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A Gene Coding for a Putative Sigma 54 Activator Is Developmentally Regulated in *Caulobacter crescentus*

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In *Caulobacter crescentus*, the alternative sigma factor σ^{54} plays an important role in the expression of late flagellar genes. σ^{54}-dependent genes are temporally and spatially controlled, being expressed only in the swarmer pole of the predivisional cell. The only σ^{54} activator described so far is the FlbD protein, which is involved in activation of the class III and IV flagellar genes and repression of the *fliF* promoter. To identify new roles for σ^{54} in the metabolism and differentiation of *C. crescentus*, we cloned and characterized a gene encoding a putative σ^{54} activator, named *tacA*. The deduced amino acid sequence from *tacA* has high similarity to the proteins from the NtrC family of transcriptional activators, including the aspartate residues that are phosphorylated by histidine kinases in other activators. The promoter region of the *tacA* gene contains a conserved sequence element present in the promoters of class II flagellar genes, and *tacA* shows a temporal pattern of expression similar to the patterns of these genes. We constructed an insertional mutant that is disrupted in *tacA* (strain SP2016), and an analysis of this strain showed that it has all polar structures, such as pili, stalk, and flagellum, and displays a motile phenotype, indicating that *tacA* is not involved in the flagellar biogenesis pathway. However, this strain has a high percentage of filamentous cells and shows a clear-plaque phenotype when infected with phage φCb5. These results suggest that the TacA protein could mediate the effect of σ^{54} on a different pathway in *C. crescentus*.

The *Caulobacter crescentus* cell cycle has a precisely controlled differentiation program in which two dissimilar daughter cells, a motile swarmer cell and a sessile stalked cell, are generated at every round of cell division (for reviews, see references 8 and 22). The coordination of all steps involved in these morphological changes is still poorly understood, but genetic evidence shows that stalk and flagellum biogenesis are related to cell division and chromosome replication (7, 13, 48, 52).

The most extensively studied process in *Caulobacter* differentiation is flagellum biogenesis. Expression of flagellar genes in the predivisional cell is cell cycle regulated and is subjected to a complex regulatory hierarchy. The first set of genes to be expressed (class II genes) is absolutely required for the expression of class III and IV genes, which code for the most external flagellar structures. Several strains with mutations in class II genes have been isolated, and all show a nonmotile phenotype as well as defects in cell division (7, 12, 24, 59).

The class II genes are all transcribed at the same time during the cell cycle, being expressed in the early predivisional cell, before any of the class III genes are expressed. The promoter sequences of some of the class II genes have been described and show a conserved element between positions −40 to −20 relative to the start site of transcription that is distinct from the consensus promoter sequences for *Caulobacter* σ^{70} and σ^{54} (3, 12, 48, 49, 53, 59–61). This conserved motif is the binding site for a signal transduction protein, CtrA, which is proposed to regulate the cell cycle expression of these genes (40).

The class II genes encode early flagellar structures (*fliF*, *fliG*, *fliN*, and *fliM*), proteins required for flagellar assembly (*fliP*, *fliQR*, and *flhA*), and also transcription factors (*rpoN* and *flbD*). The *rpoN* gene codes for an alternative sigma factor, σ^{54}, which transcribes all class III and IV flagellar genes (3, 7). σ^{54} is a specialized sigma factor in that it requires an activator protein to promote the transition from a closed transcription complex to the open state (39, 44). σ^{54} activators are phosphorylated by a histidine kinase on an aspartic residue at the amino-terminal regulatory domain, as part of a two-component regulatory system (50).

In *Caulobacter*, the only σ^{54} transcriptional activator described so far is the FlbD protein, required for the expression of the class III and IV flagellar genes, which are preferentially transcribed from the chromosome of the swarmer pole of the predivisional cell (23, 41). This pole-specific activation of flagellar gene transcription is due to temporally and spatially controlled phosphorylation of FlbD by its cognate histidine kinase FlbE (55, 57).

