Stand-Alone EAL Domain Proteins Form a Distinct Subclass of EAL Proteins Involved in Regulation of Cell Motility and Biofilm Formation in Enterobacteria

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ABSTRACT The second messenger cyclic dimeric GMP (c-di-GMP) is almost ubiquitous among bacteria as are the c-di-GMP turnover proteins, which mediate the transition between motility and sessility. EAL domain proteins have been characterized as c-di-GMP-specific phosphodiesterases. While most EAL domain proteins contain additional, usually N-terminal, domains, there is a distinct family of proteins with stand-alone EAL domains, exemplified by *Salmonella enterica* serovar Typhimurium proteins STM3611 (YhjH/PdeH), a c-di-GMP-specific phosphodiesterase, and the enzymatically inactive STM1344 (YdiV/CdgR) and STM1697, which regulate bacterial motility through interaction with the flagellar master regulator, FlhDC. We have analyzed the phylogenetic distribution of EAL-only proteins and their potential functions. Genes encoding EAL-only proteins were found in various bacterial phyla, although most of them were seen in proteobacteria, particularly enterobacteria. Based on the conservation of the active site residues, nearly all stand-alone EAL domains encoded by genomes from phyla other than proteobacteria appear to represent functional phosphodiesterases. Within enterobacteria, EAL-only proteins were found to cluster either with YhjH or with one of the subfamilies of YdiV-related proteins. EAL-only proteins from *Shigella flexneri*, *Klebsiella pneumoniae*, and *Yersinia enterocolitica* were tested for their ability to regulate swimming and swarming motility and formation of the red, dry, and rough (rdar) biofilm morphotype. In these tests, YhjH-related proteins S4210, KPN_01159, KPN_03274, and YE4063 displayed properties typical of enzymatically active phosphodiesterases, whereas S1641 and YE1324 behaved like members of the YdiV/STM1697 subfamily, with *Yersinia enterocolitica* protein YE1324 shown to downregulate motility in its native host. Of two closely related EAL-only proteins, YE2225 is an active phosphodiesterase, while YE1324 appears to interact with FlhD. These results suggest that in FlhDC-harboring beta- and gammaproteobacteria, some EAL-only proteins evolved to become catalytically inactive and regulate motility and biofilm formation by interacting with FlhDC.

IMPORTANCE The EAL domain superfamily consists mainly of proteins with cyclic dimeric GMP-specific phosphodiesterase activity, but individual domains have been classified in three classes according to their functions and conserved amino acid signatures. Proteins that consist solely of stand-alone EAL domains cannot rely on other domains to form catalytically active dimers, and most of them fall into one of two distinct classes: catalytically active phosphodiesterases with well-conserved residues of the active site and the dimerization loop, and catalytically inactive YdiV/CdgR-like proteins that regulate bacterial motility by binding to the flagellar master

Received 13 March 2017 Accepted 16 June 2017

Accepted manuscript posted online 26 June 2017

Citation El Mouali Y, Kim H, Ahmad I, Brauner A, Liu Y, Skurnik M, Galperin MY, Römling U. 2017. Stand-alone EAL domain proteins form a distinct subclass of EAL proteins involved in regulation of cell motility and biofilm formation in enterobacteria. *J Bacteriol* 199:e00179-17. <https://doi.org/10.1128/JB.00179-17>.

Editor George O'Toole, Geisel School of Medicine at Dartmouth

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regulator, FlhDC, and are found primarily in enterobacteria. The presence of apparently inactive EAL-only proteins in the bacteria that do not express FlhD suggests the existence of additional EAL interaction partners.

KEYWORDS cyclic di-GMP phosphodiesterase, flagellar regulon, motility, protein-protein interaction, FlhDC

Cyclic dimeric GMP (c-di-GMP) is a ubiquitous secondary messenger in bacteria that is involved in the transitions between motility and sessility and between acute and chronic infections (1, 2). c-di-GMP turnover is mediated by diguanylate cyclases, which contain the GGDEF domain, and c-di-GMP-specific phosphodiesterases (PDEs), which include either the EAL or the HD-GYP domain (1, 3). Along with the GGDEF domain, the EAL domain is one of the most common domains encoded in bacterial genomes (4, 5). Recent studies of the EAL domain sequences and structures provided significant insights into the mechanism of its PDE activity (1, 6, 7). These studies characterized a number of EAL domains that functioned as c-di-GMP-specific PDEs and also some inactivated domains that were devoid of enzymatic activity (8–10). Based on the conservation of the active-site motifs and the dimerization loop (11), EAL domains have been categorized into three main classes: (i) bona fide c-di-GMP PDEs, (ii) EAL domains with a degenerate loop 6 that might or might not have PDE activity, and (iii) EAL domains that lack the key catalytic residues and have no enzymatic activity (11, 12). The latter class could be further subdivided into those EAL domains that have retained the ability to bind c-di-GMP and serve as c-di-GMP receptors and those domains that do not even bind c-di-GMP (9, 10, 13, 14).

Most EAL domain proteins contain N-terminal signaling domains that regulate the formation of catalytically active dimers (6, 15, 16). However, some EAL-containing proteins do not include any other domains and therefore represent stand-alone EAL domains. In fact, the first reports on the EAL-containing proteins all dealt with stand-alone EAL domains, including (i) an unnamed open reading frame, URF2, next to the mercury resistance operons of plasmid R100 and transposon Tn501 (17), (ii) ToxR/RegA, a positive regulator of exotoxin A expression in *Pseudomonas aeruginosa* (18, 19), and (iii) BvgR, a regulator of virulence-related genes in *Bordetella pertussis* (20) (Fig. 1). However, in each of these cases, the regulatory mechanisms remain obscure.

The discovery of the multidomain PAS-GGDEF-EAL proteins of *Acetobacter xylinum* (now *Komagataeibacter xylinus*) participating in c-di-GMP turnover (21) stimulated detailed studies of the enzymatic properties of the EAL domains and their interactions with c-di-GMP (reviewed in reference 1). Stand-alone EAL domain proteins attracted far less attention. Still, an EAL-only protein, PA2133, from *Pseudomonas aeruginosa* (22, 23), two such proteins encoded in *Escherichia coli* K-12, and three encoded in *Salmonella enterica* serovar Typhimurium have been experimentally characterized (Fig. 1; see also Fig. S1 in the supplemental material). The *E. coli* protein YhjH (recently renamed PdeH [24]) and its close homolog STM3611 from *S. Typhimurium* (79% identity at the protein level) have been shown to be active c-di-GMP-specific PDEs involved in the regulation of cell motility (9, 25, 26). Similar effects of YhjH orthologs have been reported in *Aeromonas veronii*, *Dickeya dadantii*, and *Klebsiella pneumoniae* (27–29). More recently, three EAL-only proteins from the Gram-positive pathogen *Listeria monocytogenes* were shown to possess c-di-GMP PDE activity (30).

