Genetic reductionist approach for dissecting individual roles of GGDEF proteins within the c-di-GMP signaling network in Salmonella

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Bacteria have developed an exclusive signal transduction system involving multiple diguanylate cyclase and phosphodiesterase domain-containing proteins (GGDEF and EAL/HD-GYP, respectively) that modulate the levels of the same diffusible molecule, 3′-5′-cyclic diguanylic acid (c-di-GMP), to transmit signals and obtain specific cellular responses. Current knowledge about c-di-GMP signaling has been inferred mainly from the analysis of recombinant bacteria that either lack or overproduce individual members of the pathway, without addressing potential compensatory effects or interferences between them. Here, we dissected c-di-GMP signaling by constructing a Salmonella strain lacking all GGDEF-domain proteins and then producing derivatives, each restoring 1 protein. Our analysis showed that most GGDEF proteins are constitutively expressed and that their expression levels are not interdependent. Complete deletion of genes encoding GGDEF-domain proteins abrogated virulence, motility, long-term survival, and cellulose and fimbiae synthesis. Separate restoration revealed that 4 proteins from Salmonella and 1 from Yersinia pestis exclusively restored cellulose synthesis in a c-di-GMP–dependent manner, indicating that c-di-GMP produced by different GGDEF proteins can activate the same target. However, the restored strain containing the STM4551-encoding gene recovered all other phenotypes by means of gene expression modulation independently of c-di-GMP. Specifically, fimbiae synthesis and virulence were recovered through regulation of csgD and the plasmid-encoded spvAB mRNA levels, respectively. This study provides evidence that the regulation of the GGDEF-domain proteins network occurs at 2 levels: a level that strictly requires c-di-GMP to control enzymatic activities directly, restricted to cellulose synthesis in our experimental conditions, and another that involves gene regulation for which c-di-GMP synthesis can be dispensable.

biofilm formation | Salmonella virulence | signal transduction system cellulose | STM4551

Recently, a signal transduction network has been recognized that so far is restricted to bacteria. This network uses a cyclic dinucleotide, 3′-5′-cyclic diguanylic acid (c-di-GMP), to relay the signal (1–6). In c-di-GMP signaling, the sensor protein domain seems to react to the stimulus by activating an output domain, usually fused in the same polypeptide, that triggers changes in c-di-GMP levels. The only known effector domain that synthesizes c-di-GMP (designated as diguanylate cyclase activity, DGC) is the GGDEF domain. Two domains, EAL and HD-GYP, hydrolyze c-di-GMP (designated as phosphodiesterase activity, PDE) into linear 5′-phosphoguananylyl-(3′-5′)-guanosine (pGpG) or guanosine monophosphate (GMP), respectively (7–13). Many DGC proteins also contain an RxxD conserved motif 5 amino acids upstream from the GGDEF motif. The RxxD motif binds c-di-GMP and provides allosteric control of DGC (14, 15).

The mechanisms of c-di-GMP recognition and subsequent regulation are only beginning to be characterized. Several binding devices for c-di-GMP, apart from c-di-GMP metabolic enzymes, have been identified recently. These binding devices are the proteins that contain the PilZ domain (16–22), the PeID protein (23), which contains the RxxD motif, the c-di-GMP–responsive transcriptional regulator FleQ (24), and a first class of widely distributed riboswitches (25).

Finding the biochemical connection between a sensor and the proteins it regulates is a major challenge for understanding signal transduction, especially in the c-di-GMP signaling system, because all sensor GGDEF- and EAL/HD-GYP–domain proteins apparently use the same diffusible molecule, c-di-GMP, to transmit the signal. Thus, the specificity of c-di-GMP action may require selected temporal expression of GGDEF- and EAL/HD-GYP–domain proteins, co-localization of such proteins with their targets, and/or activation of these enzymes under specific environmental or intracellular stimuli (2).

The importance of the c-di-GMP signaling system is suggested by the abundance of proteins containing GGDEF and EAL/HD-GYP domains in bacterial genomes (26) and by the wide range of cellular processes it regulates: exopolysaccharide bio-synthesis, cell–cell signaling, developmental transitions, biofilm formation, motility, and virulence of animal and plant pathogens (for reviews, see refs 1–3, 5, and 27). Overall, it appears that high levels of c-di-GMP promote sessility, biofilm formation, and aggregative behavior, whereas low c-di-GMP levels promote motility and synthesis of virulence factors. However, all conclusions about c-di-GMP signaling have been inferred from analysis of recombinant bacteria that either lack or overproduce individual members of the c-di-GMP metabolic pathway. Compensatory effects or interferences from the remaining members of the system have not been addressed. In this context, we have studied c-di-GMP signaling through a genetic strategy requiring 2 sequential steps: (i) construction and analysis of a bacterial strain lacking genes for all known proteins that make c-di-GMP to determine the biological processes regulated by c-di-GMP signaling, and (ii) chromosomal restoration of individual genes encoding GGDEF-domain proteins for analysis of the specific contribution of each GGDEF-domain protein to the signaling network. We demonstrate that c-di-GMP synthesized by physi-
Consequences of total removal of GGDEF-domain proteins. Biofilm formation capacity in (A) LB medium conditions and (B) adherence test medium (ATM) conditions. (C) Cellulose production on calcofluor agar plates. (D) Cellulose and fimbriae production on Congo red agar plates. (E) Swarming motility. (F) Swimming motility.

