Cyclic diguanylic acid (c-di-GMP; cGpGp) is a global second messenger controlling motility and adhesion in bacterial cells. Intracellular concentrations of c-di-GMP depend on two opposite activities: diguanylate cyclase, recently assigned to the widespread GGDEF domain, and c-di-GMP-specific phosphodiesterase, associated with proteins harboring the cyclase, recently assigned to the widespread GGDEF domain, and c-di-GMP. The intracellular levels of c-di-GMP are regulated by the opposing diguanylate cyclase gene dos and c-di-GMP-specific phosphodiesterase activity. A Δdos::kan mutation rendered the cells unable to divide properly, suggesting that dos and yddV may be part of a fine-tuning mechanism for regulating the intracellular levels of c-di-GMP.

Both prokaryotes and eukaryotes use selected small molecules, commonly referred to as second messengers, to regulate cell function. The best known second messengers besides calcium are cyclic adenosine- and guanosine monophosphates, cAMP and cGMP, respectively. Prokaryotes and eukaryotes use cAMP, whereas cGMP is used primarily by eukaryotes. Recently, the guanine nucleotide, cyclic dinucleotide GMP (c-di-GMP; 3′,5′-cyclic diguanylic acid, cGpGp) (Fig. 1) was identified as a novel intracellular bacterial signaling molecule whose structure is known and consists of two cGMP molecules joined by a 3′,5′-phosphodiester bond (1, 2) (Fig. 1). c-di-GMP was first identified in Gluconacetobacter xylinus (formerly Acetobacter xylinum) and was shown to regulate cellulose production in this species (2–4), in which c-di-GMP binds to and activates the cellulose synthase BcsB. However, increasing evidence has demonstrated that c-di-GMP acts as an important signaling molecule in a variety of bacterial species influencing various aspects of physiology and behavior, such as motility, biofilm formation, virulence, and cell-cell interactions (for a review, see Ref. 5). These findings indicate that c-di-GMP signaling is widespread and that, like cAMP and cGMP, it is a ubiquitous signaling molecule. However, little is known about the targets of this molecule in the cell or if it affects transcriptional regulation of certain genes.

The intracellular levels of c-di-GMP are regulated by the opposing activities of two types of enzymes, diguanylate cyclases (DGC) and c-di-GMP phosphodiesterases (PDE) (3, 6, 7). DGC converts two molecules of GTP via the linear intermediate diguanylosediphosphate (pppG3′p′G5′) into c-di-GMP, and, by contrast, PDE hydrolytically cleaves the cyclic compound into GMP via the intermediate pG3′p′G (2, 6, 7). The identification of two domains, GGDEF (the name of this domain is due to the conserved GG(D/E)(D/E)F amino acid sequence pattern) and EAL, shared by all of the DGC and PDE isoforms suggested that either or both domains were involved in the diguanylate cyclase activity (8). Remarkably, the GGDEF domain is very abundant in the genomes of free living bacteria, but most of the GGDEF proteins have not yet been experimentally characterized (9). Recent evidence by the groups of Römling and Gomelsky (7, 10, 11) unequivocally showed that GGDEF and EAL protein domains are involved in the c-di-GMP synthesis and hydrolysis, respectively. In addition, three very recent reports demonstrated that EAL domains are indeed Mg2+- or Mn2+-dependent cyclic diguanylate phosphodiesterases whose activity is inhibited by Ca2+ or Zn2+ (6, 7, 12). Interestingly, GGDEF and EAL domains are found only in bacteria and are not present in Archaea or Eukarya (13), strongly suggesting that c-di-GMP-mediated signaling is an exclusive bacterial trait. It is noteworthy that proteins with GGDEF or EAL domains are reiterated in bacterial genomes; for example, Escherichia coli K-12 bears 19 proteins with the GGDEF domain and 17 with the EAL domain, but importantly, the GGDEF and EAL domains are usually found in multidomain proteins at the COOH-terminal end of often multiple sensory and signal transduction domains (5).
motility. Based on these results, a phenotypic analysis of E. coli mutant strains with different levels of c-di-GMP was performed and revealed the existence of a mechanism for fine-tuning c-di-GMP levels.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Phages, and Cell Growth**—All of the strains used were E. coli K-12 derivatives with the exception of TOB1 strain, a fecal isolate (14). The genotypes and sources of the relevant bacterial strains and plasmids are given in supplemental Table I. Luria-Bertani broth (LB) was prepared as previously described (15). A$_{600}$ was determined in an Ultraspec 3100 Pro Amersham Biosciences spectrophotometer. Aerobic cultures were grown at 37 °C with shaking (200 rpm) in 250-ml flasks containing 10 ml of medium. The following antibiotic concentrations were used unless otherwise noted: ampicillin (200 μg ml$^{-1}$) and kanamycin (100 μg ml$^{-1}$).

**Cloning of yddV for Overexpression Experiments**—Primers YddV-Ncol-5′ (5′-CCC AGC CTT ATA AGG GCG ATG GAG ATG TAT-3′) and YddV-Sacl-3′ (5′-CTC TGA GCT CGG CAT CTA AAG ACT GGC-3′) were used to PCR-amplify the whole yddV open reading frame to be inserted in frame into plasmid pSE420 (Invitrogen) previously cut with NcoI and SacI. Using this plasmid, the cloned yddV is under the regulation of the IPTG-inducible P$_{lac}$ promoter. To verify the cloned gene, the DNA sequence of all constructs was determined at the Molecular Biology Unit of the Cellular Physiology Institute (National University of Mexico).

**High Performance Liquid Chromatography**—Cells were harvested at the indicated times from LB plus 1 mM IPTG cultures of strains with different levels of c-di-GMP was performed and revealed the existence of a mechanism for fine-tuning c-di-GMP levels.

