATGGCTTGCATTACCGCTGCAATTAGTGA	
N85A_reverse	GCGGTAATGCAAGCCATGCAATAAGTTTATGC	
F144A_forward	CTGGCAAAATGCTGGGGCAGGCGAGGCG	
F144A_reverse	TGCCCCAGCATTTGCCAGCATTAATGGGA	
D165A_forward	GTTCATGTTGGCTAAAAAATTTATTCAGCAGCGAG	
D165A_reverse	TAAAAATTTTAGCCAACATGACCTGTTGAAAA	
F168A_forward	TTGGATAAAAAATGCTATTTCAGCAGC	
F168A_reverse	CTGCTGAATAGCATTTTATCCAAC	
RT-PCR		
recA_forward	GGCGAAATCGGCGACTCT	
recA_reverse	CATACGGATCTGGTTGATGAAAATC	
STM1703_forward	GGCGCAGGAGCTCTCTTT	
STM1703_reverse	GCAATGACTGAGTTGGCTTCTG	
STM3611_forward	CTGCTGCTGCAACTGATGAAC	
STM3611_reverse	CTCCACGCCCTCGACAAT	
csgD_forward	ACGCTACTGAAAGACCAGGAAC	
csgD_reverse	GCATTCGCCACGCAGAATA	
Sequencing		
pBAD_MCS_Start	ATCGCAACTCTCTACTGTTTC	
pBAD_MCS_Stop	CTGATTTAATCTGTATCAGGC	

^a SPA tag-specific priming sequences are underlined only, the FLAG tag sequence is in bold and underlined, restriction sites are shown in italics, and nucleotide exchanges leading to amino acid replacement are shown in bold.

coli DH5 α was transformed with the plasmids, which were passed through the restriction-deficient strain *S. Typhimurium* LB5010 before being introduced into *S. Typhimurium* UMR1 derivatives by electroporation.

Construction of mutants. Epitope tagging of STM1344, STM1703, and STM3611 was performed by the λ Red recombination method (5). The tag was a sequential peptide affinity (SPA) tag (consisting of a calmodulin binding peptide, a tobacco etch virus protease cleavage site, and three FLAG tags) PCR amplified from plasmid pJL148 (46). Briefly, approximately 300 ng of processed PCR product was introduced by electroporation into *S. Typhimurium* UMR1 containing pKD46, which expresses λ Red recombinase. Recovered colonies were purified at least twice on LB medium containing appropriate antibiotics.

Phage transduction was carried out with phage P22 HT105/1 *int-201*, a high-frequency generalized transducing mutant of phage P22. Transductants were colony purified twice on LB agar plates containing EGTA (10 mM) and appropriate antibiotics. All constructed mutants were verified by PCR with control primers flanking the deleted open reading frame or the inserted DNA fragment. Gene fusions were sequenced to ensure the in-frame insertion of the SPA tag sequence, leading to the production of a fusion protein.

Protein techniques. For Western blot analysis, 5 mg (wet weight) of cells was harvested, resuspended in sample buffer, and heated to 95°C for 10 min. The protein content was analyzed by staining with a Coomassie blue solution (20% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue G). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% resolving gel with 4% stacking gel and were transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore). The detection of CsgD was carried out as described previously (30) using the polyclonal anti-CsgD peptide antibody (1:5,000) as the primary antibody and goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:2,000;

Jackson ImmunoResearch Laboratories Inc.) as the secondary antibody. The detection of FLAG tags was done using anti-FLAG antibody (1:2,000) and anti-mouse immunoglobulin G conjugated with horseradish peroxidase (1:2,000; Jackson ImmunoResearch Laboratories Inc.) as the secondary antibody. Chemiluminescence from the Lumi-Light WB substrate (Roche) was recorded using the LAS-1000 system (Fujifilm) and quantified using ImageQuant software (version 5.2).

The enrichment of curli fibers was performed as described previously (29). CsgA subunit expression was detected by colloidal Coomassie blue staining after depolymerization of the curli fibers and separation on gels (4% stacking gel and 15% separating gel).

Phenotypic evaluation. (i) **Congo red and calcofluor white binding assays.** Samples of 5 μ l of an overnight culture suspended in water (to an optical density at 600 nm [OD₆₀₀] of 5) were spotted onto LB agar plates lacking NaCl and supplemented with Congo red (40 μ g ml⁻¹) and Coomassie brilliant blue (20 μ g ml⁻¹) or calcofluor white (fluorescence brightener 28; 50 μ g ml⁻¹). Plates were incubated at 28°C for 48 h or at 37°C for 24 h. The development of the colony morphology and dye binding were analyzed over time.

(ii) **Analysis of swimming motility.** Swimming motility on 0.3% LB agar plates at 37°C was observed for 6 h. The plates were inoculated with 5- μ l aliquots of an overnight culture resuspended at an OD₆₀₀ of 5. The diameter of the area covered by swimming bacteria was measured after 1 to 6 h.

(iii) **Analysis of swarming motility.** Swarming motility on 0.5% LB agar plates supplemented with 0.5% glucose was analyzed. The plates were inoculated with 5- μ l aliquots of an overnight culture (OD₆₀₀ = 5) and incubated at 37°C. The radius from the point of inoculation to the edge of the swarming zone was measured every hour for up to 6 h.