In this report, we describe the isolation of a new gene, named *tacA*, coding for a putative σ^{54} activator protein. The deduced amino acid sequence of the TacA protein contains all of the conserved residues present in the family of σ^{54} activators, including the aspartate residue that is phosphorylated in this class of proteins. The promoter region of *tacA* was characterized, and the transcription start site was determined. We show that the *tacA* promoter shares a conserved sequence element with the flagellar class II gene promoters, and the temporal expression of *tacA* follows the same pattern as found for the flagellar class II genes. We have constructed a null mutant of the *tacA* gene by the gene disruption procedure. This mutant strain has an increased percentage of cells with cell division defects and displays a clear-plaque morphology when infected with phage φCb5.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Caulobacter* cells were grown at 30°C in either PYE medium or minimal M2-glucose medium (14).

TABLE 1. Bacterial strains used in this study

Strain	Genotype and/or phenotype	Reference(s)
E. coli		
S17-1	Rp4-2,Tc::Mu Km::Tn*7*	46
TG-1	*supE* Δ*hsd5 thi*Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	20
C. crescentus		
NA1000	Synchronizable CB15 derivative	17
LS176	Δ*bla* Δ*rsa*	1a
SC1055	*rpoN*::Tn*5 proA str-140*	7, 15
SP2016	*tacA*::pUC19 Δ*bla* Δ*rsa*	This work
bNY30	Hyperpiliated, nonmotile, φCb5 sensitive	31, 38a

Strains of *C. crescentus* and *Escherichia coli* used are listed in Table 1. The *C. crescentus* synchronizable strain NA1000 was used as the wild-type strain. When assaying transcriptional activity, we used the parental strain LS176 for comparison to strain SP2016. Antibiotics used for *E. coli* were tetracycline at 12.5 μg/ml and ampicillin at 100 μg/ml; those used for *Caulobacter* were tetracycline at 1 μg/ml and nalidixic acid at 20 μg/ml. Strain SP2016 was grown in the presence of ampicillin at 50 μg/ml in plates and 10 μg/ml in liquid media.

Reporter plasmids pMV101 and pMV102 were constructed by ligating a 1.0-kb *Xho*I fragment containing the promoter regions of *tacA* and *orf1* in both orientations in plasmid pRK*lacZ*/290 (21). pMV05 is a *Hin*dIII/*Sal*I 0.5-kb fragment containing a shorter version of the *tacA* promoter in pRK*lacZ*/290 (Fig. 1). The reporter plasmids were introduced into several *C. crescentus* strains by conjugation, using *E. coli* S17-1 as the donor. Assays of β-galactosidase activity were