**Microarray Procedures and Data Analysis**—For microarray analyses, cDNA was synthesized and labeled using a protocol similar to that described by Rosenow et al. (19). The labeled cDNA was hybridized to Affymetrix Gene Chip E. coli antisense genome arrays as recommended by the manufacturer (on the World Wide Web at www.affymetrix.com). Following 16 h of hybridization at 45 °C, the washing and stain of the array was automated by a GeneChip Fluidics Station controlled by Affymetrix Microarray Suite 5.0 Software. For each condition, two biological replicates were done. The whole procedure was carried out at the Microarray facility of the National University of Mexico. Normalization of the data resulting from each array was performed using the Gen Arise software package (available on the World Wide Web at www.ifc.unam.mx/genarise/). The statistically significant variation of expression in response to high levels of c-di-GMP was obtained by filtering out those genes that had a log$_2$ (expression ratio) higher or lower than two S.D. values of the average.

**Optical and Electron Microscopy**—For optical microscopy, the samples were observed with a Nikon E-600 differential interference contrast microscope mounted with a Hamamatsu orca II camera using × 600 magnification. All images were processed using the Wasabi 1.4.0.2 and Adobe Photoshop 7.0 programs. For transmission electron microscopy, bacterial cell suspensions were applied on Formvar-coated grids. Samples were negatively stained with 1% uranyl acetate and observed with a JEOL-1200EXII electron microscope (JEOL, Tokyo, Japan).

**Biofilm and Motility Assays**—Biofilm assays were carried out at 37 °C using 96-well, non-tissue culture-treated polystyrene dishes (Costar, Cambridge, MA) or in borosilicate glass, as previously reported (20, 21). Every experiment was performed in triplicate at least three times to confirm the results. To prevent evaporation, microtiter dishes were covered with a plastic box. Visualization of attached cells was performed by removing the cell culture, staining the wells with 1.0% crystal violet for 20 min, and rinsing the wells three times with distilled water. Similar results were obtained when nondistilled water or fresh LB medium was used for rinsing. Motility assays were performed on tryptone (1.3%), NaCl (0.7%), glucose (0.2%), agar (0.3%) plates as previously described (22).

**DNA Manipulation**—Bacterial genomic DNA was obtained using the Genomic DNA isolation kit (Promega). Plasmid DNA was purified using the Miniprep kit (Qiagen). DNA fragments were amplified by PCR using E. coli chromosomal DNA as template.

**Mutagenesis and Genetic Techniques**—P$_{1}$vir generalized transductions and transformations using the CaCl$_2$ protocol were performed as described by Miller (15). In the gene inactivation experiments, the left flanking region of dos (1.1 kb) was PCR-amplified using primers MM11 (5′-GAT CGG GAA TTC TAC AAT TGT GAG ATG TAT-3′) and MM12 (5′-AGC CAG CAT GCC GCG CAT CTA AAG ACT GGC-3′). The right flanking region of dos (1.0 kb) was PCR-amplified using primers MM13 (5′-GAT CAT GCC ATC TCT CCG GAG ATG AC-3′) and MM14 (5′-GTT CGG ACT GCA GCG CGG ACA AAC TTC AG-3′). The two PCR products were gel-purified using the QIAquick kit (Qiagen), digested with the appropriate restriction enzymes, repurified, and subjected to a four-fragment ligation mixture.

**Transcriptional Responses to High Levels of c-di-GMP**

**FIGURE 1. Chemical structure of c-di-GMP.**

Grown to midexponential phase on LB plus ampicillin (A$_{600}$ = 0.3), IPTG (1 mM) was then added to the culture. After 2 h of incubation, cells were harvested by centrifugation, and total RNA was then extracted from the pellets using the hot acid phenol/chloroform method and treated with DNase I essentially as described on the World Wide Web at www.microarrays.org. Isolated RNA was quantified on the basis of its absorption at 260 nm, visualized on an agarose gel to check quality, and stored at −70 °C until further use.
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FIGURE 2. Cloning and overexpression of yddV. A, a 1.4-kb fragment containing the entire yddV structural gene was cloned into pSE420. In this construct, yddV is under the regulation of a Pruv promoter (IPTG-inducible), & Coomasie staining of a SDS-PAGE of E. coli cells bearing the pyddV plasmid. A culture of this strain was grown in LB to exponential phase and then split in two flasks: 1 mL IPTG was added to one culture (lane 2), and the other was left untreated (lane 1). Cultures were harvested after 2 h of treatment and subjected to SDS-PAGE. The arrow indicates the protein YddV.

with a 1.3-kb BamHI-BamHI fragment containing the kanamycin resistance gene cassette from plasmid pUC4K-KIXX (Amersharm Biosciences; supplemental Table I) and plasmid pTZ19R or pUC18 digested with EcoRI and PstI. T4 DNA ligase (Invitrogen) was used under the conditions suggested by the manufacturer. After 18 h of incubation at 14 °C, the ligation mixture was used for transformation of late exponential phase cultures (RNAeasy kit Qiagen) of strain MM2 (5′-GTC GCC CTG ACA AAT TCC TCT CGC CCG CAC TCG CGG GTT CAG CTC GAT CTT AAC GGA CCG CCG C CC TATT CAA TAA TTA TGC GGC TGG G-3′) or MM3 (5′-CGG GTG TTA CCA CAA CAT CCG TCT CAC GGC AGA CCT CAG CGC CTC AGA AAT TCC TGTT GCC CCG TCC CGG GGG ATG AGT CGG GAG CTG C-3′) for the amplification of 0.5 kb upstream from the translational start site of the yddV-dos operon: YddV-GV-L3 (5′-GCA ATG TAA CAT CAG AGA TTT TGA GAC ACA ACG TGG CCT TCA TAA TCA CCC TTA TAA GGC TGG G-3′) and YddV-GV-L3B (5′-CGG GCC GGA CTA AAG CCG CAG CTC GAT CTT AAC G-3′) for the amplification of 0.5 kb downstream from the dos translational stop codon. The kanamycin cartridge was PCR-amplified from chromosomal DNA from strain JMH1232 (25) using Ampli-KAN-L5 (5′-GGG GGA AAG CCA CGT GTG TCT CTC A A-3′) and Ampli-KAN-L3 (5′-GGG GGC GCT GAG GTG TTG TGG GCG G-3′) and Ampli-KAN-L5B and Membrane DNA sequencing was carried out at the DNA sequencing facility of the Biomedical Research Institute (National University of Mexico).