By contrast, the *E. coli* protein YdiV (alternatively named RflP [24]) and its *S. Typhimurium* counterparts STM1344 (CdgR) and STM1697 consist of diverged EAL domains that lack the key active site residues (Fig. 1). These proteins display no detectable PDE activity, do not readily form dimers, and do not bind c-di-GMP (9, 10), falling into class 3b of the above-mentioned classification (12). Nevertheless, these proteins have been shown to regulate motility and cell adherence and affect bacterial cytotoxicity towards macrophages (9, 10, 31, 32). This regulation involves a direct interaction with the FlhD component of FlhD₄C₂, the master regulator of flagellar gene expression, to prevent the interaction of FlhD₄C₂ with DNA and to stimulate proteolysis

Enterobacterial EAL-only phosphodiesterases

YhjH_Ecoli	48	ELLTVV	104	VN	133	ELVE	158	DDFGTGMAN	171	SEVRDYDIKIARELFVMLRQSPEGRITLFSQLLHL	218	EGVE	235	QG
STM3611	48	ELLTVV	104	VN	133	ELVE	158	DDFGTGMAN	171	SEVRDYDIKVARELFVMLRQSPEGRNLTLLQL	218	EGVE	235	QG
S4210_Shig	48	ELLTVV	104	VN	133	ELVE	158	DDFGTGMAN	171	SEVRDYDIKIARELFVMLRQSPEGRITLFSQLLHL	218	EGVE	235	QG
KPN_01159	29	ELLTKD	84	LN	113	EINE	142	DDFGRGRTS	155	ERFRFDCVKVDKDYFWDKENDPALPGLLSIHTL	198	EGIE	215	QG
KPN_03274	36	ECLSRF	87	IN	115	EVTE	142	DDFGSGYAG	155	RGYHFDYVKIKDKDFFWHLMRKESGRQLMDALVTF	198	EGVE	219	QG
YE2225_Yent	29	ELLSRF	84	IN	113	EIME	142	DDFGSGNAH	155	SSGFFECVKIKDKHFFWQ----WAGSVTFDNLVTR	198	EGVE	215	QG
YE4063_Yent	45	ELLTAV	105	IN	134	ELLE	159	DDFGSGLAN	172	ISWRYEYIKVARDLFIVLQESDVGNQLFFFKLVTL	216	EGVE	236	QG

Enterobacterial EAL-only FlhD-binding proteins

YdiV_Ecoli	29	ELITHF	84	LN	116	ENYP	142	GNLGAGNST	155	FDGLFTRVMLDKSFIQQQITHRSEFFIRAIQAQ	199	GGID	219	QG
STM1344	29	EIIATF	84	LN	116	ENYP	142	ANFGAGEAS	155	FDGLFKRVMLDKNFIQQRAEMISFFPMHAIQAQ	199	AGID	219	QG
STM1697	21	EITTHF	75	VN	107	EHFS	134	GLGVGNAT	147	VCGCFSGVKLDRSFFVSQIEKMTFPLLVKHIRRV	187	GGQE	207	QG
S1641_Shig	29	ELITHF	84	LN	116	ENYP	142	GNLGAGNST	155	FDGLFTRVMLDKSFIQQQITHRSEFFIRAIQAQ	199	GGID	219	QG
YE1324_Yent	29	ELLSRF	84	IN	113	EIME	142	DDLGS GGST	155	TANI FEYVKIKDKHFFWQHKKH-TFPI LINNIKKY	194	VGVE	214	QG

EAL-only proteins – Gram-positive bacteria

Rv1357c	89	EALLRW	148	VN	181	ELAE	211	DDFGIGFSS	164	PRLPVDVVKLGKGFIECSQARLANEQITRAMIDL	272	KLVE	292	QG
Lmo0111	29	EILLRD	77	IN	116	EMTE	147	DDVSCGLNS	160	MSYLPYIIIEIKFSLIHF--KNIPLEDDLFIKAW	205	EGIE	225	QG
Lmo0131	36	EGLVRL	79	IN	122	EIIE	152	DDFGKGYSS	165	RSLPIDIVIKTDMSFIALKTDKQQIIRAIIVNL	208	EGVE	229	QG
Lmo1914	34	ESLTRL	85	VN	112	EFLE	146	DDFGSGYAD	158	FAHHSDFVKTDRLRLDLFESDGKKIFFEQLHNY	202	EGVE	222	QG
Lp_2714	64	ECLLRQ	111	IN	142	EYTP	174	DNVGASLAN	189	LLKDIDTLKCSMRSEFRKEDPSVWLDLNLQFWNQ	233	MGIE	253	QG

Other proteobacterial EAL-only proteins

EcpC_Ddan	56	ELLTAV	112	VN	121	ELVE	166	DDFGCGVAN	164	SLTKYDCIKIARELFIMLLKTEQGRQLFPYLITL	223	EGVE	243	QG
ECA3549	34	EALARW	87	FN	120	EISE	150	DDFGAGNLQ	163	SLNFNFDTIKIDRSIIDGIGKEETKSQKLKVLNKL	208	EGVE	227	QG
URF2_Ecoli	43	EALMRW	101	IN	133	ELTE	162	DDFGTGYS	175	KCCPITTLKIDQS FVARLPDDARDQTIIVRAVIQL	---	---	---	---
ToxR_Paer	43	KLSLRE	96	IP	129	LLAI	161	QRIDTDARQ	175	DAGLCDYLGIDARLLAPSTRNLQRKSIEYLNRL	221	LNVD	241	HG
BvgR_Bpert	66	ATTIQW	123	IQ	156	AVSP	187	TVSTFDAES	200	DALEICGIALMPELMYALRQGGRAEACRAIAQQ	244	AGVV	264	LG

Reference EAL domains

PDB: 3N3T	59	EALVRW	119	VN	152	EITE	182	DDFGTGYS	195	SQLPFHGLKIDQS FVRKIPAHPSSETQIVTTILAL	238	EGIE	259	QG
cd01948	31	EALLRW	88	VN	121	EITE	151	DDFGTGYS	164	KRLPVDYLIKIDRS FVRDIETDPEDRAIVRAIAL	208	EGVE	228	QG
PF00563	32	EALLRW	87	VN	121	EITE	151	DDFGTGYS	161	SRLPPDYIKIDRSFIKD-LSDPESRALLRALIAL	204	EGVE	224	QG