RNA extraction. The extraction of total RNA was performed using the SV total RNA isolation system according to the protocol of the manufacturer (Pro-



FIG. 1. Comparison of the amino acid sequence of the EAL-like protein STM1344 to that of the most similar EAL domain protein, the phosphodiesterase STM3611 in *S. Typhimurium* UMR1. Both proteins have only an EAL domain, with the name-giving EAL motif changed to EII in STM1344 and ELL in STM3611. Amino acid alignment was performed using the FFAS03 pairwise alignment server (14) and manual adjustment. Amino acids conserved in STM1344 and STM3611 are shown with a black background. The consensus motifs for functional phosphodiesterases are displayed below the alignment and shown with a gray background. The asterisks indicate alanine replacement of amino acids in STM1344, whereby only F₁₆₈ was shown to have a functional role.

mega) with minor modifications. Prior to RNA extraction, the bacterial cells were incubated for ≥ 30 min in a mixture of ice-cold 5% (vol/vol) phenol and 95% (vol/vol) ethanol on ice to stabilize the RNA. The samples were centrifuged at 4,000 rpm for 10 min, and the supernatant was discarded. Subsequently, the pellet was resuspended in 100 μ l of lysis buffer (50 mg/ml lysozyme in Tris-EDTA buffer) and incubated for 5 min at room temperature. The following steps of RNA purification, including on-column DNase I treatment, were performed according to the instructions of the RNA isolation system manufacturer. RNA concentrations were determined using the NanoDrop system (Thermo Scientific), and the quality of the RNA was assessed via gel electrophoresis or similar methods.

Quantitative real-time RT-PCR. The expression of target genes was evaluated by two-step real-time reverse transcription (RT)-PCR using the Power Sybr green PCR master mix (Applied Biosystems) and the 7500 real-time PCR system (Applied Biosystems). First-strand cDNA synthesis from RNA samples (1 μ g) was performed with the high-capacity cDNA RT kit (Applied Biosystems). Relative transcript abundance was determined by the $2^{-\Delta\Delta Ct}$ method (20) using 7500 SDS software v1.3.1 (Applied Biosystems). The *recA* gene was used as an endogenous control for internal normalization. All experiments were performed as biological and technical replicates using the mean expression from quadruplicates per real-time PCR assay relative to a calibrator value (the value for *S. Typhimurium* UMR1). Statistical significance levels (*P* values) were determined with a paired *t* test using GraphPad Prism software.

Quantification of c-di-GMP. Nucleotide extracts were prepared essentially as described previously (39). Bacteria were suspended in 0.19% ice-cold formalin, heated to 95°C for 10 min, and extracted by ethanol treatment. Nucleotide extracts equivalent to 10 mg (wet weight) were subjected to high-performance liquid chromatography (HPLC) separation using a Hypersil octadecyl silane (ODS) reversed-phase column (5- μ m particle size; Hypersil-Keystone). Runs were carried out with a multistep gradient using 0.1 M triethylammonium acetate (pH 6.0) at 1 ml min⁻¹ with increasing concentrations of acetonitrile. Relevant fractions were collected, lyophilized, and resuspended in 10 μ l of water. Fractions containing c-di-GMP were pinpointed by matrix-assisted laser desorption/ionization-time of flight analysis and pooled. Synthetic c-di-AMP was added to the pooled fractions at a concentration suitable for use as an internal standard. A standard curve was established using fractions spiked with known amounts of c-di-GMP, with a fixed amount of synthetic c-di-AMP as an internal control. The peak areas for c-di-GMP and c-di-AMP were calculated, and the ratio was determined. c-di-GMP measurements were done in triplicate in two independent experiments.

Purification of proteins. STM1344, YcgR (34), and WspR*, constitutively active WspR (18), were purified as soluble C-terminal His₆ tag fusions by nickel affinity chromatography essentially according to the protocol of the chromatography system manufacturer (Qiagen). STM1344 was eluted in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM MgCl₂, and 250 mM imidazole. Soluble STM3611 with an N-terminal FLAG tag fusion was purified using monoclonal anti-FLAG M2 affinity gel according to the protocol of the manufacturer (Sigma-Aldrich). Buffer exchange and the concentration of proteins were done by centrifugation in Amicon ultrafiltration units (Millipore) with a cutoff of 10 kDa. STM1344 and STM3611 were concentrated to 1 mg/ml in a solution of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10 mM MgCl₂ and used directly in assays or stored for up to 2 days at 4°C. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce).

c-di-GMP binding assays. (i) **UV cross-linking of c-di-[³²P]GMP.** Purified protein (10 μ M) was incubated with c-di-GMP (100 μ M) in binding buffer 1 (50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 0.5 mM EDTA, 10% glycerol) (34) or binding buffer 2 (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM MgCl₂) for 20 min at room temperature. Samples were irradiated at 254 nm for 5 to 15 min on ice in a Hoefer UVC 500 UV cross-linker with a UVG-54 mineral light lamp (UVP) at a distance of 3 cm (intensity, $\sim 14,000 \mu$ W cm⁻²). After irradiation, samples were treated with 2 \times SDS-PAGE sample buffer (40% glycerol, 8% SDS, 2% β -mercaptoethanol, 40 mM EDTA, 0.05% bromophenol blue, 250 mM Tris-HCl, pH 6.8) and boiled for 5 min. Proteins were separated by SDS-PAGE (with 15% separating gel) and detected by storage phosphorimaging with a Typhoon scanner (Amersham Bioscience) and Coomassie blue staining.

(ii) **Equilibrium dialysis.** c-di-GMP binding to STM1344 was assessed using the rapid equilibrium dialysis (RED) device disposable chambers and plate according to the instructions of the manufacturer (Pierce). In short, at the start of the assay, 200 pmol of the protein to be tested was added to the binding buffer in the RED sample chamber, which resulted in a 2 μ M concentration of protein. Two hundred picomoles of c-di-GMP was added to 300 μ l of binding buffer in the control chamber to give a starting c-di-GMP concentration of 667 nM. The chambers were covered with parafilm and incubated for 16 h at room temperature to allow equilibrium between the two chambers to be reached. The c-di-GMP concentrations in both chambers were determined and normalized against the c-di-GMP concentrations in the chambers of the RED device insert in which no protein had been added to the sample chamber. The amount of bound c-di-GMP was calculated according to the protocol of the manufacturer, as follows: % bound c-di-GMP = $100 \times (1 - [c-di-GMP]_{\text{Buffer chamber}}/[c-di-GMP]_{\text{Sample chamber}})$, where $[c-di-GMP]_{\text{Buffer chamber}}$ is the concentration of c-di-GMP in the control chamber and $[c-di-GMP]_{\text{Sample chamber}}$ is the concentration of c-di-GMP in the sample chamber.