DNA Sequence Analyses—The GCG Wisconsin Package, NCBI BLAST (available on the World Wide Web at www.ncbi.nlm.nih.gov:80/BLAST/6), and the Colibri server (available on the World Wide Web at genolist.pasteur.fr/Colibri/) facilities were used for DNA sequence analyses.

Isolation of RNA and RT-PCR Experiments—Total RNA was purified from late exponential phase cultures (RNAeasy kit Qiagen) of strain MC4100 grown aerobically on LB medium. Pure RNA preparations were treated with Sau3AI restriction enzyme for 1 h at 37 °C, and the RNA was repurified. cDNA was synthesized using the OMNIScript kit (Qiagen) with primers MM1 (5′-GGG GCC CCA CCA CAA CAA CAT CCG TCT CAC GGC AGA CCT CAG CAG CTC AGA AAT TCC TGTT GCC CCG TCC CGG GGG ATG AGT CGG GAG CTG C-3′) and MM3 (5′-CGG GCC GGA CTA AAG CCG CAG CTC GAT CTT AAC GGA CCG TCT CAC GGC AGA CCT CAG CGC CTC AGA AAT TCC TGTT GCC CCG TCC CGG GGG ATG AGT CGG GAG CTG C-3′) (Fig. 6A). RT-PCR amplification was carried out using primers MM2 (5′-GGG GCC CCA CCA CAA CAT CCG TCT CAC GGC AGA CCT CAG CAG CTC AGA AAT TCC TGTT GCC CCG TCC CGG GGG ATG AGT CGG GAG CTG C-3′) and MM4 (5′-CGG GCC GGA CTA AAG CCG CAG CTC GAT CTT AAC GGA CCG TCT CAC GGC AGA CCT CAG CAG CTC AGA AAT TCC TGTT GCC CCG TCC CGG GGG ATG AGT CGG GAG CTG C-3′) as described under “Results” (Fig. 6A). RT-PCR products were analyzed in a 1% agarose TBE 1× gel electrophoresis (Fig. 6B).

RESULTS

Identification of yddV as a Diguanylate Cyclase—Genomic studies have identified 19 proteins with a GGDEF domain in E. coli (supplemental Table IV) (9). Taking the amino acid sequence of DGC1 (diguanylate cyclase) from G. xylinus as template, we found that one of the closest matches (31% identical in the GGDEF domain) to this protein in E. coli was the product of the gene yddV, also named ylck or b1490 (Fig. 2A). The main difference between YddV and DGC1 is the presence of a PAS domain in DGC1 (2, 8). Interestingly, two recent reports provided biochemical and genetic evidence that YddV has diacylglycerol cyclase activity, since it complemented a celB mutant of Rhizobium leguminosarum (8), and high levels of c-di-GMP were detected when a plasmid bearing yddV under the regulation of its native promoter was transformed into Salmonella enterica (11). Then we decided to clone yddV under the control of an inducible promoter (Puv) in order to manipulate the intracellular c-di-GMP levels. yddV was cloned as described under “Experimental Procedures,” and the DNA sequence of the cloned fragment was determined to check for any possible inserted mutations due to the PCR procedure (data not shown). As shown in Fig. 2B, an increase in the expression of YddV was evident when 1 mM IPTG was added to the medium (lane 2). We determined that maximal YddV induction (as judged by the increase in the intensity of the band corresponding to YddV on a 10% SDS-PAGE) was observed when an exponentially growing culture (A600 = 0.3) was treated with 1 mM IPTG for 2 h (data not shown). To validate an increase in c-di-GMP levels, we performed HPLC of the cell extracts using chemically synthesized c-di-GMP as a standard (see “Experimental Procedures” and Fig. 3A). As depicted in Fig. 3B, there was a clear increase of c-di-GMP levels in cell extracts of the E. coli strain overexpressing YddV. To confirm the iden-
tity of the major peak in our sample, we mixed the cell extract with the standard, and only one peak with a retention time of 8.7 was observed (Fig. 3C). No signals were detected when cell extracts from cultures of strains harboring the control vector were used (data not shown). To analyze the kinetics of c-di-GMP synthesis, we performed a similar HPLC analysis using extracts from cultures of strains bearing the plasmid vector pSE420 or the pYddV construct at different given times after the induction by IPTG. As shown in Fig. 4, a progressive accumulation of c-di-GMP was evident at 0.5, 1, 3, and 5 h after the addition of IPTG; by contrast, no c-di-GMP signal was detected in extracts from the strain bearing the control vector even after 5 h of induction (Fig. 4). Taken together, these results strongly suggest that our cloned gene yddV is indeed a diguanylate cyclase and that the induction of the gene yddV provokes an increase in c-di-GMP intracellular levels. In order to determine the transcription profile of E. coli under high levels of c-di-GMP, we decided to extract total RNA from cultures treated with IPTG for 2 h, since they were still in the exponential phase of growth (Fig. 1). We decided to extract total RNA from cultures treated with IPTG for 2 h, since they were still in the exponential phase of growth (Fig. 1). We then microarray analyses were normalized and filtered for those genes with increased transcription of known transcriptional factors such as Fur may indicate on one side that genes involved in iron uptake are highly repressed; accordingly, two of the most Fur-negatively regulated genes, gltA and the operon including sufA, sufB, and sufS, are highly repressed (30) (supplemental Table III; see “Discussion”). On the other hand, the induction of SoxS, the positive transcriptional regulator of the superoxide response, may suggest a stress condition caused by the elevated levels of c-di-GMP. Surprisingly, SoxS-regulated genes, such as sodA, are not considerably up-regulated; this apparent discrepancy has already been reported (31). Very interestingly, ynlF, nadR, xapR, and zraR, encoding putative transcriptional regulators, are also induced; the significance of this fact remains unknown, but it is interesting to note that ZraR is involved in metal tolerance-resistant processes (32). Finally, it is noteworthy that ftsT (b1570) and ftsX (b3462), involved in cell division processes, were highly up-regulated.