FIG 1 Conservation of the active site, dimerization loop, and the FlhD-binding residues among EAL-only proteins. The first two blocks represent enterobacterial EAL-only PDEs and enterobacterial catalytically inactive FlhD-binding EAL-only proteins from strains investigated in this work. These proteins from *S. enterica* serovar Typhimurium ATCC 14028, *E. coli* K-12, *S. flexneri* 2a, *K. pneumoniae*, and *Y. enterocolitica* are listed under their names in the NCBI protein database; the respective GenBank accession numbers are listed in Table S4 in the supplemental material. Experimentally characterized EAL-only proteins of Gram-positive *Actinomyces* and *Firmicutes* come from *Mycobacterium tuberculosis* (Rv1357c [73, 74]), *Listeria monocytogenes* (Lmo0111, Lmo0131, and Lmo1914 [30]), and the catalytically inactive Lp_2714 from *Lactobacillus plantarum* (75). Other proteobacterial EAL-only proteins include experimentally characterized proteins from *Dickeya dadantii* (EcpC_Ddan [28]), ECA3549 from *Pectobacterium atrosepticum* (76), and the first sequenced stand-alone EAL proteins URF2 from *E. coli* (GenBank accession no. AAA83542), ToxR/RegA from *Pseudomonas aeruginosa* (AAG04096), and BvgR from *Bordetella pertussis* (AAC23902) (17–20). Reference EAL domain sequences are from the *Thiobacillus denitrificans* protein TBD1265 (PDB entry 3N3T), NCBI's Conserved Domain Database entry cd01948, and the Pfam domain PF00563 (7, 39, 60). The residues that form the active site of c-di-GMP PDEs (7, 8) are shown in white on a red background; conserved residues located in the vicinity of the active site are in red or magenta. The hydrophobic residues that participate in YdiV-FlhD binding (35) are shaded yellow, and charged residues at that interface are in blue. Names of previously experimentally characterized proteins are in bold.

by the ClpXP protease (10, 33, 34). The structures of stand-alone YdiV and the YdiV-FlhD complex have been solved, providing an insight into the mechanism of this regulatory interaction (35). Three more EAL-only proteins, the *S. Typhimurium* protein STM1697 and the *L. monocytogenes* PDEs Lmo0111 and Lmo0131 (Fig. 1), have been structurally characterized.

In addition to experiments in mice (10, 31, 36) and cytotoxicity data from the Chinese hamster ovary cell line (22), several other observations support the involvement of EAL-only proteins in bacterial pathogenesis. An EAL-only-encoding gene, *sfaY*, is part of a pathogenicity island in a newborn meningitis *E. coli* isolate and its expression in *Vibrio cholerae* has been shown to affect biofilm formation (37). Similar EAL-only proteins PdeW, PdeX, and PdeY have been found encoded in the genomes of various enteropathogenic and enterotoxigenic strains of *E. coli* (38).

In *S. Typhimurium*, we have observed that EAL-only proteins, despite a significant divergence in functionality (Fig. S1), are phylogenetically more closely related to each other than to EAL proteins with an additional N-terminal and/or GGDEF domain (see Fig. S2). We were therefore wondering to what extent macro- and microevolution occur

in EAL-only proteins beyond *S. Typhimurium*. Thus, this work extends the characterization of evolutionary relationship versus functionality of stand-alone EAL domain proteins beyond *S. Typhimurium* to include analyses of such proteins from several other enterobacterial pathogens.

RESULTS

Phylogenetic distribution of EAL-only proteins. According to the Pfam database (39), almost one-fifth of the EAL-containing proteins (Pfam family PF00563) listed in UniProt (40) do not include any other (known) domains. The NCBI's CDART tool (41) reports similar numbers. However, many of these proteins are not stand-alone EAL domains, as they contain long unannotated segments, which likely represent uncharacterized protein domains. Thus, the recently identified CSS motif domain is found in five formerly EAL-only proteins in *E. coli* K-12 (24) and also in five EAL proteins in *S. Typhimurium*. However, even limiting the protein lengths in the NCBI protein database to 280 amino acid residues and filtering out truncated fragments still leaves ~20,000 sequences that represent true stand-alone EAL domain proteins. This is in sharp contrast to the GGDEF domain-containing proteins, where fewer than 200 (~0.03% of the total) represent GGDEF-only proteins. EAL-only domain proteins are encoded in the genomes of representatives of most well-sequenced bacterial phyla, including *Actinobacteria*, *Aquificae*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, and *Spirochaetes* (see Table S4a in the supplemental material). The largest numbers of EAL-only proteins are found in gammaproteobacteria, particularly in enterobacteria, where they often comprise more than 10% of all EAL-containing proteins (see Table S4b). In some bacterial groups, such as lactobacilli and listeria, all EAL-containing proteins appear to be stand-alone EAL domains (see Table S4c). It should be noted that the active site residues responsible for the PDE activity (Fig. 1) are remarkably well conserved in the majority of EAL-only proteins expressed by groups other than proteobacteria (see Fig. S3). The sequences of the dimerization loop are also conserved, albeit deviating somewhat from the consensus pattern described previously (11).

Clustering of enterobacterial EAL-only proteins. The chromosome of *S. Typhimurium* ATCC 14028 (42) encodes a total of 20 GGDEF and/or EAL domain proteins, which regulate, for example, the red, dry, and rough (rdar) biofilm phenotype, biofilm formation, bistability, motility, and virulence properties in cell culture systems, mammals, and plants (10, 43–47). Of these twenty, 15 proteins contain EAL domains and three of them, STM1344, STM1697 and STM3611, represent stand-alone EAL domains that are all involved in the regulation of motility in *S. Typhimurium* (10, 46). We have previously reported that STM1344 (YdiV) and STM3611 (YhjH) proteins were more closely related to each other than to other EAL domains of *S. Typhimurium* and branched together on the phylogenetic tree of the EAL domains (46). This observation has now been confirmed by constructing a maximum likelihood-based phylogenetic tree of all EAL domains of *S. Typhimurium* (Fig. S2). In addition to these 15 EAL-containing proteins, the genome of *S. Typhimurium* strain ATCC 14028 encodes two truncated EAL domains, STM0551 (77) and plasmid-encoded PSLT032, which have not been studied in this work.