Results and Discussion

Consequences of Removing all GGDEF-Domain Proteins in a Single Strain. Because most GGDEF domain proteins were constitutively expressed under all conditions tested (SI Text and Fig. S1A), we reasoned that the only way to evaluate the role of c-di-GMP signaling in Salmonella biology was to construct a strain in which all genes encoding for putative DGCs were deleted. Accordingly, we developed a markerless gene replacement method that permitted us to delete sequentially all 12 genes encoding GGDEF-domain proteins in a single clinical S. Enteritidis strain. The resulting strain, ΔXII, displayed levels of c-di-GMP that were undetectable by reversed-phase HPLC and MALDI-TOF analysis (data not shown). ΔXII exhibited growth rates indistinguishable from the wild type under all environmental conditions tested, including acidic conditions, high osmolality, and starvation. However, ΔXII showed a drastic reduction in long-term survival during desiccation in the absence of nutrients (Fig. S2).

Next, we analyzed phenotypes typically associated with c-di-GMP production (Figs. 1 and S3). ΔXII lost the capacity to form a biofilm, did not fluoresce on calcofluor agar plates, and formed smooth, white colonies on Congo red agar plates, indicating the inability to produce cellulose and fimbriae (28, 29). Surprisingly, and contrary to the accepted idea that low c-di-GMP levels promote motility, our results showed that complete absence of GGDEF-domain proteins negatively and severely affected cell motility.

We compared the virulence of the ΔXII and the wild-type strain by performing survival assays in BALB/c mice, ileal loop co-infection experiments, and colonization and invasiveness tests. ΔXII was totally avirulent. None of the mice infected with up to 10^9 cfu of ΔXII died during the course of an oral infection model (Fig. 2A). ΔXII also showed a significantly reduced capacity to adhere and invade the intestinal epithelium (Fig. 2C) and a drastic reduction in the degree of multiplication inside the spleens and livers of i.p. infected mice at 72 h after infection (Fig. 2D).

Together, these results demonstrate that the GGDEF-domain protein signaling network is not essential for cell viability. However, this network is crucial for long-term survival of Salmonella in the environment, for cell motility, and for all phenotypes involved in multicellular behavior. Furthermore, our results reveal that 1 or several GGDEF-domain proteins are essential for virulence of Salmonella at several stages of the infection process in mice.

GGDEF-Domain Protein Signaling Regulon. We next questioned whether the phenotypes displayed by ΔXII were associated with a specific modification in the global transcriptional profile. Microarray expression data (Table S1) and subsequent validation by RT-PCR (Fig. S4) showed that the incapacity of ΔXII to produce a biofilm under LB conditions and to fluoresce on calcofluor plates, along with colony morphology and color on Congo red plates, correlated with lower mRNA levels of the transcriptional regulator CsgD, which controls cellulose and fimbriae production (29). In addition, the attenuation of virulence in ΔXII also correlated with lower transcription levels of the plasmid-encoded spvAB genes. Contrary to expectation, reduced motility of ΔXII coincided with increased mRNA levels of flagellar body genes. Despite such overexpression, ΔXII presented a severe defect in the exportation of flagellin out of the cell, as established by Western blot analysis (data not shown). Taken together, global transcriptional data indicate that the GGDEF-domain protein signaling network directly or indirectly regulates mRNA levels of genes essential for cellular processes such as virulence, motility, and multicellular behavior.

One GGDEF Protein, One Strain Strategy. We created a collection of 12 strains, derivatives of ΔXII, each of which contained the chromosomal copy of a single gene encoding GGDEF-domain
protein in the original wild-type genomic location. Because the c-di-GMP synthesis then would depend on a single GGDEF-domain protein, analysis of these strains gave insight into the role and specificity of action of each protein in the c-di-GMP signaling network. We generated this collection in duplicate. In 1 set, each of the 12 ΔXII derivative strains was constructed to produce a single GGDEF-domain protein tagged with a 3xFlag epitope, facilitating analysis of the expression of each protein in the absence of the rest of the members of the system. Western blot experiments showed that, with the exception of AdrA, expression levels were not interdependent (Fig. S1B).