Genes Whose Transcription Is Repressed under High Levels of c-di-GMP—Analysis of the genes induced by high c-di-GMP levels indicated that cell wall-modifying proteins were the highest induced under these culture conditions (supplemental Table II). Particularly interesting is the rcsA gene, encoding the transcriptional auxiliary protein RcsA involved in later stages of biofilm development and capsular polysaccharide synthesis (26, 27); the acrA gene, encoding a lipoprotein involved in cell wall biogenesis; b2056 (wcaD), a gene regulated by RcsA, involved in the regulation of colanic acid production (28); and nlpA, encoding for a putative surface antigen. This transcriptional profile may imply a dramatic change in the properties of the cell surface as a response to high levels of c-di-GMP (supplemental Table II). This is consistent with previous observations in S. enterica and Caulobacter crescentus (11, 29).

Increased transcription of known transcriptional factors such as Fur may indicate on one side that genes involved in iron uptake are highly repressed; accordingly, two of the most Fur-negatively regulated genes, gltA and the operon including sufA, sufB, and sufS, are highly repressed (30) (supplemental Table III; see “Discussion”). On the other hand, the induction of SoxS, the positive transcriptional regulator of the superoxide response, may suggest a stress condition caused by the elevated levels of c-di-GMP. Surprisingly, SoxS-regulated genes, such as sodA, are not considerably up-regulated; this apparent discrepancy has already been reported (31). Very interestingly, ynlF, nadR, xapR, and zraR, encoding putative transcriptional regulators, are also induced; the significance of this fact remains unknown, but it is interesting to note that ZraR is involved in metal tolerance-resistant processes (32). Finally, it is noteworthy that ftsT (b1570) and ftsX (b3462), involved in cell division processes, were highly up-regulated.

Genes Whose Transcription Is Repressed under High Levels of c-di-GMP—Analysis of the most repressed genes under our experimental conditions revealed that genes involved in sugar metabolism were predominant; isocitrate lyase (aceA), citrate synthase, (gltA), citrate lyase (citF), ribokinase (yeiC), succinate dehydrogenase (sdhA), proteins of the sugar phosphotransferase system (agaD and b2387), and a putative transcriptional regulator of sugar metabolism, yglH, were drastically repressed (supplemental Table III). Interestingly, several permease-related encoding genes are heavily shut down: uhlpC, b1688, b1966, b1683, ber, yjiI, oppA, yfcA, and likewise membrane-associated protein genes (yngE, b2430, yhlL, yhbA, glpG, b1966, yhpA, b2433, ycdP, and ompC; supplemental Table III). These results indicate that the main effect triggered by the elevation of c-di-GMP intracellular levels is a cell
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FIGURE 4. Kinetics of c-di-GMP formation. c-di-GMP amounts were monitored by HPLC in cell extracts from cultures of strain MG1655 bearing the pYddV or the control pSE420 plasmid after the addition of 1 mM IPTG. Samples were taken at different times and subjected to HPLC analysis. For clarity, since the control experiments did not display any c-di-GMP signal, only the 5-h trace is shown (vector).

surface change. Intriguingly, two genes encoding cooperative transcriptional regulators, GadX (yhiX) and GadE (yhiE), are severely repressed; it is known that high expression of these genes leads to increased resistance of E. coli strains under high acid conditions (supplemental Table III) (33). Other transcriptional regulator-encoding genes, such as yfhiH, yneA, ygbi, ydeW, and yieP, are also repressed. The meaning of this repression has yet to be elucidated, since the target genes and the role of these transcriptional factors are unknown. Moreover, genes involved in motility are highly repressed (yfcP and hofF; supplemental Table III).

Effect of High Intracellular Levels of c-di-GMP in E. coli Physiology—In light of our genome-wide transcriptional analyses, we reasoned that processes like cell shape, motility, and cell division may be altered by an abrupt increase in the intracellular c-di-GMP levels. In order to test this idea, we first checked if cells with high levels of c-di-GMP were affected in cell morphology by performing optical microscopy. Surprisingly, cells carrying the pYddV plasmid displayed an abnormal length, either in the exponential or stationary phase of growth; this was even more evident when 1 mM IPTG was added (Fig. 5A). Apparently, cell division stopped in more than the 85% of the population, and cells started to elongate progressively. Interestingly, those elongated cells that could divide made it only at one pole, releasing a cell the size of which was the equivalent of a wild-type cell. These results suggest that an increase in c-di-GMP levels has a dramatic effect on cell division. To further explore this phenomenon, we carried out transmission electron microscopy with samples from cultures of the strains bearing the control vector or the pYddV plasmid, both treated with 1 mM IPTG for 2 h. As shown in Fig. 5B, cells bearing the control vector showed a normal rod shape and an average length of 1.0–1.8 μm. By contrast, 85% of cells carrying the pYddV plasmid showed a filamentous shape and a length from 5 to 15 μm, again, suggesting that cells with high levels of c-di-GMP are impaired in cell division.

Recent reports have suggested that high levels of the second messenger c-di-GMP in S. enterica serovar Typhimurium promote sessility (11) and biofilm formation (34). In order to test if a similar pattern was observed in E. coli when yddV is overexpressed, strain W3110 was transformed with our pYddV construct, and biofilm and swimming assays were carried out (Fig. 5, C and D). It was evident that a significant increase in biofilm formation was observed in LB cultures of W3110 transformants bearing the plasmid pYddV in comparison with those transformants bearing the control vector pSE420 (Fig. 5C). When motility assays were carried out, a clear decrease in motility was observed concomitantly with the increase of c-di-GMP levels, suggesting that the higher the c-di-GMP levels, the lesser the ability to swim (Fig. 5D). Finally, taking into account that c-di-GMP was identified in G. xylinus as a second messenger involved in cellulose production, we decided to check whether or not under our experimental conditions, high levels of c-di-GMP resulted in higher amounts of cellulose. To test this idea, we transformed the E. coli strain TOB1, a fecal isolate able to produce cellulose (14) with the control vector pSE420 or the plasmid pYddV; we then streaked the transformants on calcifluor plates, an indicator of cellulose production (Fig. 5E) (14). TOB1 transformants bearing the control vector pSE420 displayed basal levels of fluorescence, indicating low cellulose production; by contrast, the sole presence of plasmid pYddV clearly enhanced cellulose production as judged by the sharp increase in fluorescence. The addition of 1 mM IPTG to the plates increases cellulose production (Fig. 5E). Taken together, these results strongly indicate that YddV is indeed a diguanylate cyclase and that elevated intracellular levels of c-di-GMP affect the bacterial cell physiology.