BLAST searches of enterobacterial genome sequences showed that they encode from zero to eight EAL-only proteins (Table S4b). Remarkably, EAL-only proteins from *S. Typhimurium*, *E. coli* K-12, *Shigella flexneri*, *Klebsiella pneumoniae*, and *Yersinia enterocolitica* formed a well-defined branch on the phylogenetic tree of the EAL domain sequences from these bacteria, separate from those of the EAL domains coming from multidomain proteins (Fig. 2; see also Table S5). Furthermore, the EAL-only cluster was still preserved after the addition of EAL domain proteins from a variety of other enterobacteria (see Fig. S4). While most of these proteins clearly fell into either YhjH (STM3611 and PdeH) or YdiV (STM1344, CdgR, or RfIP) subfamilies, additional subfamilies showed up, particularly in such organisms as *Citrobacter koseri*, *Enterobacter asburiae*, and *Shigella boydii*, which encode more than three EAL-only proteins. Based on the conservation of the active site residues (Fig. 1), members of the YhjH/PdeH

FIG 2 Phylogenetic tree of enterobacterial EAL domain proteins. EAL domain sequences from *S. Typhimurium*, *E. coli*, *S. flexneri*, *K. pneumoniae*, and *Y. enterocolitica* (see Fig. S4 for the complete tree with accession numbers and domain boundaries) were aligned with MUSCLE (61) and a maximum likelihood tree was built using PhyML (63), with automatic selection of the best-fit substitution model and branch support values estimated using the aBayes algorithm (64). The tree was rooted using the sequence of an EAL domain from *Petrotoga mobilis*, a member of the phylum *Thermotogae*. The tree is drawn to scale, with branch lengths corresponding to the numbers of substitutions per site, and visualized with MEGA7 (62). The branches leading to the multidomain proteins with identical domain compositions have been collapsed and marked with the names of their *E. coli* members and domain architectures as listed in reference 24. Names of previously experimentally characterized proteins are in bold. Those with demonstrated phosphodiesterase activity against c-di-GMP are marked with asterisks; those shown not to hydrolyze c-di-GMP are in italic font. Enterobacterial stand-alone EAL domains characterized in this work are shown in normal font (blue, PDE activity; red, catalytic activity is lacking).

subfamily could be predicted to function as enzymatically active PDEs (class I EAL-only domain), whereas members of the YdiV/CdgR/RflP subfamily had active site substitutions incompatible with PDE activity (class III EAL-only domain). At the same time, the residues identified in the crystal structure of the YdiV-FlhD complex (35) as important for the interaction between these two proteins are well conserved among the members of the YdiV family (Fig. 1). Finally, several stand-alone EAL domains have substitutions in both the active site residues and the residues at the YdiV-FlhD interface, making them unlikely to either have the PDE activity or be able to bind FlhD (Fig. 1).

Functionality of the EAL-only proteins. To test functional assignments of the EAL-only proteins expressed in other enterobacteria, representative strains from three different species with completely sequenced genomes, *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578, *Shigella flexneri* 2a strain 24570^T, and *Yersinia enterocolitica* subsp. *enterocolitica* 8081 (48, 49), were chosen. The first two organisms are nonmotile, while *Y. enterocolitica* is motile. Their chromosomes encode, respectively, two (KPN_01159 and KPN_03274), two (S1641 and S4210), and three (YE1324, YE2225, and YE4063) EAL-only proteins (see Table S5 for accession numbers). The plasmid-encoded EAL-only protein [KPN_pKPN4p07065](#) of *K. pneumoniae* was not a subject of this investigation. These proteins were classified as follows: S4210, KPN_01159, KPN_03274, and YE4063 belong to the YhjH/STM3611 subfamily, and S1641 belongs to the YdiV/STM1344 subfamily (Fig. 1 and 2; see also Fig. S4). The closely related proteins YE2225 and YE1324 do not fall into either of these subfamilies; the former contains the full set

FIG 3 Effect of EAL-only protein expression on *S. Typhimurium* UMR1 motility and rdar morphotype formation. (A) Effect of EAL-only proteins on motility in 0.3% LB agar incubated at 37°C for 5 h measured by colony diameter. Data from three independent experiments were averaged and the standard errors of the means are presented. ***, $P < 0.001$; ns, not significant. S1641 and YE1324 were overexpressed in both a UMR1 wild type and an $\Delta STM1697$ background, while S4210 and YE2225 were overexpressed in the wild type and a $\Delta STM3611$ background. VC (vector control) is pSRKGm. All genes were cloned in pSRKGm. (B) Effect of EAL domain proteins on rdar morphotype formation in *S. Typhimurium* UMR1. Congo red agar plates were incubated for 48 h at 28°C.

of active site residues, whereas the latter lacks a catalytically important Glu residue (Fig. 1).

We started the functional characterization of these proteins by assessing the phenotype changes in biofilm formation and motility upon their overexpression in well-established models of *S. Typhimurium*. As described previously (50), the expression of enzymatically active PDEs is indicated by a downregulation of the rdar morphotype biofilm on Congo red agar plates and a minor upregulation of motility in the wild-type background (Fig. 3; see also Fig. S5). Accordingly, the expression of non-PDE YdiV/STM1344/STM1697-like proteins that interact with FlhD is indicated by opposite phenotypes, namely, an upregulation of the rdar biofilm morphotype and a downregulation of motility in the wild-type background. As the phenotypic change in motility in the wild type is not always informative, the functionality of PDEs was also assessed in deletion mutants. Deletion of the PDE-encoding STM3611 (*yjhH* or *pdeH*) gene resulted in significantly suppressed motility, which could be robustly restored upon overexpression of a functional PDE. On the other hand, catalytically nonfunctional EAL proteins were expected to robustly suppress motility in the $\Delta STM1697$ mutant background. To summarize these tests, the expression of KPN_01159, KPN_03274, S4210, and YE4063 proteins led to the downregulation of the rdar morphotype biofilm in the wild type and

FIG 4 Mutant analysis of *Y. enterocolitica* subsp. *enterocolitica* 8081 YE2225 PDE. Effects of mutants YE2225_{E29A} and YE2225_{E195A} on motility and biofilm formation. (A) Motility assay in *S. Typhimurium* was performed in 0.3% LB agar generate. Single clones were stabbed into the agar and plates were incubated at 37°C for 5 h. YE2225 overexpression in the Δ STM3611 mutant complemented the reduced-motile phenotype, while neither YE2225_{E29A} nor YE2225_{E195A} rescued the reduced-motile phenotype of the Δ STM3611 mutant. Data from three independent experiments are averaged, and the standard errors of the means are presented. ***, $P < 0.001$; ns, not significant. (B) Effect of YE2225 on rdar morphotype expression in *S. Typhimurium*. Congo red plates were incubated for 24 h at 28°C. VC, vector control pSRKGm. All genes were cloned in pSRKGm.

restoration of motility in the Δ STM3611 mutant background (Fig. 3; see also Fig. S5). Thus, in full accordance with their placement on the phylogenetic tree (Fig. 2), these proteins displayed behaviors typical of active PDEs. The expressed S1641 behaved like an YdiV/STM1344-type protein (Fig. 3), as it downregulated motility and upregulated the rdar morphotype (Fig. 3), a phenotype that again correlates with its phylogenetic position.