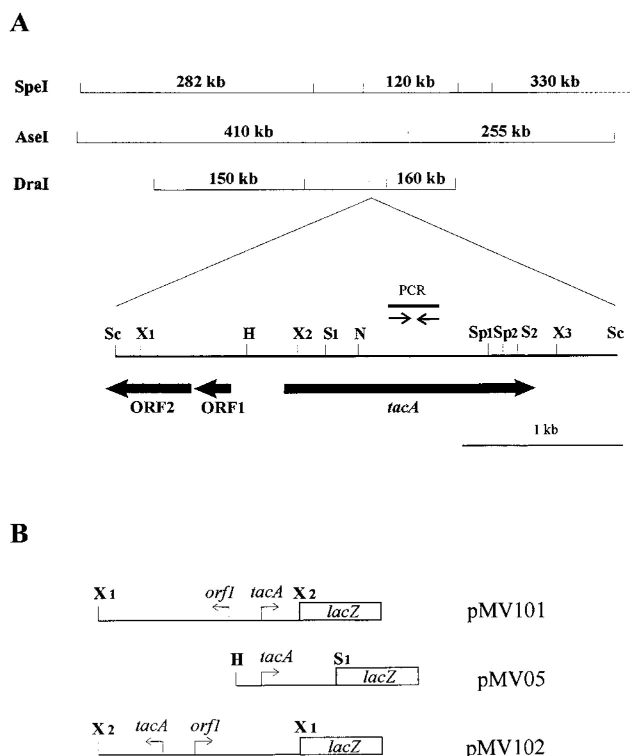

FIG. 1. (A) Schematic representation of the localization of the *tacA* gene on the *C. crescentus* genomic map. Restriction fragments of part of the *Caulobacter* chromosome corresponding to DNA digested with *Ase*I, *Spe*I, and *Dra*I are depicted with their approximate sizes (15). A partial restriction map of a 3.2-kb *Sac*I fragment containing the *tacA* gene is indicated, with letters representing the restriction sites as follows: H, *Hin*dIII; N, *Nco*I; S, *Sal*I; Sc, *Sac*I; Sp, *Sph*I; and X, *Xho*I. The arrows represent the coding regions of the *tacA* gene and the divergent *orf1* and *orf2* genes. (B) Transcriptional fusions of the *tacA* and *orf1* promoter regions to *lacZ* reporter gene. The restriction sites used in the constructions are as described for panel A.

performed as described by Miller (33), using mid-logarithmic-phase cells grown in PYE medium containing tetracycline.

Phage spot tests were performed with *Caulobacter* phages φCbK and φCb5 (>10^{10} PFU/ml). One hundred microliters of each culture grown overnight was plated in PYE plates with 3 ml of PYE medium containing 0.3% agar. The plates were allowed to dry for 30 min, then 3 μl of φCbK and φCb5 phage lysates was spotted on each plate, and the plates were incubated at 30°C.

PCR amplification and cloning of the *tacA* gene. Amplification of the central domain of σ^{54} activator proteins from total *C. crescentus* DNA was carried out by PCR. The nondegenerate oligonucleotides used for the amplification reaction were 5′-GAGCTCGGATCCGAGTCGGAGCTGTTCGGCCAC-3′ and 5′-GGTACCGAATTCGACGTTCAGGCGGTAGTACAG-3′. These oligonucleotides correspond to the deduced amino acid sequences ESELFGH and LYYRLNV (opposite strand), respectively, from the central domain of the *flbD* gene (41), based on the codon preference of *Caulobacter* (45). We also used a second pair of oligonucleotides which were identical to those mentioned above except that they were partially degenerate at the third positions of the codons according to the codon bias. The PCRs were carried out in 50 μl of 10 mM Tris-Cl buffer (pH 8.4) containing 50 mM KCl, 100 μg of gelatin per ml, 200 μM each dATP, dCTP, dTTP, and dGTP, 1 μM each primer, 1 μg of *Caulobacter* DNA, 1 to 3 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Promega). DNA was amplified by 30 cycles in a DNA Thermal Cycler (Perkin-Elmer), with the following settings: 90 s at 94°C, 2 min at 37°C, and 1 min at 72°C, followed by a 7-min extension step at 72°C. The reactions were performed in the presence of either the pair of nondegenerate oligonucleotides or the pair of degenerate ones. Every reaction was carried out in at least three independent tubes, varying the $MgCl_2$ concentration from 1 to 3 mM, conditions that were experimentally determined to be the most adequate for amplification.

The restriction sites at each end of the amplified fragments were used to clone the PCR products into the vector Bluescript II SK (Stratagene). The resulting subclones (123 clones) were analyzed through electrophoresis in 6% polyacrylamide gels, and the DNAs of those with the expected insert size (300 bp; 33 clones) were partially sequenced. Two different groups of sequences that coded for peptides conserved among σ^{54} activators were recognized; one group corresponded to the central domain of the *flbD* gene (9 clones), and the other group corresponded to a new sequence (14 clones). The other clones did not contain sequences that encoded conserved amino acid residues of central domain of σ^{54} activators.