Genetic Location of yddV—Looking up at the E. coli genetic map, we realized that yddV is located at position 33.71 min. The genomic region of yddV includes the gene dos (b1489), which is located immediately downstream of yddV (Figs. 2A and 7; on the World Wide Web, see genolist.pasteur.fr/Colibri/). The gene dos has been extensively studied (see Ref. 35 and references therein), and interestingly, it encodes for the EcDos protein composed of two domains, an NH₂-terminal heme sensor-PAS-PAS-containing domain and a COOH-terminal GGDEF-EAL catalytic phosphodiesterase domain. The domain composition of the EcDos protein (PAS-PAS-GGDEF-EAL) may imply that it may work as a diguanylate cyclase (due to the presence of a GGDEF domain) as well.
levels of c-di-GMP caused by overexpressing yddV (7). Optical microscopy of a strain of W3110 with the vector plasmid (left) or with the pYddV plasmid in the absence (middle) or presence of IPTG (right) were prepared for optical microscopy. B, transmission electron microscopy was carried out on cells bearing the pSE420 control vector (top) or the pYddV construct (bottom) in the presence of 1 mM IPTG. Different amplifications and fields are shown. C, aerobically cultures of transformed W3110 strain with the vector plasmid (first tube) or with the pYddV plasmid in the absence (middle) or presence of IPTG (right). E, TO81 strains transformed with the vector plasmid (left) or with the pYddV plasmid were streaked on calcofluor plates in the absence (two left panels) or in the presence of 1 mM IPTG (right panel). Plates were incubated at 30 °C for 48 h and then exposed to short wave UV to check for fluorescence. A clear and elegant example of the role of c-di-GMP in a different bacterial process is its role as a cofactor of the protein PleD, a transcriptional regulator involved in the transition of stacked to a motile cell in Caulobacter crescentus (37). On the other hand, c-di-GMP has been associated with the transition from sessility to motility in S. enterica serovar Typhimurium (11) and with the process of biofilm formation in Yersinia pestis (38) and Staphylococcus aureus (39). Genetic studies had predicted that GGDEF domains are DGC and that the EAL domains should harbor the c-di-GMP-specific PDE activity. Apparently, GGDEF domains are a bacterial trait, since they are absent in Archea and Eukarya. Despite a great effort to elucidate the role of c-di-GMP in bacterial metabolism, limited information is available on the activities and possible targets of c-di-GMP and on the specific biochemical properties of enzymes involved in synthesis and hydrolysis of c-di-GMP.

The present work had the goal of studying the genome-wide transcriptional profile of E. coli under a growth condition with high levels of c-di-GMP. The information provided by this transcriptional profile would allow us to explain some of the phenotypes in which c-di-GMP has been involved as well as to possibly identify transcriptional regulators responding to this second messenger. High levels of c-di-GMP have been associated with increased biofilm formation (34); this was in line with our findings, since several genes involved in exopolysaccharide synthesis and membrane-associated genes are heavily induced (supplemental Table II); on the other hand, we could confirm that high levels of c-di-GMP result in higher biofilm formation (Fig. 5).

**DISCUSSION**

There is increasing evidence for a broader role of c-di-GMP in bacterial metabolism than originally anticipated when it was shown for A. xylinus and S. enterica that c-di-GMP levels regulate cellulose production (high levels of c-di-GMP increase the production of cellulose (2, 36)). A clear and elegant example of the role of c-di-GMP in a different bacterial process is its role as a cofactor of the protein PleD, a transcriptional regulator involved in the transition of stacked to a motile cell in Caulobacter crescentus (37). On the other hand, c-di-GMP has been associated with the transition from sessility to motility in S. enterica serovar Typhimurium (11) and with the process of biofilm formation in Yersinia pestis (38) and Staphylococcus aureus (39). Genetic studies had predicted that GGDEF domains are DGC and that the EAL domains should harbor the c-di-GMP-specific PDE activity. Apparently, GGDEF domains are a bacterial trait, since they are absent in Archea and Eukarya. Despite a great effort to elucidate the role of c-di-GMP in bacterial metabolism, limited information is available on the activities and possible targets of c-di-GMP and on the specific biochemical properties of enzymes involved in synthesis and hydrolysis of c-di-GMP.

**Characterization of ΔyddV-dos::kan and ΔEAL-dos::kan Mutant Strains—** In order to study the contribution of both of these genes to the regulation of c-di-GMP intracellular levels, we constructed a ΔyddV-dos::kan and a ΔEAL-dos::kan mutant strains (see “Experimental Procedures” and Fig. 7F). Optical microscopy of a ΔyddV-dos::kan culture showed elongated cells in 60% of the culture (Fig. 7C). This phenotype resembles that of high levels of c-di-GMP caused by overexpressing yddV (Fig. 5, A and B); however, it is important to note that the size of these elongated cells was significantly shorter. On the other hand, a ΔyddV-dos deletion does not affect cell size (Fig. 7F). These results indicate that yddV deletion probably contributes significantly to the c-di-GMP intracellular levels. As shown in Fig. 6B, a 1.0-kb PCR product was evident (lane 3). To confirm these data, primers MM3 and MM4 were used for RT-PCR (Fig. 6A). As shown in lane 2 of Fig. 6B, a 0.7-kb PCR product was observed. When primer MM3 was used for cDNA synthesis, we observed a 0.7-kb PCR product when MM4 and MM3 primers were used for RT-PCR (lane 6), and no PCR products were amplified when the combinations MM1 + MM4 or MM1 + MM2 were used (lanes 9 and 7, respectively). Moreover, a 294-bp PCR product was observed when primers MM1 and MM2 were used for RT-PCR only when the cDNA was synthesized using primer MM1 (compare lanes 1 and 7). Negative control experiments for possible DNA contamination were carried out by running a PCR using RNA as template with primers MM1 and MM4 (lane 13), and positive control experiments were carried out by using the same primers but with chromosomal DNA as template (lane 15). Similar experiments were carried out with RNA extracted from a ΔEAL-dos::kan mutant strain. In all cases, no PCR products were observed (lanes 4–6, 10–12, and 14). Taken together, these results strongly suggest that dos and yddV are part of a bicistronic operon.