The two closely related *Yersinia* proteins, YE1324 and YE2225, which formed a separate branch in the YhjH (active PDEs) part of the tree (Fig. 2), were investigated in more detail. YE2225 showed a typical PDE phenotype; when expressed in the Δ STM3611 mutant, it restored the motility defect and downregulated the rdar morphotype. This phenotypic pattern is in accordance with the conservation of all the core active site residues (Fig. 1 and 3). To confirm that the phenotypic effects of YE2225 depended on its ability to function as a PDE, the catalytically important Glu29 and Glu195 of the EAL and EGVE motifs, respectively, were replaced by alanines, two changes that were previously shown to generate catalytically inactive EAL domains (7). Indeed, the expression of the resulting YE2225_{E29A} and YE2225_{E195A} mutant proteins in *S. Typhimurium* did not restore the motility defect of an *S. Typhimurium* Δ STM3611 (Δ yhjH) mutant and, accordingly, did not cause a downregulation of the rdar morphotype in the wild-type strain or the Δ STM3611 (Δ yhjH) mutant (Fig. 4), consistent with YE2225 functioning as a PDE *in vivo*.

The other *Yersinia* protein, YE1324, which had amino acid replacements in the catalytically important Glu194 of the EGVE motif and in several amino acid residues at

the EAL-FlhD interface (Fig. 1), upregulated the *rdar* morphotype and showed a trend in downregulation of motility, although not statistically significant (Fig. 3). This finding indicates that EAL-only proteins may have additional regulatory modes that differ from the YhjH and YdiV paradigm.

Functionality of proteins in their respective hosts. Although the phenotypes of EAL-only proteins in *S. Typhimurium* suggest a certain functionality, a different functionality in their respective native hosts could not be excluded. Therefore, we performed experiments in *Shigella* and *Y. enterocolitica*.

Shigella is considered to be nonmotile; however, remnants of a flagellar regulon with partially functional components can still be present in certain strains (51, 52). Indeed, limited expression of flagella and motility has been reported in *Shigella* isolates (53). Using *S. flexneri* 2a strain 24570^T and 10 *Shigella* sp. clinical isolates, we observed apparent regain of motility in low percentage soft agar, which was, however, not consistent among the isolates (see Fig. S6). We investigated whether protein S1641, which effectively inhibited motility in *S. Typhimurium*, and S4210, with predicted PDE activity, had impacts on the apparent motility of clinical isolates. In two different clinical isolates with a motility phenotype, the overexpression of S1641 and S4210 did not lead to phenotypic alterations (Fig. S6 and data not shown). Why these two proteins retained functionality, while the flagellar regulon is nonfunctional in *Shigella*, remains to be investigated.

Y. enterocolitica subsp. *enterocolitica* 8081 is motile at 25°C. Thus, we tested the impacts of YE1324, YE2225, and YE4063 on motility in the native host at this temperature (Fig. 5). While YE1324 suppressed, and YE2225 upregulated, swimming and swarming motility, YE4063 did not discernibly change the phenotype of *Y. enterocolitica* at 25°C. For YE2225, these data are consistent with the predicted function and the phenotype as observed in *S. Typhimurium*. The lack of a phenotype in motility for YE4063 in *Y. enterocolitica* compared with that in *S. Typhimurium* suggests a function other than motility for this PDE in its native host. On the other hand, the statistically significant repression of motility by YE1324 in *Y. enterocolitica* suggests interference with the flagellar regulon. The role of PDE YE2225 in motility was further investigated. In agreement with the phenotypes displayed in *S. Typhimurium*, YE2225 upregulated motility, but the mutant proteins YE2225_{E29A} and YE2225_{E195A} did not alter motility in the *Y. enterocolitica* wild type, consistent with YE2225 being a PDE that affects motility through its c-di-GMP phosphodiesterase activity but not as a protein that interacts with FlhD of the flagellar master regulator (Fig. 5C).

Potential molecular mechanism of YE1324 regulating motility. YE1324 downregulated motility in *Y. enterocolitica* when overexpressed, suggesting that YE1324 interacts with the *Y. enterocolitica* FlhDC (FlhDC_{YE}). To verify the function of YE1324 on the chromosomal expression level, we constructed a YE1324 deletion. Indeed, the Δ YE1324 mutant showed enhanced swimming motility (Fig. 6A), and a trend for enhanced swarming motility (Fig. 6B) at 25°C.

Since deletion of YE1324 relieves motility repression at 25°C, we wondered whether YE1324 and/or c-di-GMP signaling is involved in motility repression in *Y. enterocolitica* at 37°C. However, motility repression was not relieved in the Δ YE1324 mutant, even when the PDE YE2225 was overexpressed (data not shown).

How does YE1324 affect motility? A reasonable hypothesis is that, similar to EAL-only proteins STM1344 and STM1697 in *S. Typhimurium*, YE1324 inhibits motility by interacting with FlhD to interfere with FlhDC functionality in *Y. enterocolitica*. To this end, we investigated whether YE1324 and FlhD_{YE} could interact. We assessed protein-protein interactions by a bacterial two-hybrid system, an approach based on the restoration of adenylate cyclase activity upon the interaction of fusion proteins. Indeed, blue colonies due to cAMP-dependent β -galactosidase activity and the restoration of growth on maltose-supplemented minimal medium indicated that YE1324 and FlhD_{YE} could interact with each other (Fig. 7).

FIG 5 Motility phenotypes upon YE2225, YE1324, and YE4063 overexpression in *Y. enterocolitica* subsp. *enterocolitica* 8081. (A) Swimming motility assay was performed in 0.2% TB0 agar supplemented with 10 mM glucose. Single colonies were stabbed into the agar and plates were incubated at 25°C for 18 h. A representative image of motility behavior is presented. (B) Swarming motility assay was performed on 0.3% TB0 Eiken agar incubated at 25°C for 20 h. A representative image of the swarming behavior is shown. (C) Motility assay of the YE2225 PDE and YE2225_{E29A} and YE2225_{E195A} mutants. Swimming motility was assessed in 0.2% TB0 agar with 10 mM glucose. Single colonies were stabbed into the agar and plates were incubated at 25°C for 18 h. Representative image of motility behavior is presented. Data are means from three independent experiments and bars indicate standard errors. *, $P < 0.05$; ns, not significant. VC, vector control pSRKGm. All genes were cloned in pSRKGm.