One clone representative of this new sequence (clone U16) was used as a probe to screen a *C. crescentus* cosmid library (2). DNA minipreps of pooled cosmid clones (12 clones in each pool) were performed, and the DNA was blotted into nitrocellulose filters. The U16 probe was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham), using the random primed synthesis procedure (18). The filters were hybridized to the U16 probe in 5× Denhardt's solution containing 5× SSC (1× SSC is 15 mM sodium citrate plus 150 mM NaCl), 10 mM EDTA, 0.4% sodium dodecyl sulfate (SDS), and 30% formamide at 65°C for 16 h and then washed for 4 h at 60°C in 0.1× SSC–0.1% SDS. Filters were air dried and exposed to Hyperfilm (Amersham) with an enhancing screen at −80°C.

Two positive DNA pools were identified, and then individual DNA minipreps of each cosmid clone were performed. The DNA of each clone was digested with *Bam*HI, transferred to nitrocellulose, and hybridized to the U16 probe under the same conditions as described above. Two positive cosmid clones were isolated, and restriction digestion analysis showed they contained overlapping regions of the *C. crescentus* genome. A 3.2-kb *Sac*I fragment that hybridized to the probe and was common to both clones was subcloned into pUC19. This clone was shown by DNA sequencing to contain the complete *tacA* gene (see below).

DNA sequence analysis. The PCR amplification subclones in plasmid Bluescript were sequenced from DNA minipreps after denaturing the DNA with 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37°C followed by ethanol precipitation. Sequencing of the U16 clone and genomic DNA was performed after subcloning into M13BM vectors (Boehringer Mannheim Biochemicals), using a Sequenase 2.0 DNA sequencing kit (United States Biochemicals). Analyses of sequence data and sequence comparisons were performed with the SNAP program (38) and the CLUSTAL V program (27), respectively.

Mapping the 5′ end of *tacA* mRNA. *Caulobacter* RNA was isolated from mid-log-phase NA1000 cells grown in PYE medium, and the integrity of the RNA was assessed by electrophoresis in denaturing formaldehyde-agarose gels. The 18-mer oligonucleotide used as primer was complementary to nucleotides +118 to +100 of the *tacA* gene (see Fig. 3). The primer was end labeled with [α-^{32}P]ATP and T4 polynucleotide kinase and hybridized to 50 μg of RNA. The annealing reaction was carried out in 25 μl of 100 mM PIPES buffer (pH 7.0) containing 1 M NaCl and 5 mM EDTA at 56°C for 16 h. The nucleic acids were ethanol precipitated and resuspended in 40 μl of 50 mM Tris-Cl (pH 8.3) containing 5 mM $MgCl_2$, 40 mM KCl, 2 mM dithiothreitol, 0.2 mM each dATP, dCTP, dGTP, and dTTP, and 40 U of RNase inhibitor. The annealed primer was extended at 42°C for 90 min, using 25 U of avian myeloblastosis virus reverse transcriptase (United States Biochemicals). RNA was digested for 30 min at 37°C by the addition of 1 μg of RNase A (Sigma), and the extended products were analyzed by electrophoresis on denaturing sequencing gels. The same oligonucleotide was used as primer in a sequencing reaction with a M13 subclone containing the *tacA* promoter region, to be used as size marker.

Gene disruption procedure and complementation. To obtain a null mutant of the *tacA* gene, a 785-bp *Nco*I/*Sph*I fragment corresponding to positions +508 to +1293 (Fig. 1) of the *tacA* gene was subcloned into a pUC19 vector. A DNA miniprep of this subclone was washed twice with 70% ethanol, dried, and resuspended in water to a concentration of 1 mg/ml. One microgram of plasmid DNA was introduced by electroporation into *C. crescentus* LS176 cells resuspended in ice-cold 10% glycerol. The electroporation was performed in a Gene Pulser apparatus (Bio-Rad) set to 25 μF, 200 Ω, and 1.8 kV, and the cells were plated in PYE-ampicillin medium.