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very interestingly, one of the most induced genes is \textit{rcsA} (30.7-fold), coding for a transcriptional activator (RcsA), which is part of a complex phosphorelay that also includes the proteins RcsB, RcsC, and RcsD. The phosphate travels from the histidine kinase domain in RcsC to a response regulator domain in the same protein; from there to a phosphotransfer protein, RcsD; and from there to the response regulator RcsB, which in conjunction with the unstable regulatory protein RcsA, is required for capsule synthesis. Consistently, most of the genes involved in this process are induced under our experimental conditions (\textit{wzc}, \textit{wcaD}, \textit{wza}, \textit{gmd}, \textit{wcaB}, \textit{wcb}, \textit{manB} or \textit{cpsG}, \textit{wcaG}, and \textit{wcaE}).

\textbf{FIGURE 6.} RT-PCR analysis of the \textit{dos} gene. A, diagram showing the RT-PCR strategy. CDNA was synthesized with primer MM1 or MM3. Primers MM4 and MM2 were used for RT-PCR. The expected sizes of the PCR-generated fragments are indicated below the primers. B, gel electrophoresis of the RT-PCR products generated using RNA from cells grown aerobically at 37°C. Lanes 1–6, PCR products from CDNA generated with primer MM1. Lanes 7–12, PCR products from CDNA generated using primer MM3. RNA from MC4100 (wild type (wt)) strain (lanes 1–3 and 7–9) and from a \textit{Δdos::kan} mutant (lanes 4–6 and 10–12) were used. Control experiments were carried out using RNA from the wild type (lane 13) and \textit{Δdos::kan} mutant strains (lane 14) and chromosomal DNA from the wild type strain (lane 15) for PCR amplification. Lane 16, 1-kb ladder. Pairs of primers used for PCR are indicated by a plus sign.

\textbf{FIGURE 7.} Construction of \textit{Δdos::kan} or \textit{ΔyddV-dos::kan} mutant strains and effect of the mutations on cell shape. A and D, diagram showing the \textit{yddV-dos} chromosomal region. The arrows indicate the BamHI (B), EcoRI (E), or DraI (D) restriction sites of the W3110 strain and the \textit{Δdos::kan} or \textit{ΔyddV-dos::kan} mutant strains. B and E, Southern blot analysis of chromosomal DNA extracted from wild type (W3110), a \textit{Δdos::kan}, or a \textit{ΔyddV-dos::kan} mutant strain digested with EcoRI and BamHI (for the \textit{dos} mutant) or DraI (for the \textit{yddV-dos} mutant). The 0.9- or 0.5-kb probes used are depicted as well as the expected sizes of the fragments (A). C and F, optical microscopy of cultures of the wild-type (W3110), \textit{Δdos::kan}, and \textit{ΔyddV-dos::kan} mutant strains. \textit{wt}, wild type.
TABLE 1
Genes regulated by SoxS, Fur, and RyhB and their responses to high levels of c-di-GMP

| Gene type          | SoxS-regulated genes | Fur-regulated genes | RyhB-regulated genes |
|--------------------|----------------------|---------------------|----------------------|
| Gene               | Regulation by SoxS | -Fold change in high levels of c-di-GMP | Gene               | Regulation by Fur | -Fold change in high levels of c-di-GMP | Gene               | Regulation by RyhB | -Fold change in high levels of c-di-GMP |
| fur                | +                    | 25.63               | tdcD                | +                    | 20.1               | kgtP                | +                    | 3.82               |
| aacA               | +                    | 5.56                | espB                | +                    | 7.35               | hybF                | +                    | 2.47               |
| yggX               | +                    | 4.84                | nusC                | +                    | 5.58               | nuoC                | −                    | 5.58               |
| fimU               | +                    | 3.19                | ldcf                | +                    | 4.54               | frdA                | −                    | 2.25               |
| mdaA               | +                    | 3.05                | exhB                | +                    | 3.73               | nusL                | −                    | 2.13               |
| fpr                | +                    | 2.38                | narW                | +                    | 3.05               | ydhC                | −                    | 2.04               |
| acsA               | +                    | 1.82                | frdA                | +                    | 2.25               | acnA                | −                    | 1.82               |
| flora               | +                    | 1.58                | ydhC                | +                    | 2.23               | bfr                 | −                    | 1.29               |
| pqmB               | +                    | 1.49                | nusL                | +                    | 2.13               | frdB                | −                    | 1.08               |
| marA               | +                    | 1.44                | acnA                | +                    | 1.82               | fumA                | −                    | 1.06               |
| inia               | +                    | 1.43                | cut                 | −                    | 1.29               |                     |                      |                    |
| zwf                | +                    | 1.25                | fdd                 | +                    | 1.08               |                     |                      |                    |
| pqmA               | +                    | 1.12                | fumA                | +                    | 1.06               |                     |                      |                    |
| soxS               | −                    | 4.55                | entA                | −                    | 5.06               |                     |                      |                    |
| ompF               | −                    | 1.19                | fob                 | −                    | 3.58               |                     |                      |                    |
|                    |                      |                     | sufE                | −                    | 1.78               |                     |                      |                    |
|                    |                      |                     | flhF                | −                    | 1.50               |                     |                      |                    |
|                    |                      |                     | sufD                | −                    | 1.22               |                     |                      |                    |
|                    |                      | −1.12               | sufB                | +                    | −1.18              | oppC                | +                    | −2.77              |
| nfo                |                    | −1.15               |                    |                      |                     |                      |                      |                    |
| sodA               | +                    | −1.15               | fua                 | +                    | −1.32              | oppA                | +                    | −3.37              |
| flfA               | +                    | −1.67               | frdD                | +                    | −1.38              | oppB                | −                    | −4.97              |
| ribA               | −                    | −2.08               | sdhD                | +                    | −1.41              | sodB                | −                    | −1.18              |
|                    |                      |                     | fucA                | +                    | −1.47              | acnA                | −                    | −1.32              |
|                    |                      |                     | sdhA                | +                    | −1.92              |                     |                      |                    |
|                    |                      |                     | exhD                | +                    | −2.06              | frdD                | −                    | −1.38              |
|                    |                      |                     | fccA                | +                    | −2.13              | sdhC                | −                    | −1.41              |
|                    |                      |                     | fccD                | +                    | −2.15              | frdC                | −                    | −1.47              |
|                    |                      |                     | pqmL                | +                    | −2.67              | sdhA                | −                    | −1.92              |
|                    |                      |                     | ccrA                | +                    | −3.08              | nusF                | −                    | −2.05              |
|                    |                      |                     | gusU                | +                    | −3.70              | nusD                | −                    | −2.06              |
|                    |                      |                     | ycaN                | +                    | −3.58              | pcpB                | −                    | −3.01              |
|                    |                      |                     | ccmA                | +                    | −4.05              | yggG                | −                    | −3.16              |
|                    |                      |                     | nuoB                | +                    | −4.45              |                     |                      |                    |
|                    |                      |                     | fucA                | +                    | −8.74              |                     |                      |                    |
|                    |                      |                     | sufC                | −                    | −1.20              |                     |                      |                    |
|                    |                      |                     | flhB                | −                    | −2.21              |                     |                      |                    |
|                    |                      |                     | flhC                | −                    | −3.00              |                     |                      |                    |
|                    |                      |                     | espF                | −                    | −3.15              |                     |                      |                    |
|                    |                      |                     | flhD                | −                    | −3.2               |                     |                      |                    |
|                    |                      |                     | nikB                | −                    | −3.59              |                     |                      |                    |
|                    |                      |                     | nirC                | −                    | −3.87              |                     |                      |                    |
|                    |                      |                     | sufB                | −                    | −6.30              |                     |                      |                    |
|                    |                      |                     | sufA                | −                    | −8.25              |                     |                      |                    |
|                    |                      |                     | sufS                | −                    | −8.26              |                     |                      |                    |