Assessment of phosphodiesterase activity by a riboswitch-based assay. Although crystal structures for some EAL-only proteins are available (see Table S6), we observed that predicted catalytically active EAL-only proteins, but not EAL-only proteins without predicted catalytic activity, formed inclusion bodies upon overexpression (data not shown). Thus, we chose *c*-di-GMP riboswitch-controlled β -galactosidase activity as a readout to assess the catalytic function of EAL-only proteins (54). The Vc2 riboswitch is on at infinitesimal *c*-di-GMP levels and turns off upon the elevation of the *c*-di-GMP concentration. *E. coli* TOP10 with the Vc2 riboswitch on the chromosome exhibited already substantial β -galactosidase activity, indicating that *c*-di-GMP levels in these cells were too low to reliably assess PDE activity by enhanced β -galactosidase activity and accompanying colony color change (Fig. 8 and data not shown). Therefore, we expressed the diguanylate cyclase AdrA in *E. coli* TOP10::Vc2*lacZY*, and the β -galactosidase activity was repressed. Subsequent overexpression of the PDE STM3611 (YhjH) but not its active site mutant STM3611_{E136A} relieved the repression, clearly demonstrating the PDE activity of STM3611. Similarly, expression of YE2225 but not its active site mutants YE2225_{E29A} and YE2225_{E195A} led to a decrease in *c*-di-GMP concentration and

FIG 6 Effect of YE1324 on swimming and swarming motility in *Y. enterocolitica*. A YE1324 deletion led to a significant upregulation of swimming (A) and swarming (B) motility in *Y. enterocolitica* subsp. *enterocolitica* 8081. Swimming motility was assessed in 0.2% TB0 agar. Single colonies were stabbed into the agar and plates were incubated at 25°C for 18 h. The swarming motility assay was performed on 0.3% TB0 Eiken agar supplemented with 10 mM glucose and incubated at 25°C for 20 h. Representative images of motility behavior are shown. Data are means from three independent experiments and bars indicate standard errors. **, $P < 0.01$; ns, not significant.

enhanced β -galactosidase activity. By contrast, YE1324 did not cause any color change, indicating a lack of PDE activity and further indicating that the role of YE1324 on motility might be primarily through its interaction with FlhD.

DISCUSSION

The c-di-GMP signaling network is to date the most complex secondary messenger signaling network in bacteria. Turnover of the c-di-GMP second messenger is performed by GGDEF domain diguanylate cyclases and EAL/HD-GYP domain PDEs, whereby the number of c-di-GMP turnover proteins encoded in a genome is generally correlated with genome size within the different bacterial classes or phyla (http://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html [1]). So far, c-di-GMP turnover proteins have been subdivided according to their domain compositions (GGDEF, EAL, and GGDEF-EAL domain proteins), the nature of the N-terminal signaling domains (phosphotransfer receiver domain, small molecule sensing PAS or GAF domain, light sensing BLUF domain, and so on), and the conservation of the signature motifs (class I, II, and III EAL domains and active and inactive GGDEF domains with a conserved inhibitory I site). Here, we analyzed the properties of the stand-alone EAL domains, a distinct class of EAL domain proteins expressed in various bacterial phyla (see Table S4 in the supplemental material). In such proteins, there are no N-terminal domains that could potentially assist in dimerization and thus in enhancement of activity. Therefore, EAL-only proteins cannot fall into class II of Rao and colleagues (11) and must be either

FIG 7 Assessment of protein-protein interaction between FlhD_{YE} and YE1324 by bacterial two-hybrid assay. YE1324 could interact with FlhD_{YE} (YE2581) from *Y. enterocolitica* subsp. *enterocolitica* 8081. BTH101 reporter cells expressing the indicated proteins fused to the T18 or T25 domain of the *Bordetella* adenylate cyclase were patched on plates with X-Gal indicator and M63/maltose minimal medium. Blue colony color on the X-Gal plate and growth on the M63 medium plate indicate protein interaction. In pKT25 and pUT18C, the heterologous proteins are fused to the C-terminal ends of T25 and T18, respectively. In pUT18, the heterologous protein YE1324 is fused to the N-terminal end of T18. The two-hybrid experiment was performed as described in Materials and Methods.

active PDEs (class I) or enzymatically inactive molecules (class III) that participate in regulation solely through protein-protein interactions. Given that FlhC and FlhD are only expressed in certain beta- and gammaproteobacteria (see, e.g., families PF05280 and PF05247 in the Pfam database), it is hardly surprising that most EAL-only domains found in phyla other than proteobacteria show remarkable conservation of the active site residues (Fig. 1; see also Fig. S2) and appear to be catalytically active PDEs, as has

FIG 8 Analysis of PDE activity of *Y. enterocolitica* proteins YE2225 and YE1324 using a chromosomally encoded Vc2-off riboswitch system. (A) Schematic model of the Vc2-off riboswitch system. The Vc2-off riboswitch system is composed of a riboswitch transcriptionally fused to *lacZY*. Upon c-di-GMP binding, the expression of the downstream gene is turned off. Thus, high levels of c-di-GMP result in repression of *lacZY* expression, while low levels of c-di-GMP result in *lacZY* derepression. In *E. coli* TOP10 cells (WT), there is a residual amount of c-di-GMP, resulting in a light blue colony. Expression of diguanylate cyclase, such as AdrA, results in a white colony, while expression of a PDE enhances color development (not informative in the *E. coli* TOP10 background). (B) β-Galactosidase reporter gene assays indicate PDE activity of YE2225 but not of YE2225 mutants YE2225_{E29A} and YE2225_{E195A} and YE1324. Expression of EAL genes in the pSRKGm vector was induced by 0.75 mM IPTG, while expression of the diguanylate cyclase AdrA in pBAD30 was induced by 0.003% L-arabinose. The cell-spotted plate also contained a X-Gal (80 μg/ml) and was incubated at 28°C for 1 to 2 days.

been demonstrated for three *L. monocytogenes* proteins (Fig. 1) (30). At the same time, the dimerization loop, which follows the conserved Asp151-Asp152 motif (cd01948 numbering) (Fig. 1; see also Fig. S1) of strand 6, showed a clear conservation of three residues (Phe153, Gly/Ser154, and Gly156) but not the other three. It appears that the requirement for the DFG(A/S/T)(G/A)(Y/F)(S/A/T)(S/A/G/V/T) motif in this loop, advocated by Rao and colleagues (11), was unnecessarily restrictive. Examples of enzymatically active PDEs from *L. monocytogenes* and *Mycobacterium tuberculosis* (Fig. 1) showed that these proteins can form catalytically active dimers despite having fairly divergent sequences in this loop and no assistance from any upstream domains (although interaction with independently encoded protein domains which affect activity cannot be excluded).

In phylogenetic trees, EAL-only proteins formed well-defined branches, separate from those of the EAL domain sequences coming from EAL-containing multidomain proteins of the same organisms (Fig. 2; see also Fig. S3 and S4). This suggests that (at least some) EAL-only domains represent an ancient protein lineage rather than a product of a recent domain fission. In general, EAL domain sequences clustered mostly according to their domain composition (Fig. S4), indicating that the formation of the principal domain architectures preceded the divergence of the key enterobacterial lineages. It also seems likely that the coexistence of domains within a conserved domain architecture restricts sequence diversification. These conclusions are consistent with the notion that the origin of c-di-GMP-mediated signaling occurred at an early stage of bacterial evolution (1, 55).