Total DNA of several colonies was purified as described by Chen and Kuo (10), and the presence of the integrated plasmid was assessed by Southern blot followed by hybridization. We isolated 18 clones that had the *tacA* gene disrupted by insertion of a single copy of pUC19 DNA, as confirmed by Southern analysis using both the *Nco*I/*Sph*I fragment and pUC19 DNA as probes in duplicate filters. One representative clone was chosen to be further characterized, generating strain SP2016.

A complementing plasmid containing the *tacA* gene was obtained by PCR amplification of a 1.8-kb DNA fragment containing the promoter and the whole coding region of the *tacA* gene. The amplified fragment was cut at the endogenous *Hin*dIII site and at a *Sac*I site created by PCR immediately after the transcription stop site of the *tacA* gene and then subcloned into plasmid pMR20 (cloning vector derivative of pMR4). The promoter region downstream of the *Hin*dIII site was sufficient to provide normal levels of transcription. The recombinant plasmid was introduced into *Caulobacter* SP2016 by conjugation with *E. coli* S17-1.

Cell cycle expression assay. *Caulobacter* strain NA1000 harboring plasmid pMV101 was grown on M2-glucose medium to an optical density at 600 nm of 1.0, and swarmer cells were isolated by centrifugation through a Ludox density gradient (17) (DuPont Company). Progress through the cell cycle was monitored by light microscopy. Proteins were pulse-labeled with 3 μCi of [^{35}S]methionine (Amersham) per ml for 10 min at various times throughout the cell cycle. Labeled proteins were immunoprecipitated with monoclonal anti-β-galactosidase antibody (Sigma) or a polyclonal antiflagellin antibody as described previously (25).

Nucleotide sequence accession number. The DNA sequence of *tacA* has been deposited in GenBank under accession no. AF010585.

RESULTS

Cloning and sequencing of a putative σ^{54} activator in *C. crescentus*. We designed oligonucleotides that correspond to the codons of two amino acid sequences highly conserved among all σ^{54} activators (34, 37). These oligonucleotides were used in PCRs to amplify sequences from the *Caulobacter* genome that corresponded to genes coding for putative σ^{54} activator proteins. Two types of amplified sequences containing all conserved amino acids found within this domain of σ^{54} activators, one corresponding to the *flbD* gene (41) and a second distinct from *flbD*, were obtained in the same proportion. A clone from this latter type (clone U16) was then used as a probe to screen a *Caulobacter* genomic library constructed in a cosmid vector, and two clones that hybridized to the probe were identified (not shown). These clones were shown by restriction mapping to overlap. A 3.2-kb *Sac*I fragment that hybridized to the U16 probe in both clones was further subcloned and sequenced (Fig. 1A). This fragment contained the entire gene corresponding to the U16 clone, and this gene was named *tacA*, for transcriptional activator A.

To determine the position of the *tacA* gene on the *C. crescentus* genome, pulsed-field gel electrophoresis of chromosomal DNA digested with *Ase*I, *Spe*I, or *Dra*I was performed, followed by Southern blotting and hybridization to a ^{32}P-labeled U16 probe. The hybridizing bands corresponded to a 120-kb *Spe*I fragment and a 410-kb *Ase*I fragment (Fig. 1A). No *Dra*I fragments hybridized to the probe, suggesting that the fragment containing *tacA* is smaller than 50 kb and probably ran off the gel. These results indicate that the *tacA* gene maps around position 2300 of the *C. crescentus* physical map, close to the *hunG* locus (15). This region of the chromosome is distant from the major flagellar gene clusters.