* Data taken from Ref. 31.
* Data obtained from this work.
* Data taken from Ref. 30.
* Data taken from Ref. 36.

A gene involved in phospholipid synthesis and in the gene wcaD coding for a colanic acid polymerase (supplemental Table II). Then a clear change in cell surface properties is occurring, and this may be involved in higher biofilm formation (26) (Fig. 5C).

Another result involving the Rcs system in the response to high levels of c-di-GMP was the fact that two genes, flhD and flhC, that are negatively regulated by the RcsA/RcsB complex, are also repressed under the experimental conditions tested. About 50 genes are involved in flagellar synthesis, motility, and chemotaxis in E. coli. They are expressed in a hierarchical manner and have been organized into three classes according to the level in the hierarchy at which they are expressed (40).

At the lowest level, class III contains the flagellin-encoding gene fliC and the genes involved in chemotaxis. Class II contains the α factor-encoding gene flaA, required for the expression of the genes of class III, the anti-α protein-encoding gene fliM, and the genes required for the synthesis of the flagellum basal body. Only two genes, flhD and flhC, organized into an operon, define class I. They code for a transcriptional activator required for the expression of genes of class II. Thus, activation of the entire set of motility and chemotaxis genes depends on the expression of the master operon flhDC. Therefore, the decrease in the expression of this operon by high levels of c-di-GMP may explain our observations of a decreasing motility concomitantly with an increase in yddD expression (Fig. 5D). To further support this point, fliC and fliM (class III) and flaA (class II) are both down-regulated by RcsA/RcsB (41) and by high levels of c-di-GMP (−1.32, −2.97, and −1.53, respectively) (this work). More studies on the role of c-di-GMP in the RcsA regulatory network are necessary to understand this phenomenon.

Intriguingly, high levels of c-di-GMP triggered the transcription of more than 50 genes of unknown function. On the other hand, it is important to note that the transcription of 27 genes encoding membrane-associated proteins is dramatically decreased. This clearly suggests a change in cell shape; consistently, cells overexpressing yddD are...
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elongated in shape and apparently are affected in cell division processes; notably, genes involved in cell division such asftsT andftsX are altered under this condition. The transcription of 10 genes encoding transcriptional regulators was clearly diminished; in particular, two cooperative regulators, GadE (yhiE) and GadX (yhiX), are apparently shut down. Overexpression of yhiE, similarly to overexpression of yhiX, a known regulator of glutamate decarboxylase expression, leads to increased resistance ofE. colistrains under high acid conditions, suggesting that YhiE is a regulator of gene expression in the acid response (33). The significance of the decrease in the transcriptional activity ofboth genes upon exposure to high levels of c-di-GMP warrants further investigation.

Thefur gene, encoding the transcriptional repressor Fur, is highly induced, implying a repression of the iron uptake system; accordingly, genes involved in iron uptake, such as the induced, implying a repression of the iron uptake system; accordingly, YhiE is a regulator of gene expression in the acid response (33). The reported several genes that are up-regulated by Fur; this complex regul-

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mucoid phenotype inA. xylinum (23). Other operons as

opposite activities of DGC1 (c-di-GMP synthase) and PDEA1 (c-di-

GMP phosphodiesterase); however, oxygen is the master regulator, because PDEA1 activity is dependent on the redox status of its heme-

based PAS domain (35, 43). Therefore, A. xylinum produces cellulose only under aerobic growth conditions (45). The genetic arrangement here described foryddVand dos may suggest that inE. coli a similar key role is played by the oxygen, since the phosphodiesterase activity of EcDos is lost upon exposure to oxygen (35, 43). Studies are in progress to establish the role ofc-di-GMP under other environmental conditions such as anoxic growth conditions. This study has revealed that, despite previous work, there is still a great deal to be discovered concerningc-di-GMP metabolism inE. coliK-12.