Synthesis of c-di-[³²P]GMP. c-di-[³²P]GMP was synthesized essentially as described previously (21) by incubating 50 μ g of purified His₆-WspR* (18) with 200 μ Ci of [³²P]GTP (Amersham Biosciences) at room temperature in 100 μ l of reaction buffer (75 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM KCl, 10 mM MgCl₂). Residual [³²P]GTP was dephosphorylated by the addition of 5 U of calf intestinal phosphatase (New England Biolabs) to the reaction mixture, followed by 15 min of incubation at room temperature. The protein was precipitated by heating the sample to 95°C for 5 min and centrifuging at 16,000 \times g. The c-di-[³²P]GMP was passed through an Ultrafree-MC column (Millipore) with a cutoff of 5 kDa by centrifugation for 30 min at 5,000 \times g. The conversion of [³²P]GTP to c-di-[³²P]GMP was analyzed by thin-layer chromatography on polyethyleneimine-cellulose plates (Sigma) using 1.5 M KH₂PO₄, pH 3.65, as the mobile phase. The ³²P-labeled nucleotides were visualized and quantified by storage phosphorimaging with a Typhoon scanner (Amersham Bioscience).

Enzymatic assay. The c-di-GMP-synthesizing activity was tested by incubating 5 μ M protein and 2 μ M GTP in 100 μ l of reaction buffer (75 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM KCl, 10 mM MgCl₂) for 1 h. Phosphodiesterase activity was assayed by incubating 1.0 μ M protein and 100 μ M c-di-GMP in 100 μ l of reaction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM MgCl₂) for 1 h. The reactions were stopped by heating the sample to 95°C for 5 min, and protein was precipitated by centrifugation at 16,000 \times g for 5 min. The supernatant was analyzed by reversed-phase HPLC on a Hypersil ODS C₁₈ column with the dimensions 50 by 4.6 mm and a 3- μ m particle size and a Hypersil ODS guard column (10 by 4 mm; 5- μ m particle size). The mobile phase was buffer A (0.1 M trimethylammonium acetate, pH 6.0) and buffer B (80% acetonitrile and

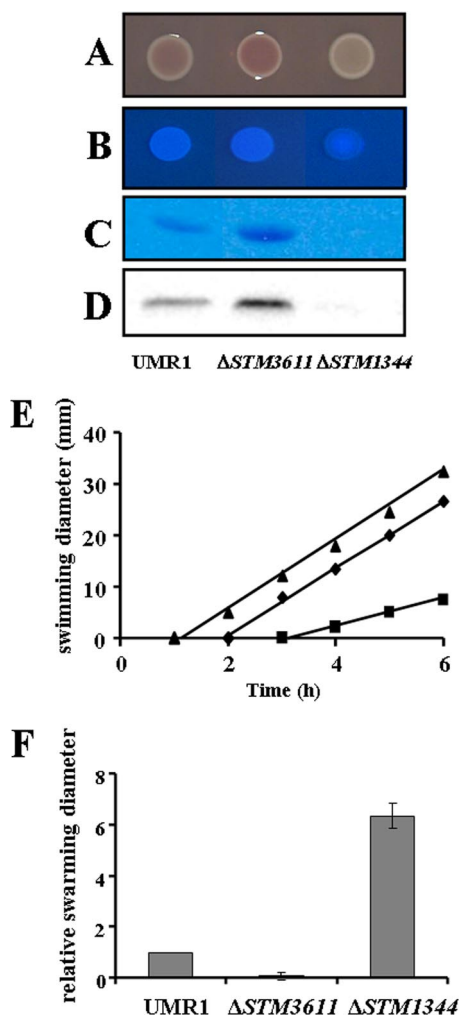


FIG. 2. Phenotypes of the STM1344 knockout mutant in comparison with those of wild-type *S. Typhimurium* UMR1 and the c-di-GMP-specific phosphodiesterase STM3611 knockout mutant. (A to D) The expression of the rdar morphotype (A), calcofluor white binding capability (indicative of cellulose expression) (B), extracellular matrix component curli fimbriae (C), and the master regulator of rdar morphotype expression, CsgD (D), in the Δ STM1344 mutant was downregulated in comparison to that in wild-type *S. Typhimurium* UMR1. Cells were grown on plates of LB agar without salt supplemented with Congo red (A) or calcofluor white (B) at 28°C for 20 h. The expression of CsgD was evaluated by Western blot analysis. Curli fimbriae were visualized by Coomassie brilliant blue staining of protein gels. (E and F) The swimming (E) and swarming (F) motilities of the Δ STM1344 mutant were upregulated in comparison to those of wild-type *S. Typhimurium* UMR1. Diameters of the zones covered by swimming and swarming bacteria were measured. The regulation of all phenotypes of the Δ STM1344 mutant was opposite that of the phenotypes of the STM3611 knockout mutant. Plates were incubated at 37°C for up to 6 h. In panel E, diamonds represent the wild-type strain UMR1, squares represent the STM3611 mutant, and triangles represent the STM1344 mutant.

20% buffer A) at a flow rate of 1 ml/min in a gradient from 0 to 10% buffer B in 5 min. c-di-GMP eluted at 3.35 min.