These results validated experiments with the next collection of strains, constructed using the markerless gene replacement method. In this second set, each of the 12 ΔXII derivative strains contained a chromosomal copy of a single gene encoding for 1 GGDEF-domain protein without the 3xFlag tag to avoid possible undesirable effects of the tag upon protein activity. Phenotypic analysis of the second collection showed that only 1 strain, ΔXII+strm4551, partially recovered the wild-type phenotypes shown for calcofluor and Congo red plates, indicating that this strain produces fimbriae and not cellulose (Fig. 3A and B). In addition, ΔXII+strm4551 fully recovered the level of survival of the wild-type strain during desiccation in the absence of nutrients (Fig. S2). Three strains, ΔXII+STM1987, ΔXII+yegE, and ΔXII+yfnN, recovered the capacity to produce a biofilm under ATM conditions and thus were able to synthesise cellulose under this condition (Fig. 3D, Bottom) (30). Motility assays revealed that the swimming capacity of ΔXII reached wild-type levels when restored with STM4551, YfeA, and STM2503 (Fig. 3C), the latter 2 containing a degenerate catalytic GGDEF site and a conserved EAL site (14).

We also investigated the role of each GGDEF-domain protein in the virulence process of *Salmonella*. Survival experiments using BALB/c mice, orally infected with each of the 12 strains containing a single GGDEF-domain protein were performed (Fig. 2B). ΔXII+strm4551 was the only member of the collection that recovered the virulence of the wild-type strain. Further analysis showed that ΔXII+strm4551 recovered the capacity to adhere and invade the intestinal epithelium and to multiply within spleens and livers of i.p. and orally infected mice (Fig. 4).

In short, these 1-protein, 1-strain data show, first, that the expression of most GGDEF-domain proteins is regulated neither by c-di-GMP nor by the activity of other GGDEF-domain proteins. The only exception is AdrA, which previously has been shown to be transcriptionally activated by CsgD in a process that depends on the activity of 2 other DGCs (31). Second, the data stand in opposition to the model in which localized modification depends on the activity of other DGCs. Third, a unique strain, ΔXII+strm4551, restores most of the phenotypes missing in ΔXII (virulence, motility, fimbriae synthesis, and long-term survival), suggesting that a variety of biological processes can be regulated by the activity of a single GGDEF-domain protein.

Alternatively, the phenotypes missing in ΔXII might result from...
were inserted in the chromosome of c-di-GMP produced by each GGDEF-domain protein, indicating that the 
stm4551 promoter so that gene expression was obtained (Fig. 5). The c-di-GMP synthesis. (Fig. 5) and equal virulence restoration (Fig. 4). RT-PCR assays confirmed the relationship between phenotypes associated with the presence of STM4551 and recovery of csgD, csgA, and spvAB expression to levels similar to those displayed by the wild-type strain. Similar results were obtained in the 
∆XII+stm4551 GGGSF strain, indicating that an active GGDEF domain is not required for regulation of target mRNA levels (Fig. S4). Third, we generated a ∆XII derivative encoding for an STM4551 protein with an inactive I site (GxxE) to exclude the possibility that an additional GGDEF protein hypothetically present in the genome of the 3934 clinical isolate could potentially produce c-di-GMP and affect STM4551 activity. Against this hypothesis, the resulting strain, 
∆XII+stm4551 GxxE, displayed in vitro phenotypes identical to the wild-type strain (data not shown). Its avirulent phenotype, identical to that shown by ∆XII, indicated that re-establishment of the expression of SpvAB is responsible for the 
stm4551 virulent behavior.

Therefore, our results show first that restoration of virulence, motility, fimbriae expression, and long-term survival in ∆XII containing the gene encoding STM4551 occurs through a mechanism that does not depend on c-di-GMP synthesis. These results are consistent with 2 recent studies showing that regulatory activities of 2 GGDEF domain proteins, CsrD from Escherichia coli (33) and GdpS from Staphylococcus aureus (34), do not require the synthesis of c-di-GMP. In contrast to STM4551, however, these 2 proteins do not present DGC activity. Second, our findings strongly suggest that, under the experimental conditions tested, cellulose synthesis is the only enzymatic activity of the biological processes under study that strictly requires the presence of c-di-GMP.

Motility assays showed the high level of complexity in GG-DEF-domain protein-dependent regulation. On one hand, this process is regulated independently of the production of c-di-GMP, as demonstrated by the restoration of motility displayed by the strains producing either STM4551 or STM4551-GGGSF and also by 2 phosphodiesterase-like proteins containing a degenerate GGDEF site (YfeA, and STM2503). However, a c-di-GMP component that controls this process also exists, because the individual presence of AdrA or HmsT in ∆XII blocked motility in a manner that depended on the presence of an active GGDEF site. The latter finding is in agreement with previous studies that correlate high levels of c-di-GMP and sensibility (12, 35–38).