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REFERENCES

1. Jenal, U. (2004) Curr. Opin. Microbiol. 7, 185–191
2. Ross, P., Mayer, R., and Benzmüller, M. (1991) Microb. Rev. 55, 35–58
3. Ross, P., Mayer, R., Weinhouse, H., Amikam, D., Huggirit, Y., and Benzmuller, M. (1990) J. Biol. Chem. 265, 18933–18943
4. Amikam, D., and Benzmuller, M. (1989) J. Bacteriol. 171, 6695–6669
5. Romling, U., Gomelsky, M., and Galperin, M. (2005) Mol. Microbiol. 57, 629–639
6. Tamayo, R., Tischler, A., and Camilli, A. (2005) J. Bacteriol. 187, 4774–4781
7. Zogaj, X., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benzmuller, M., and Lindberg, M. (2001) FEMS Microbiol. Lett. 204, 163–167
8. Galperin, M., Nikolskaya, N., and Koonin, E. (2001) FEMS Microbiol. Lett. 203, 11–21
9. Ryjenkov, D., Tarutina, M., Moskvin, O., and Gomelsky, M. (2005) J. Bacteriol. 187, 1792–1798
10. Simm, R., Moro, M., Kader, A., Nimtz, M., and Romling, U. (2004) Mol. Microbiol. 53, 1123–1134
11. Christen, M., Christen, B., Folcher, M., Schauter, A., and Jenal, U. (2005) J. Biol. Chem. 280, 30829–30837
12. Galperin, M. (2004) Environ. Microbiol. 6, 552–567
13. Zogaj, X., Nimtz, M., Rohde, M., Bekranz, W., and Romling, U. (2001) Mol. Microbiol. 39, 1452–1463
14. Miller, J. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Amikam, D., Steinberger, O., Shkolnik, T., and Ben-Ishai, Z. (1995) Biochem. J. 311, 921–927
16. Weinhouse, H., Sapir, S., Amikam, D., Shilo, Y., Volman, G., Ohana, P., and Benzi-

man, M. (1997) FEBS Lett. 416, 207–211
17. Hayakawa, Y., Nagata, R., Hirata, A., Hyodo, M., and Kawai, R. (2003) Tetrahedron 59, 6465–6471
18. Rosenow, C., Saxena, R. M., Durst, M., and Gingeras, T. R. (2001) Nucleic Acids Res. 29, E112
19. O’Toole, G., Pratt, L., Watnick, P., Newman, D., Weaver, V., and Kolter, R. (1999) Methods Enzymol. 310, 91–109
20. Corona-Izquierdo, F., and Membrillo-Hernández, J. (2002) FEMS Microbiol. Lett. 211, 105–110
21. Takeda, S., Fujisawa, Y., Matusbara, M., Aiba, H., and Mizuno, T. (2001) Mol. Microbiol. 40, 440–450
22. Winans, S., Elledge, S., Krueger, J., and Walker, G. C. (1985) J. Bacteriol. 161, 1219–1221
23. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24. Chaverioche, M., Ghigo, J., and d’Enfert, C. (2000) Nucleic Acids Res. 28, E97
25. Majdalani, N., and Gottesman, S. (2005) Annu. Rev. Microbiol. 59, 379–405
26. Majdalani, N., Heck, M., Stout, V., and Gottesman, S. (2005) J. Bacteriol. 187, 6770–6778
27. Wehland, M., and Bernhard, F. (2000) J. Biol. Chem. 275, 7013–7020
28. Prall, R., Weiser, S., Arnott, N., Chan, C., Schirmer, T., Giese, B., and Jenal, U. (2004) Gen. Dev. 18, 715–727
Transcriptional Responses to High Levels of c-di-GMP

30. McHugh, J., Rodriguez-Quinones, F., Abdul-Tehrani, H., Svirzhenko, D., Poole, R. K., Cooper, C., and Andrews, S. C. (2003) J. Biol. Chem. 278, 29478–29486
31. Pomposiello, P., Bennik, M., and Demple, B. (2001) J. Bacteriol. 183, 3890–3902
32. Leonhardtberger, S., Huber, A., Lottspeich, F., and Bock, A. (2001) J. Mol. Biol. 307, 93–105
33. Hommais, F., Krin, E., Copper, J., Lacroix, C., Yeramian, E., Danchin, A., and Bertin, P. (2004) Microbiology 150, 61–72
34. Solano, C., García, R., Valle, J., Berasain, C., Ghigo, J., Gamazo, C., and Lasa, I. (2002) Mol. Microbiol. 43, 793–808
35. Sasakura, Y., Hirata, S., Sugiyama, S., Suzuki, S., Taguchi, S., Watanabe, M., Matsui, T., Sagami, I., and Shimizu, T. (2002) J. Biol. Chem. 277, 23821–23827
36. D’Argenio, D., and Miller, S. (2004) Microbiology 150, 2497–2502
37. Chan, C., Paul, R., Samoray, D., Amist, N., Giese, B., Jenal, U., and Schirmer, T. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17084–17089
38. Kirillina, O., Fetherston, J., Bobrov, A., Abney, J., and Perry, R. (2004) Mol. Microbiol. 54, 75–88
39. Karaolis, D., Rashid, M., Chythanya, R., Luo, W., Hyodo, M., and Hayakawa, Y. (2005) Antimicrob. Agents Chemother. 49, 1029–1038
40. Chilcott, G. S., and Hughes, K. T. (2000) Microbiol. Mol. Biol. Rev. 64, 694–708
41. Francez-Charlot, A., Laugel, B., Van Gemert, A., Dubarry, N., Wiorowski, F., Castanié-Cornet, M. P., Gutierrez, C., and Cam, K. (2003) Mol. Microbiol. 49, 823–832
42. Massé, E., and Gottesman, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4620–4625
43. Delgado-Nixon, V., Gonzalez, G., and Gilles-Gonzalez, M. (2000) Biochemistry 39, 2685–2691
44. García, B., Latasa, C., Solano, C., García-del Portillo, F., Gamazo, C., and Lasa, I. (2004) Mol. Microbiol. 54, 264–277
45. Tal, R., Wong, H., Calhoon, R., Gelfand, D., Fear, A., Volman, G., Mayer, R., Ross, P., Amikam, D., Weinhouse, H., Cohen, A., Sapir, S., Ohana, P., and Benziman, M. (1998) J. Bacteriol. 180, 4416–4425