RESULTS

Identification of STM1344 as an unconventional EAL domain protein. Recently, a knockout library of genes in the *S.*

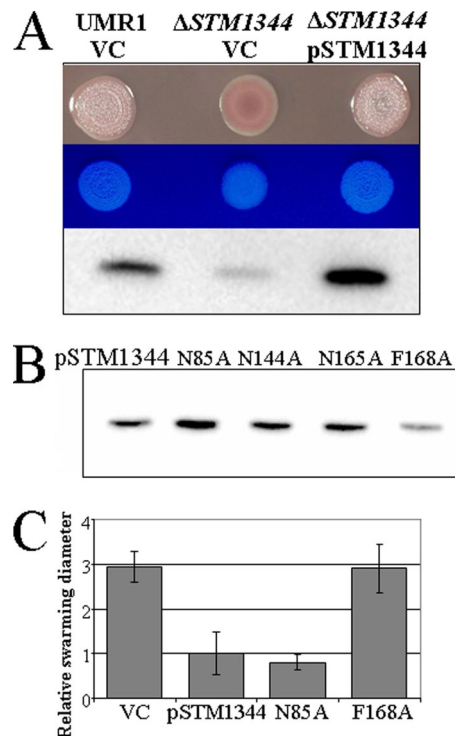


FIG. 3. Complementation of an *S. Typhimurium* UMR1 Δ STM1344 mutant with wild-type and variant STM1344-His₆ proteins. The complementation of Δ STM1344 with a pBAD30 vector expressing STM1344-His₆ (pSTM1344) leads to the upregulation of the rdar morphotype. (A) Calcofluor white binding and CsgD expression. (B) Complementation of Δ STM1344 with STM1344-His₆ variants. Only the F168A mutant of STM1344 did not complement CsgD expression. The N85A STM1344 variant produced a hypercomplementation phenotype. For Western blot analysis (A and B), cells were grown for 20 h on plates of LB agar without salt at 28°C. Pictures of plate-grown cells were taken after 48 h to make morphotype changes strongly visible. (C) Repression of swarming motility by the overexpression of STM1344-His₆ and selected variants. While the N85A variant of STM1344 repressed swarming motility as did wild-type STM1344, the F164 variant of STM1344 had no effect. VC, pBAD30 vector control.

Typhimurium chromosome known to encode EAL domain proteins was created (37). The bioinformatic analysis identified STM1344 (also called YdiV) as a single-domain EAL domain protein with a high overall degree of similarity (43%) to the phosphodiesterase STM3611 yet missing several consensus residues required for phosphodiesterase activity (Fig. 1) (26).

In order to elucidate the function of STM1344, if any, in rdar morphotype expression, an STM1344 knockout mutant of *S. Typhimurium* UMR1 was created. Surprisingly, rdar morphotype expression in the Δ STM1344 mutant was downregulated compared with that in UMR1 (Fig. 2A). In line with the downregulation of the rdar morphotype, CsgD, the major regulator of rdar morphotype expression (9), was downregulated (Fig. 2D), as were extracellular matrix components cellulose and curli fimbriae (Fig. 2B and C; also data not shown), which are commonly activated by CsgD (26).

Motility is another major phenotype regulated by c-di-GMP signaling (38), and wild-type *S. Typhimurium* cells show swimming and swarming motilities. The knockout of STM1344 created a mutant that swam at the same speed as, but started to

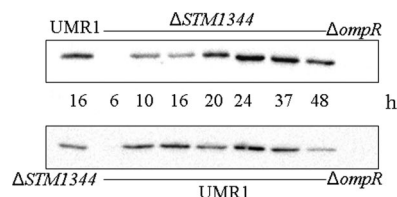


FIG. 4. Western blot analysis of growth phase-dependent expression of CsgD in the Δ STM1344 mutant of *S. Typhimurium* UMR1. As in wild-type *S. Typhimurium* UMR1, there is growth phase-dependent expression of CsgD in the Δ STM1344 mutant. The expression of CsgD in the Δ STM1344 mutant is reduced approximately 50% compared to that in the wild type. The reference was *S. Typhimurium* UMR1 or the Δ STM1344 mutant grown for 16 h; the negative control was *S. Typhimurium* MAE46 (Δ ompR) grown for 24 h.

swim earlier than, the wild type (Fig. 2E). On the other hand, the swarming motility of the STM1344 knockout mutant was highly enhanced compared to that of the wild type (Fig. 2F). In summary, the phenotypes of the STM1344 knockout mutant are opposite to the phenotypes of the c-di-GMP-specific phosphodiesterase STM3611 knockout mutant, which shows up-regulation of rdar morphotype expression and downregulation of motility (Fig. 2) (37).

Complementation analysis of Δ STM1344. The Δ STM1344 mutant was complemented by STM1344 expressed from a plasmid as a C-terminally His-tagged protein. Complementation studies revealed that, at 28°C, STM1344-His₆ overexpression enhanced rdar morphotype expression slightly over wild-type levels and downregulated swimming and swarming motilities (Fig. 3A and C; also data not shown). The temperature regulation of rdar morphotype expression was not overcome by the overexpression of STM1344-His₆ since no rdar morphotype expression at 37°C was observed (data not shown).

To elucidate which amino acids are critical for the function of STM1344, alanine replacement of selected amino acids was carried out. Amino acids highly conserved in experimentally confirmed c-di-GMP-dependent phosphodiesterases (25, 36) were chosen, in particular E₂₉, N₈₅, F₁₄₄, D₁₆₅, and F₁₆₈. The F168A replacement had an effect on the function of STM1344, as the altered protein did not complement the expression of CsgD and the rdar morphotype and did not repress swimming and swarming motility compared to the wild-type STM1344 protein (Fig. 3B and C). On the other hand, the N85A variant showed a gain of function (Fig. 3B and C). While the E29A replacement in STM1344 could not be obtained without secondary mutations, F144A and D165A did not have an effect on STM1344 functionality (Fig. 3B; also data not shown). All proteins were expressed at the same level as wild-type STM1344-His₆, with the exception of the N85A variant, which was expressed at a lower level (data not shown).