Sequence analysis of 630 bp downstream of *tacA* showed no open reading frame which follows the codon preference of *Caulobacter*. Although we cannot rule out the possibility of *tacA* being part of an operon, so far we have no indication of other genes downstream. Two open reading frames, ORF1 and ORF2, were found upstream, on the opposite direction of *tacA* gene (Fig. 1A). Therefore, two divergent promoter regions could be localized between *orf1* and *tacA*. The ORF2 coding region is incomplete in this clone, being interrupted at the *Sac*I site. ORF1 showed 24.8% overall identity to an open reading frame from *Mycobacterium tuberculosis* whose product is unknown. ORF2 showed no significant degree of similarity to any sequence deposited in the GenBank at the time the search was done.

The deduced amino acid sequence of *tacA* predicts a protein of 488 amino acids with a calculated molecular mass of 52.5 kDa (Fig. 2). Two putative start codons separated by 21 nucleotides are found at the amino terminus (Fig. 3A). The codon usage between the ATG in position 27 and the alternative start codon at position 51 suggests that this region is not translated. In fact, three of eight codons are the least used for their respective amino acids in *Caulobacter*. In addition, the most downstream codon is preceded by a putative ribosome-binding site (GGA) six bases upstream, and so we favor this as the most likely translation start codon.

The deduced amino acid sequence of *tacA* was subjected to a sequence similarity search in GenBank, and a good correlation was found with σ^{54} activator sequences. The highest scores of similarity were found with the *Salmonella typhimurium* and the *E. coli* hydrogenase G transcriptional regulatory protein (HydG; 40% overall identity) and the nitrogen regulatory protein NtrC from several bacteria (36.7% identity). Figure 2 shows an alignment of TacA with only one representative of each kind of activator, *S. typhimurium* HydG (11) and *Rhizobium meliloti* NtrC (51), due to their high degree of similarity. The single σ^{54} activator described so far for *C. crescentus*, the FlbD protein (41), also showed a good degree of identity (36%) with TacA (Fig. 2).

TacA possesses all important conserved amino acid residues present in other σ^{54} activators (34, 37), including an aspartic acid residue at position 54 which is analogous to Asp-54 that is phosphorylated in NtrC (43). The amino-terminal region is constituted of alternated alpha helices and beta sheets and contains the clusters of four hydrophobic residues at the proper positions compared to CheY structural domains (50). In this region there are also three aspartic acid residues (Asp-9 to Asp-11) and a lysine (Lys-104) that may correspond to Asp-13 and Lys-109, which constitute the regulatory site of the response regulator protein family (Fig. 2) (50). The central region has the proposed ATP-binding motif (GESGVGK) found in several ATP-binding proteins and the conserved motif GSFTGA that has been proposed as the site of interaction with σ^{54} (54). The carboxy terminus shows a helix-turn-helix motif which is involved in DNA binding in this class of transcriptional activators (34).

Characterization of the *tacA* promoter region. The transcription start site of the *tacA* gene was determined by a primer extension assay, using a ^{32}P-labeled 18-mer complementary to nucleotides +100 to +118 (Fig. 3A). A single extension product was obtained when the reaction was run in a urea-polyacrylamide sequencing gel, with the transcript beginning 50 nucleotides upstream of the putative initiator ATG (Fig. 4).

Figure 3A shows the promoter region of the *tacA* gene and the beginning of the divergent *orf1* gene. An imperfect inverted repeat is found close to the *orf1* start codon. Upstream of the *tacA* transcription start site there is a sequence similar to a previously described consensus sequence for class II flagellar promoters (Fig. 3B). Quon et al. proposed a new consensus for this class of promoters (TTAA-N7-TTAAC), based on the

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TacA    MTKT---VLVVDDDPTQRRLIQAVLERDGFAVSHAEGGDAAIAHLTSGAPADVILLDLVMPGLN    61
HydG    MIRGKIDILVVDDDVSHCTILQALLRGWGYNVALAYSGHDALAQV-REKVFDLVLCDVRMAEMD    63
NtrC    MTGAT--ILVADDDAAIRTVLNQALSRAGYDVRIT-SNAATLWRWIAAGDGDLVVTDVVMPDEN    61
FlbD    MR-----LLVVGKLNGQLSVAVKMAMNAGAKVSHVETTEQATNALRAGQGADLLMVDYVL-DIA    58
        *      .**  .         .         *  *         .         *... *  .