In conclusion, in this study, we have dissected bacterial c-di-GMP signaling by using a systematic approach that could be used to elucidate the connectivity between a sensor and subsequent regulated proteins in any signal transduction pathway.
This approach, carried out in 2 phases, first eliminates in the same cell all proteins responsible for the synthesis of the effector molecule, enabling assessment of the number of biological processes controlled by the signaling network. Second, it individually restores genes for proteins that synthesize the effector, permitting identification of the specific role of each of the proteins in the absence of interferences among them. Our findings support a model in which the majority of GGDEF-domain proteins are constitutively expressed and in which their expression levels are not interdependent. GGDEF-domain proteins thus are ready to be activated under appropriate environmental conditions. Once activated, they can control cellular functions at 2 different levels. One level includes enzymes whose activity can be triggered only through direct binding of c-di-GMP; in our experimental conditions, this activity was restricted to cellulose synthase. A second level includes processes that are controlled at the gene expression level and whose regulation becomes much more complex, because both c-di-GMP-dependent and c-di-GMP-independent mechanisms may coexist.

Materials and Methods

Bacterial Strains, Plasmids, and Nucleotide Primers. Bacterial strains and plasmids used in this work are listed in Table S2. The primers used are listed in Table S3.

Phenotypic Assays. Cellulose production, qualitatively assessed by the level of calcocfluor binding, and biofilm formation in a nutrient-deficient medium, ATM conditions, and in a rich-medium condition (LB) was determined and visualized as previously described (28). Colony morphology and color on Congo red agar plates (39) and swimming and swarming motility were tested at 28 °C as described (12).

Desiccation Experiments. The desiccation experiment was adapted from a described protocol (40). Briefly, 100 μl from overnight cultures grown in LB medium at 37 °C were tested immediately (initial numbers) or air dried and stored in 24-well tissue culture plates at room temperature for 12 days. After rehydration of bacteria in 500 μl PBS, pH 7.4, the number of viable cells remaining in each sample was determined by serially diluting cell mixtures and plating in duplicate. The average and SD of 3 independent assays were recorded.

Virulence Assays. The virulence of the wild-type strain and of mutant ΔXII was compared in an orphagic-infection mouse model as described (41) using animals infected with 10^9 cfu Salmonella cells in 100 μl PBS per animal. Ileal loop co-infection experiments (42), modified as described (41), were performed. Organ colonization was determined as described (41).

Gene Disruption. For disruption of yeaI, yfaE, and spvA genes, PCR-generated linear DNA fragments were used as previously described (43). Mutants in bcsA were obtained by transduction between an insertional mutant in bcsA (28) and the recipient strain.

Detection of c-di-GMP. Synthesis of c-di-GMP was carried out at the 4 μM scale on a NH4-Tentagel resin (RAPP polymer) using a described procedure and the trisopropylsilylethoxymethyl (TOM) group for the 2′-OH protection (44, 45). The crude product (11% cyclization yield) was purified by reversed-phase HPLC and characterized by MALDI-TOF MS, as discussed later. Reversed-phase HPLC analyses were carried out on Teknokroma C18 columns (10 μm, 250 × 4.6 mm), using a multistep gradient of 0.05 M aq ammonium acetate, pH 7.0, and ACN/H2O (1:1) at 1 ml/min (12, 31). MALDI-TOF MS analysis (Voyager-DERP, Applied Biosystems) was performed in the negative mode using ammonium citrate and either α-cyanohydroxynamic acid or trihydroxyacetophenone matrices: m/z 688.830 [M-H]^- (calc. monoisotopic mass for C20H23N10O14P2-: 689.087). With electrospray ionization (ESI) MS, 2 major peaks were observed in the negative mode (10 V): m/z 344.4 and 171.2, corresponding to [(M–2H)/2]^2^- (calc. mass: 344.04) and [(M–4H)/4]^4^- (calc. mass: 171.52), respectively.

Real-Time PCR. Total RNA of the wild-type strain S. Enteritidis 3934, ΔXII, ΔXII+st4551, and ΔXII+st4551 GGDSF was isolated after 72 h of incubation in LB under biofilm-forming conditions. The assay and statistical analysis used were as described (30).

Descriptions of the identification of genes encoding for putative c-di-GMP synthetases, analysis of GGDEF-domain protein expression patterns, construction of strains expressing a 3xFlag-tagged GGDEF domain protein, construction of ΔXII, construction of a collection of strains containing a single GGDEF-domain protein, microarrays, and statistical analysis are provided in SI Methods.

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