Expression of STM1344 and effect of the knockout on CsgD expression. The analysis of CsgD expression in the Δ STM1344 mutant during growth revealed that CsgD was downregulated approximately 50% during the entire time period from 10 to 48 h (Fig. 4). The growth phase-dependent pattern of CsgD expression, with a characteristic peak at 24 h, remained. As ompR is required for csgD transcription (28), the ompR mutant served as a negative control. We investigated whether the expression of STM1344 parallels the expression of CsgD.

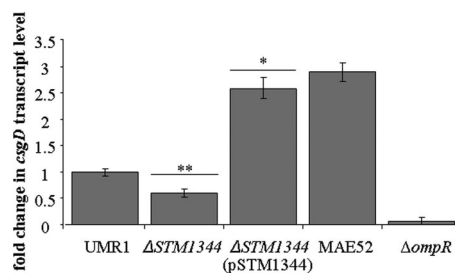


FIG. 5. Effect of STM1344 on *csgD* transcription in *S. Typhimurium* UMR1. The deletion of the STM1344 gene downregulated the *csgD* transcript level to approximately 60%, as determined by quantitative real-time RT-PCR. RNA was isolated from cells grown at 28°C for 20 h on LB agar plates without salt. Average values with standard deviations are displayed. **, $P \leq 0.01$; *, $P \leq 0.05$. The negative control was *S. Typhimurium* MAE46 Δ ompR; the positive control was MAE52, showing *csgD* expression enhanced threefold compared to that in UMR1 (31).

STM1344 was expressed at similar levels throughout the growth phase (data not shown).

Effect of STM1344 on *csgD* transcription. CsgD protein levels are downregulated in the Δ STM1344 mutant. To investigate on which level the regulation of CsgD expression occurs, the transcription of *csgD* was measured by real-time RT-PCR analysis. *csgD* transcript levels in the Δ STM1344 mutant were downregulated to 60% compared to those in the wild type after 20 h of growth, while the overexpression of STM1344-His₆ led to a 2.6-fold increase in *csgD* transcript levels compared to wild-type levels (Fig. 5).

Effect of STM1344 on intracellular c-di-GMP levels. c-di-GMP positively affects the expression of CsgD (16). Since STM1344 changed the expression of CsgD, the change in c-di-GMP levels by STM1344 was investigated. The Δ STM1344 mutant displayed a reduction in the c-di-GMP levels compared to those in wild-type UMR1, while the c-di-GMP levels in the Δ STM1344 mutant complemented with the STM1344 gene on a plasmid reached wild-type levels (Fig. 6A). The elevation of the c-di-GMP concentration by STM1344 is consistent with the phenotypes observed for a Δ STM1344 mutant, as described above. The reduction of c-di-GMP levels in a Δ STM1344 mutant suggested that STM1344 has a direct or indirect role in the c-di-GMP signaling network.

To elucidate if STM1344 had a direct role in c-di-GMP metabolism, the STM1344 gene with a sequence encoding a C-terminal His tag was cloned into the expression vector pET23a(+), overexpressed, and purified as outlined in Materials and Methods. STM1344-His₆ was obtained with over 99% purity as estimated by Coomassie blue staining of protein gels (data not shown). Functional analysis using relevant positive controls, however, showed that STM1344 was neither a c-di-GMP-dependent phosphodiesterase nor a diguanylate cyclase (Fig. 6B and C).

Surprisingly, no c-di-GMP binding by STM1344 was detected in two different assays using UV cross-linking and equilibrium dialysis (Fig. 6D and E), although the binding of c-di-GMP by the positive control YcgR, a PilZ domain-containing c-di-GMP binding protein (34), was readily detected. In addition, STM1344 did not bind related nucleotides such as cyclic GMP, cyclic AMP, or cyclic di-AMP (data not shown).

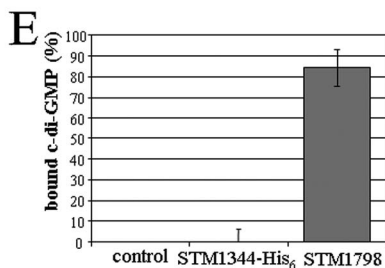
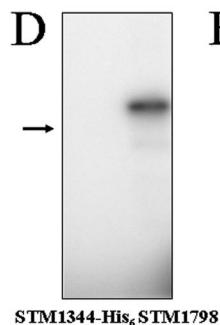
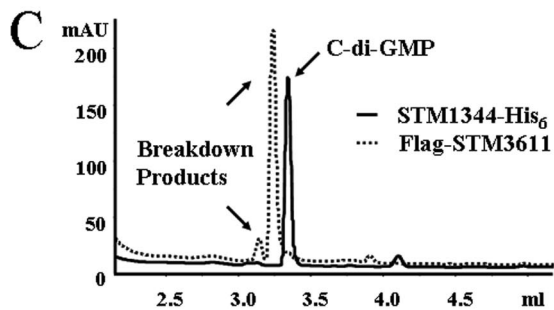
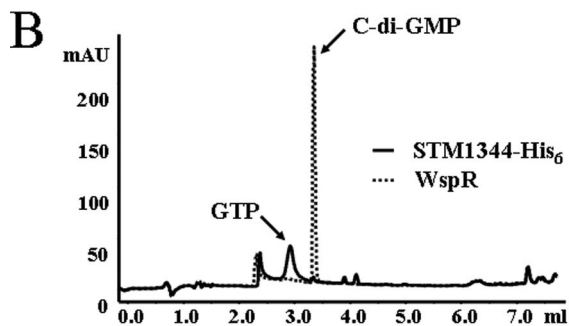
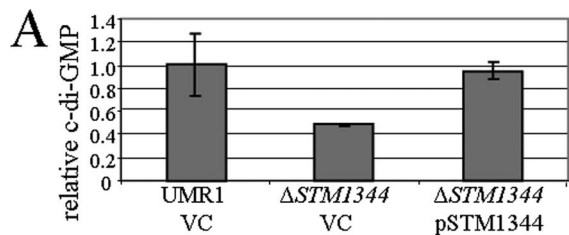