TacA    GQDALKEMRARGFNQPVIVLTASGGVDTVVKAMQAGACDFFIKPASPERITVSIRNALSMGDLK   125
HydG    GIATLKEIKALNPAIPILIMTAFSSVETAVEALKAGALDYLIKPLDFDRLQETLEKALA--HTR   125
NtrC    AFDLLPRIKKARPDLPVLVMSAQNTFMTAIKASEKGAYDYLPKPFDLTELIGIIGRALA--EPK   123
FlbD    GLIAANEAERMR--VPVVACGVDADPMRAANAIKAGAKEFIPLPPDAE-LIAAVLAAVT-----   109
                    *..             *   **  ...   *       .    .
                                                        ATP
                                                      -------
TacA    GEVERLTKRAGGKTTFADLIGASPVMTMVKRMGERAAKSGIPVLITGESGVGKELIARAVHGSS   189
HydG    ETGAEL--PSASAAQFG-MIGSSPAMQHLLNEIAMVRPSDATVLIHGDSGTGKELVARALHACS   186
NtrC    RRPSKLEDDSQDGMP---LVGRSAAMQEIYRVLARLMQTDLTLMITGESGTGKELVARALHDYG   184
FlbD    ----------DDEKP---MVVRDPAMEQVIKLADQVAPSEASILITGESGSGKEVMARYVHGKS   165
                          ..   .*  .         .   ..* *.** ***..** .*   .

TacA    DRAGKPFVAVNCGAIPENLVESILFGHEKGSFTGATDKHLGKFKEADAGTLFLDEVGELPLDMQ   253
HydG    ARSDRPLVTLNCAALNESLLESELFGHEKGAFTGADKRREGRFVEADGGTLFLDEIGDISPLMQ   250
NtrC    KRRNGPFVAINMAAIPRDLIESELFGHEKGAFTGAQTRSTGRFEQAEGGTLFLDEIGDMPMDAQ   248
FlbD    RRAKAPFISVNCAAIPENLLESELFGHEKGAFTGAMARRIGKFEEADGGTLLLDEISEMDVRLQ   229
         *   *....* .*.    *.** *******.****   .   *.* .*..***.***....    *

TacA    VKLLRALQEGEIDPIGSKRSIKVDVRIVSATNRDLQQAVSGGPFREDLFYRLNVFPIEAPSLRE   317
HydG    VRLLRAIQEREVQRVGSNQTISVDVRLIAATHRDLAEEVSAGRFRQDLYYRLNVVAIEMPSLRQ   314
NtrC    TRLLRVLQQGEYTTVGGRTPIRSDVRIVAATNKDLKQSINQGLFREDLYYRLNVVPLRLPPLRD   312
FlbD    AKLLRAIQEREIDRVGGSKPVKVNIRILATSNRDLAQAVKDGTFREDLLYRLNVVNLRLPPLRE   293
         .*** .*. *   .*.   .  ..*.......** . .   * **.** *****   .  *.**.

TacA    RREDIPALVEAFIRRFNVEEGKRVIGASPETMQLLTSFDWPGNVRQLENTVYRAIVLADAPYLQ   381
HydG    RREDIPLLADHFLRRFAERNRKVVKGFTPQAMDLLIHYDWPGNIRELENAIERAVVLLTGEYIS   378
NtrC    RAEDIPDLVRHFVQQ-AEKEGLDVKRFDQEALELMKAHPWPGNVRELENLVRRLTALYPQDVIT   375
FlbD    RPADVISLCEFFVKKYSAANGIEEKPISAEAKRRLIAHRWPGNVRELENAMHRAVLLSAGPEIE   357
        *  *.  *   *...     .               ..    .    ****.*.***  . *    *     .