In *S. Typhimurium*, the EAL domain-only proteins STM1697, STM1344, and STM3611 are an integral part of the flagellar regulon cascade, primarily restricting flagellar expression and enhancing functionality. YE1324 of *Y. enterocolitica* (78, 79) is, despite c-di-GMP network reduction, also present in *Yersinia pestis* (56), which switches from a motile to nonmotile state upon infection. YE1324 seems to have an unconventional function similar to those of STM1697 and STM13244, as its deletion upregulated flagellum-mediated swimming and swarming motility. Also, the two-hybrid system assay indicated that YE1324 could interact with FlhD from *Y. enterocolitica*. Although YE1324 hardly affected motility in *S. Typhimurium*, a preliminary two-hybrid assay suggested it interacts with *S. Typhimurium* FlhD (data not shown). The interaction of YE1324 with the FlhD of *S. Typhimurium* (FlhD_{ST}) might not interfere with FlhD_{ST} functionality. Alternatively, this interaction with YE1324 could be weaker than in its native host, as the sequence of FlhD_{YE} differs from that of FlhD_{ST} (data not shown). However, upregulation of the *rdar* biofilm formation does not exclude the possibility that YE1324 has another, yet unknown, binding partner in *S. Typhimurium*. As the amino acids mediating interactions with FlhD are not strictly conserved in YE1324 (Fig. 1), the molecular mechanisms behind this unconventional phenotype of YE1324 need to be investigated further. In general, noncanonical EAL-only proteins can have at least two protein-protein interaction interfaces: one that is interacting with FlhD (32, 35) and another one which targets FlhDC to the ClpXP protease (33).

The functionality of YE1324 in *S. Typhimurium* in the upregulation of *rdar* biofilm formation also indicates that these EAL-only proteins can readily acquire biological significance when horizontally transferred. Indeed, several of the EAL-only proteins of *E. coli* are encoded by plasmids (Fig. S4). Genes for an additional group of EAL-only proteins, including Sfa_{II}, are integral parts of operons harboring pilus biosynthesis genes, which are horizontally transferred into uropathogenic *E. coli* strains (37). However, of note, Sfa_{II} is only distantly related to STM3611, STM1344, and STM1697 (data not shown).

Surprisingly, nonmotile *Shigella* spp. have conserved functional YdiV and YhjH family homologs, although the flagellar regulon cascade does not seem to be intact or, as in *S. flexneri* 2a strain 24570^T, is even missing a full-length FlhD protein, the target of the catalytically inactive EAL-only proteins of *S. Typhimurium* and *E. coli*. Thus, we hypothesize that motility is not the only physiological output of these proteins. The nonmotile whooping cough agent *Bordetella pertussis* still carries certain chemotaxis and flagellar genes, including *flhD*, which are negatively regulated by the EAL-only

protein BvgR (20, 57). Although BvgR has a sequence quite distinct from that of STM1344/STM1697 (Fig. 1), it still could interact with *B. pertussis* FlhD (data not shown), which would explain some of its regulatory effects. In other species that do not possess the FlhDC regulon and/or are nonmotile, EAL domain-only proteins have diverged in sequence and function. The *K. pneumoniae* MrkJ PDE (identical to KPN_03274) degrades the c-di-GMP that is required for type 3 fimbria expression (29, 58). Finally, judging from the sequence of *P. aeruginosa* virulence regulator ToxR/RegA (18, 19) (Fig. 1), this protein cannot have PDE activity nor can it interact with FlhD, as FlhD is not encoded in *P. aeruginosa*. Such proteins must have alternative interaction partners that still remain uncharacterized. Some obvious candidates would be those containing the S helix, whose interaction with the enzymatically inactive EAL domain of the LapD protein has been characterized in detail (59). In summary, macro- as well as microevolution of the phylogenetically distinct EAL-only proteins extends beyond *S. Typhimurium*. FlhD is a common interaction partner, whereby catalytic activity and interaction with FlhD by EAL-only proteins seem to be mutually exclusive. However, EAL-only proteins are predicted to interact with an additional unknown protein(s). Thus, the diversity in function of the stand-alone EAL domain-only protein subfamily is only starting to be discovered.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables S1 and S2 in the supplemental material, respectively. *S. Typhimurium* strain UMR1 (ATCC 14028 Nal^r), *Shigella flexneri* 2a strain 24570^T (ATCC 29903^T), and *Klebsiella pneumoniae* subsp. *pneumoniae* strain MGH 78578 (ATCC 700721) were either cultured in Luria-Bertani (LB) liquid medium with shaking at 200 rpm or grown on LB agar plates at 37°C supplemented with 100 $\mu\text{g} \cdot \text{ml}^{-1}$ ampicillin, 50 $\mu\text{g} \cdot \text{ml}^{-1}$ kanamycin, and 30 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin, when required. *Yersinia enterocolitica* subsp. *enterocolitica* strain 8081-L2 was cultivated in TB broth (1% tryptone, 0.5% NaCl) at 25°C with shaking at 200 rpm supplemented with 30 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin, when required. The expression of genes cloned into pSRKGm was induced with 0.1 mM IPTG (isopropyl- β -D-thiogalactopyranoside).

Sequence analysis. The presence of EAL-only proteins in various taxa has been evaluated by searching the NCBI protein database for the EAL-containing proteins from 220 to 280 amino acid residues in length (the “EAL domain AND 220:280[SLen]” search pattern) with additional filtering by taxonomy, such as, for example, “AND Aquificae[ORGN]” and limiting the source databases to GenBank/EMBL/DBJ. Protein domain architectures were either taken from reference 24 or determined by comparing the sequences against the Pfam (39) and CDD/SPARCLE (60) databases. Sequence alignments of EAL domains for phylogenetic analysis were generated using MUSCLE (61) as implemented in MEGA7 (62). Highly divergent sequences were aligned by comparing them to the CDD profile cd01948 (60) with relaxed parameters. Phylogenetic trees were constructed using PhyML (63) with branch support values estimated using the aBayes algorithm (64). Graphical visualization and editing of the trees were performed in MEGA7 (62). The sequence logo was constructed using WebLogo (65).

Plasmid construction. Seven genes of interest and that encoding STM3611 were amplified by PCR (primers are listed in Table S3) and cloned into the IPTG-inducible expression vector pSRKGm (66) with a 6 $\times$ His tag at the C-terminal end. The PCR products and vectors were digested with NdeI and NheI, replacing *lacZ α* of the plasmid with EAL genes. Site-directed mutants were obtained by assembly PCR and subsequent cloning into pSRKGm (primers listed in Table S3). The integrity of all sequences was confirmed by DNA sequencing on a StarSEQ platform. The constructs were transformed into *E. coli*, *S. Typhimurium*, and *S. flexneri* by electroporation (Bio-Rad); >100 ng of plasmid DNA was electroporated with one pulse of 1.3 kV for 7 ms in 1-mm cuvettes.