FIG. 6. Role of STM1344 in c-di-GMP metabolism in *S. Typhimurium* UMR1. (A) The total cellular c-di-GMP concentration is downregulated in the Δ STM1344 mutant compared to those in wild-type *S. Typhimurium* UMR1 and the Δ STM1344 mutant complemented with the STM1344-His₆ sequence cloned into pBAD30. Cells were grown for 20 h on LB agar plates without salt at 28°C. Averages with standard deviations, which are based on two independent experiments with three technical replicates, are displayed. VC, pBAD30 vector control. (B to E) Purified STM1344 does not exhibit c-di-GMP-metabolizing and c-di-GMP binding activities. (B) Purified STM1344 does not exhibit diguanylate cyclase activity, in contrast to the positive control, constitutively active WspR* (18). Protein at 5 μ M was incubated with 100 μ M GTP for 1 h. The HPLC elution profiles of the reaction products are as follows: c-di-GMP elutes at 3.35 ml, whereas GTP elutes before 3 ml. The dashed-line curve represents the WspR* sample, whereas the solid-line curve represents the STM1344 sample. (C) Purified STM1344 does not exhibit c-di-GMP-specific phosphodiesterase activity, in contrast to the positive control, STM3611 (38). Protein at 1 μ M was incubated with 100 μ M c-di-GMP for 1 h at 37°C.

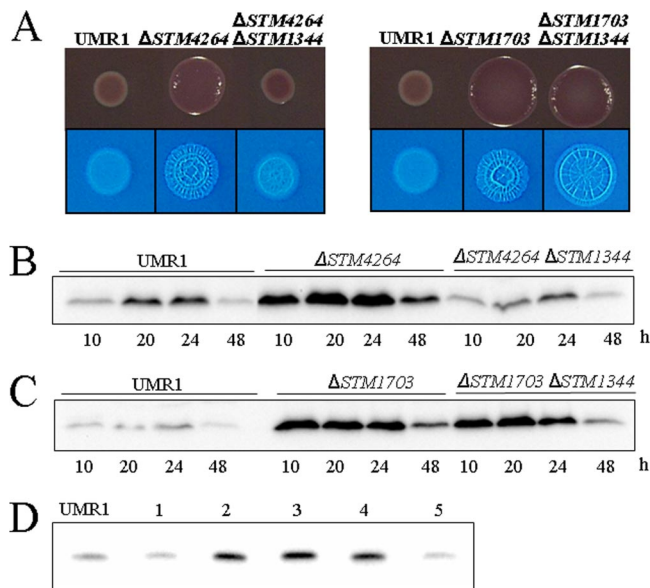


FIG. 7. Position of STM1344 in the c-di-GMP signaling network regulating CsgD expression in *S. Typhimurium* UMR1. (A) Expression of the rdar morphotype (top) and the calcofluor white binding phenotype (bottom) in wild-type *S. Typhimurium* UMR1, the corresponding mutants with knockouts of the STM4264 and STM1703 phosphodiesterase genes, and the respective double mutants with STM1344 deleted. While the knockout of STM1344 downregulated rdar morphotype expression in the STM4264 mutant background, only slightly altered rdar morphotype expression in the STM1703 background was observed. Cells were grown for 20 h at 28°C on plates of LB agar without salt, supplemented with Congo red or calcofluor white. (B to D) Effects of STM1344 deletion on CsgD expression in the STM4264 (B), STM1703 (C), STM3611 (D), and STM1827 (D) mutant backgrounds. CsgD was detected by Western blot analysis. Cells were grown on LB agar plates without salt at 28°C. In the analysis presented in panel D, cells were grown for 20 h. Lanes: 1, Δ STM1344 mutant; 2, Δ STM3611 mutant; 3, Δ STM1344 Δ STM3611 double mutant; 4, Δ STM1827 mutant; 5, Δ STM1344 Δ STM1827 double mutant.

Role of STM1344 in the c-di-GMP signaling network. To analyze the position of STM1344 in the c-di-GMP signaling network, double mutants with the STM1344 knockout and mutations in genes coding for phosphodiesterases previously identified to affect rdar morphotype and CsgD expression, namely, STM1703, STM1827, STM3611, and STM4264, were constructed. While mutations in single genes upregulated rdar

The HPLC elution profiles of the reaction products are as follows: c-di-GMP elutes at 3.35 ml; the first breakdown product, GpGp, elutes at 3.25 ml; and GMP elutes at 3.15 ml. The dashed-line curve represents the STM3611 sample, whereas the solid-line curve represents the STM1344 sample. mAU, milli-absorbance units. (D and E) Purified STM1344 does not show c-di-GMP binding, in contrast to the positive control, STM1798 (YcgR) (34), as determined by UV cross-linking (D) and equilibrium dialysis (E). The arrow in panel D indicates the position of STM1344-His₆. UV cross-linking was performed with c-di-[³²P]GMP. Protein at 10 μ M was incubated with 100 μ M c-di-GMP. For equilibrium dialysis, 200 pmol of c-di-GMP or other tested nucleotides was mixed with the purified proteins (200 pmol) in a dialysis cell surrounded by a membrane with an 8-kDa molecular mass cutoff. Equilibrium was reached after 16 h at room temperature with slow shaking. Average values with standard deviations are displayed.

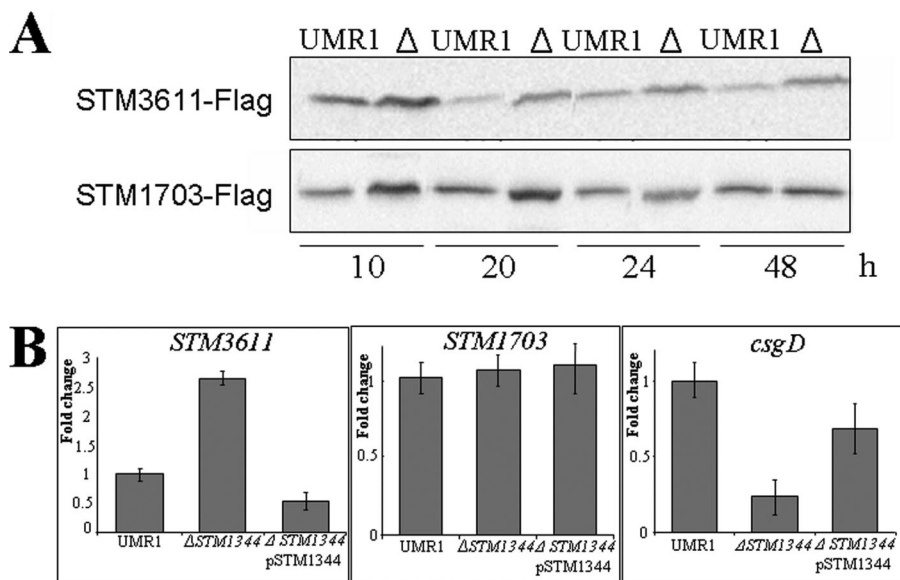


FIG. 8. Effect of STM1344 knockout in *S. Typhimurium* UMR1 on the expression of the phosphodiesterases STM3611 and STM1703. (A) STM1344 downregulates the expression of STM3611 and STM1703 throughout the growth phase, as upregulated expression of STM3611 and STM1703 in the Δ STM1344 mutant is observed. The STM3611 and STM1703 genes on the chromosome were linked with a FLAG tag sequence, and the proteins were detected with a FLAG antibody by Western blot analysis. Cells were grown on LB agar plates without salt at 28°C for the indicated times. Δ indicates the STM1344 knockout mutant of *S. Typhimurium* UMR1. (B) STM1344 affects the STM3611 gene transcript level but does not affect the STM1703 gene transcript level as determined by quantitative real-time RT-PCR. The *csgD* transcript was monitored as a positive control. RNA was isolated from cells grown for 16 h on LB agar plates without salt at 28°C. Average values with standard deviations are displayed.

morphotype and CsgD expression as reported previously (37), the knockout of STM1344 had different effects in different strain backgrounds (Fig. 7; also data not shown). Upon the deletion of STM1344 in the STM4264 and STM1827 mutant backgrounds, the downregulation of rdar morphotype and CsgD expression to the level in the UMR1 wild type was observed, while in the STM1703 and STM3611 mutant backgrounds, CsgD expression remained at the levels in the corresponding single mutants (Fig. 7; also data not shown). On the other hand, swarming motility was partially restored by the deletion of STM1344 in the STM3611 mutant background, suggesting that STM1344 affects swarming motility through STM3611 and through another pathway (data not shown). Consequently, STM1344 acts upstream of STM1703 and STM3611 in the regulation of CsgD, whereas STM1344 is either located downstream of STM4264 and STM1827 or is part of an independent pathway overriding CsgD regulation by STM4264 and STM1827.

As STM1344 exerts its effect on CsgD expression through STM1703 and STM3611, we tested whether STM1344 has an effect on the expression of STM1703 and STM3611. To monitor the expression of the STM1703 and STM3611 proteins, sequences encoding C-terminally FLAG-tagged fusion proteins were constructed on the chromosome. Subsequent Western blot analysis revealed that, indeed, the expression of STM1703 and STM3611 was elevated in the Δ STM1344 mutant (Fig. 8A). Quantitative real-time RT-PCR analysis revealed that STM1344 downregulated STM3611 transcript levels, but not STM1703 transcript levels (Fig. 8B). Therefore, STM1344, directly or indirectly, represses STM1703 and STM3611 expression, yet on different levels.

DISCUSSION

In this study, a function for the EAL-like protein STM1344 in the transition between sessility and motility in *S. Typhimurium* was assigned. STM1344 positively regulated the expression of the multicellular rdar morphotype behavior and its major regulator CsgD and suppressed motility. STM1344,

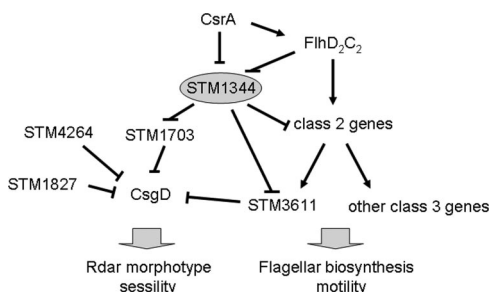


FIG. 9. Model illustrating the position of STM1344 in the c-di-GMP signaling network regulating the transition between sessility and motility. STM1344 positively affects the expression of the major regulator of the multicellular rdar morphotype behavior, CsgD, through the downregulation of the expression of the phosphodiesterases STM1703 and STM3611 (37). However, the regulation of STM3611 by STM1344 is probably indirect, as STM1344 represses not only STM3611, but also transcription from class 2 and class 3 promoters of the flagellar regulon (45). On the other hand, the expression of CsrA and the FlhD₂C₂ activator complex (expressed by the class 1 promoters) at the top of the flagellar regulon hierarchy downregulates STM1344 expression (45; Jonas et al., unpublished). In addition, CsrA positively affects FlhD₂C₂ (44). STM4264 and STM1827 are two other phosphodiesterases that downregulate the expression of CsgD independently of STM1344 (37).

however, does not seem to play a direct role in c-di-GMP metabolism but regulated the rdar morphotype and motility indirectly by affecting the expression of the c-di-GMP-dependent phosphodiesterases STM1703 and STM3611 (Fig. 9).