In the case of *Y. enterocolitica*, pSRKGm constructs were introduced by conjugation (67). Overnight cultures of *Y. enterocolitica*, *E. coli* with helper plasmid pRK2013, and *E. coli* TOP10 carrying the pSRKGm construct of interest were mixed in a 1:1:1 ratio. The mixture was spotted on LB agar plates and incubated overnight at 37°C. The bacteria were resuspended in 10 mM MgSO₄ and plated on *Yersinia*-selective cefsulodin-Irgasan-novobiocin (CIN) agar (Oxoid, UK) containing 30 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin. Positive clones were selected after an overnight incubation at 37°C.

Mutant construction. Primers ye1324-f and ye1324-r were used to amplify a 409-bp internal fragment of the gene encoding YE1324 of *Y. enterocolitica* strain 8081. The primers introduced BamHI restriction sites for cloning into the suicide vector pSW23T (68) to obtain plasmid pSW23T-1324. The plasmid was then mobilized from *E. coli* strain ω 7249::pSW23T-1324 into *Y. enterocolitica* strain 8081-L2, a restriction-negative derivative of strain 8081 (69). The resulting Cm^r colonies were tested for the correct integration of pSW23T-1324 to obtain the 8081 Δ YE1324 mutant strain (8081 Δ YE1324::pSW23T-1324). PCR was performed using primers ye1324-f2 and ye1324-r2, which amplify a 701-bp fragment from the intact gene encoding YE1324 and a 2.8-kb fragment from the inactivated gene from 8081 Δ YE1324::pSW23T-1324.

Phenotypic evaluation. (i) Swimming motility. For *S. Typhimurium*, the swimming motility test was performed in 0.3% LB agar plates supplemented with 30 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin and 0.1 mM IPTG. Single colonies were inoculated into the agar and the motility diameter was measured after 5 h at 37°C. For

Shigella strains, the motility test was performed in 4 ml of 0.18% LB agar added to a glass tube. A single colony was inoculated into the agar, and tubes were incubated for 72 h at 37°C (53). The motility of *Y. enterocolitica* was assessed in 0.2% TB0 (TB medium without salt [1% tryptone, 0.2% agar]) agar supplemented with 10 mM glucose. Single colonies were inoculated into the agar and incubated at 25°C and 37°C for 18 h (70). At least three independent experiments with three technical replicates were performed for all assays. To determine statistical significance, a two-sided Student *t* test was used.

(ii) Swarming motility. The *Y. enterocolitica* swarming assay was performed on 0.3% TB0 Eiken agar supplemented with 10 mM glucose. Two microliters of an overnight culture was spotted on the plate and incubated at 25°C and 37°C for 20 h (70).

(iii) rdar morphotype assessment. To assess the rdar morphotype formation, 5 μ l of an overnight culture (optical density at 600 nm [OD₆₀₀] of 4.0 to 5.0) was spotted onto an LB without salt agar plate supplemented with 40 μ g $\cdot$ ml⁻¹ Congo red and 20 μ g $\cdot$ ml⁻¹ Coomassie brilliant blue. Plates were incubated at 28°C for up to 72 h with phenotype assessment every 12 h.

Construction of a Vc2 riboswitch strain. The Vc2 riboswitch along with *lacZY* were amplified from the recombinant pRS414 vector (gift from R. R. Breaker, Yale University). The PCR product and pGRG25 vector were digested with *PacI* and *NotI* (New England BioLabs, Ipswich, MA). Digested pGRG25 was dephosphorylated by Antarctic phosphatase (New England BioLabs) and then ligated with the digested PCR product by T4 DNA ligase (Roche Life Science). The resulting product was transformed into chemically competent *E. coli* TOP10 cells grown at 30°C. The transgene was inserted into the chromosomal Tn7 attachment site as described previously (71).

Vc2 riboswitch-based phosphodiesterase assessment assay. The diguanylate cyclase *AdrA* gene cloned in pBAD30, EAL-only genes cloned into pSRKGm, and vector controls were cotransformed into *E. coli* TOP10 cells carrying the chromosomal Vc2 riboswitch-*lacZY* transcriptional fusion. Individual colonies were grown overnight at 37°C with shaking in LB medium. Next, 3 μ l of culture grown to an OD₆₀₀ of 0.6 was spotted onto an LB agar plate containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 80 μ g/ml), 0.75 mM IPTG, 0.003% L-arabinose (to induce the diguanylate cyclase, *AdrA*), and relevant antibiotics. β -Galactosidase activity as assessed by colony color development was observed at 28°C for up to 2 days.

Bacterial two-hybrid system analysis. Full-length open reading frames of STM1925 (FlhD_{ST}) of *S. Typhimurium* and YE2581 (FlhD_{YE}) and YE1324 from *Y. enterocolitica* subsp. *enterocolitica* 8081 were cloned into pKT25, pNKT25, pUT18, and pUT18C as N- and C-terminal fusion proteins. Genes of interest were PCR amplified, digested with *XbaI* and *KpnI*, and ligated into *XbaI*/*KpnI*-digested pKT25, pNKT25, pUT18, and pUT18C vectors. The sequence integrity of all constructs was checked by DNA sequencing. The oligonucleotides used are listed in Table S3. The resulting constructs express fusion proteins to the T25 and T18 domains of the adenylate cyclase encoded by *cyaA* from *Bordetella pertussis*. Protein-protein interactions were assayed through functional reconstitution of the adenylate cyclase activity in the *cyaA*-deficient *E. coli* strain BTH101, with activation of β -galactosidase activity and growth as the readouts (72). On LB agar plates at 30°C supplemented with 40 μ g $\cdot$ ml⁻¹ of the substrate X-Gal and 0.5 mM IPTG, the cleavage of the substrate X-Gal resulted in a blue colony phenotype observed for up to 72 h. Growth promotion was assessed on M63 minimal medium agar at 28°C containing 0.5 mM IPTG and 0.2% (wt/vol) maltose as the sole carbon source for up to 72 h.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00179-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, PDF file, 2.5 MB.

ACKNOWLEDGMENTS

We acknowledge technical assistance of Juha Laitinen and thank Yuri Wolf (NCBI) for advice on phylogenetic analysis. We thank Ronald R. Breaker, who provided the pRS414::Vc2 riboswitch construct.

Youssef El Mouali received a scholarship from Eurolife (JPTEM program). This work was supported by the Swedish Research Council for Natural Sciences and Engineering (grant 621-2013-4809) and the Karolinska Institutet. M.Y.G. is supported by the NIH Intramural Research Program at the U.S. National Library of Medicine.

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