The closest homologue of STM1344 is the EAL domain protein STM3611. Not only do these two proteins have the same domain structure (only an EAL domain), but also, of all EAL domain proteins in *S. Typhimurium*, STM3611 has the amino acid sequence most similar to that of STM1344 (26). However, there are variations in the details. Many amino acids conserved in STM1344 and STM3611 are located outside of the motifs conserved in functional phosphodiesterases and known to be required for maximal activity against c-di-GMP (Fig. 1) (25). STM1344 is also lacking several amino acids highly conserved in functional phosphodiesterases, some of which have been shown previously to be required for c-di-GMP-dependent phosphodiesterase activity (Fig. 1) (25, 36). If present, these conserved amino acids, such as N₈₅ (Fig. 1), do not have an influence on STM1344 function. Only the F168A substitution gave a protein which had lost its regulatory function. Functionally, STM1344 and STM3611 are also the only two EAL domain proteins which influence both CsgD expression and motility in the *S. Typhimurium* UMR1 strain background, albeit in opposite manners (Fig. 2).

CsgD expression and motility are not the only phenotypes regulated by STM1344 in *S. Typhimurium*. STM1344 was originally identified as a protein required for virulence in a mouse model of systemic infection (13). In particular, STM1344 was shown to mediate resistance to hydrogen peroxide and to restrict fast killing of macrophages.

In this previous work, a sevenfold-higher c-di-GMP concentration in the Δ STM1344 mutant than in the wild type was found (13). We, however, observed the downregulation of the total cellular c-di-GMP levels in the Δ STM1344 mutant. The reason for the inconsistency in the effect of STM1344 on the c-di-GMP concentration between previous work and our study is unknown but may be methodology related. Only c-di-GMP present in the soluble fraction of a bacterial lysate was measured previously, while we extracted c-di-GMP from whole cells.

The effect of STM1344 on c-di-GMP concentrations as determined in this study is, however, in agreement with the effect of STM1344 on the multicellular and motility phenotypes, e.g., the expression of STM1344 leads to higher c-di-GMP levels, the upregulation of CsgD (as CsgD expression is activated by c-di-GMP [16]), and the inhibition of motility (as motility is inhibited by c-di-GMP [38]). The previously suggested correlation between STM1344 expression and c-di-GMP concentration contributed to the hypothesis that high c-di-GMP levels inhibit the acute infection phenotype (41). While low c-di-GMP levels are indeed required for specific virulence phenotypes (42), systemic infection of mice with *S. Typhimurium* is complex. Therefore, it is more likely that dynamic regulation of c-di-GMP concentrations is required for successful infection.

How does STM1344 exert its function? A recent study has shown that an EAL domain with noncanonical motifs can interact with a transcriptional regulator and thus influence gene expression (43). This study shows that STM1344, directly or indirectly, represses the expression of the phosphodiesterases STM1703 and STM3611. Although direct biochemical

evidence strongly suggests that STM1344 lacks c-di-GMP-metabolizing activity, STM1344 may still interact with functional GGDEF, EAL, or c-di-GMP binding domains and thus influence c-di-GMP metabolism, with subsequent expression of EAL domain proteins. Equally likely, however, STM1344 may have acquired an alternative function and interact with, e.g., a transcriptional regulator.

EAL domain proteins, like GGDEF domain proteins, form a large superfamily of bacterial proteins. However, not all the proteins have retained their core functions, namely, c-di-GMP-degrading and c-di-GMP-synthesizing activities; some have evolved alternative functions. Those functions may be tightly associated with the c-di-GMP network components, as GGDEF domains with nonconsensus GGDEF motifs located in the N-terminal direction from EAL domains in the same protein function as GTP sensors and signal transducers (3, 6). A more distant function independent of c-di-GMP network components is performed by the GGDEF-EAL domain protein CsrD, as it aids in the degradation of small RNAs by RNase E (40). As CsrD, however, regulates the activity of the carbon storage regulator A (CsrA), which affects the expression of a number of GGDEF and EAL domain proteins (15), even CsrD is still part of a c-di-GMP signaling network.

STM1344 is a novel component that contributes to the fine-tuned regulation of the transition between sessility and motility (Fig. 1 and 9). Swarming motility, a form of multicellular movement on surfaces (12), but not swimming motility is highly affected by STM1344. Indeed, STM1344 does not influence the swimming speed but does influence the onset of swimming motility. Collectively, these observations lead to the conclusion that STM1344 is specifically required for the sessility-motility transition on surfaces. STM1344 favors sessility by repressing the expression of the two phosphodiesterases, STM1703 and STM3611, required for the downregulation of CsgD expression (37). Whether the repression of phosphodiesterase expression by STM1344 is direct remains to be shown. In any case, STM1344 connects sessility with motility, as the STM3611 gene, a class 3 flagellar gene, is part of the flagellar regulon (17). The repression of STM3611 expression and motility by STM1344 is in agreement with the recently reported inhibition of flagellar class 2 and class 3 promoters by STM1344 (45). STM1344 is an integral part of the sessility-motility switch but not the most upstream component, as the expression of STM1344 is directly repressed by the binding of the carbon storage regulator CsrA, a sessility-motility switcher, to the STM1344 mRNA (K. Jonas, A. N. Edwards, I. Ahmad, A. Lamprokostopoulou, T. Romeo, U. Römling, and Ö. Melefors, unpublished results) and by the action of the master regulator of the flagellar regulon, FlhD₂C₂, when the flagellar biosynthesis machinery is switched on (45).

ACKNOWLEDGMENTS

We are grateful to Stephen Lory for providing constitutively active Wspr* and to Stephanie Kamann for experimental contributions during project work.

This work was supported by grants from the Karolinska Institutet (elitforskartjänst to U.R.), the Swedish Research Council (621-2004-3979 and 621-2007-6509), and the Carl Trygger Foundation (CTS-07-306).

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