TacA    PFDFPAISGLAAPIEAVSISPSPPPAALLQATHAAMAAAVA--EAPVRILDDRGHL-RTLEEIE   442
HydG    ERELPL-----------------------------------AIA--ATPIKT-EYSGEI-QPLVDVE   406
NtrC    -REI-IENELRSEIPDSPIEKAAARSGSLSISQAVEENMRQYFASFGDALPPSGLYDRVLAEME   437
FlbD    EFAIRLPDG-------QPMAPAPDVAVARGAQMAADAASRAF------------VGSTVAEVE   401
           .                                                         .  ..*
                                    H-T-H
                          ---------------------------
TacA    RDLIQHAIDVYAGHMSEVARRLGIGRSTLYRKVRE---QGIEV-----DMKEAG             488
HydG    KEVILAALEKTGGNKTEAARQLGITRKTLLAKLSR                                441
NtrC    YPLILAALTATRGNQIKAADLLGLNRNTLRKKIRE---LGVSV------YRSLA             482
FlbD    QQLIIDTLEHCLGNRTHAANILGISIRTLRNKLKEYSDAGVQVPPPQGGVGAAA             455
          .*   ..     *.     *  **.     **   *.
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FIG. 2. Comparison of the deduced amino acid sequence of *tacA* with sequences of several σ^{54} activators. Identical residues among all proteins are indicated by asterisks, and conserved residues are indicated by dots. HydG is from *S. typhimurium* (11), NtrC is from *R. meliloti* (51), and FlbD is from *C. crescentus* (41). Shaded residues indicate conserved hydrophobic amino acids characteristic of the amino-terminal regions of σ^{54} activators. Conserved acidic residues important for phosphorylation are boxed. ATP indicates a putative ATP-binding motif; H-T-H indicates a putative helix-turn-helix domain.

binding site for the transcription factor CtrA (40). Analysis of the *fliLM*, *fliF*, and *fliQR* promoters by site-directed mutagenesis showed that this element is essential for maximal transcription (48, 53, 60). This conserved sequence is located between nucleotides −40 and −20 of these promoters, the same relative position being found in the *tacA* gene. Two sequences that resemble this motif are found in the *tacA* promoter: one between nucleotides −40 and −20 and the other between nucleotides −85 and −70 (Fig. 3). This latter sequence is contained within a motif that is repeated three times, one inverted in relation to the other two, downstream of the *Hin*dIII site.

Temporal expression of the *tacA* gene. To determine the pattern of expression of *tacA* during the cell cycle, we constructed a transcription fusion in which the *tacA* promoter region was fused to a promoterless *lacZ* gene (pMV101). A synchronous population of *Caulobacter* strain NA1000 harboring this plasmid was pulse-labeled with [^{35}S]methionine at various times during the cell cycle, and the labeled proteins were immunoprecipitated with a β-galactosidase-specific antibody. As shown in Fig. 5, *tacA* is transcribed in swarmer cells, has a low level of transcription during the stalked-to-predivisional cell transition phase (0.2 to 0.3 cell division units) and has a peak of transcription in the predivisional cell (0.6 cell division units). This pattern of expression is similar to those for other *Caulobacter* genes that have the same conserved promoter (7, 30, 49, 53, 60). One notable difference is that, like *tacA*, only two other genes, *rpoN* and *ccrM*, have from 20 to 30% of maximal expression in the swarmer cells (7, 49). This difference may have a physiological meaning, if we consider that both DNA methyltransferase and σ^{54} may have roles in the cell that are not exclusively related to flagellar biogenesis.

Analysis of a *tacA* null mutant and its effect in *tacA* expression. To construct a mutant strain defective in the TacA protein, a 785-bp *Nco*I/*Sph*I fragment of the *tacA* gene corresponding to the central domain of the protein was subcloned into the vector pUC19. The recombinant plasmid was electroporated into *Caulobacter* LS176 (*bla*) cells, and integration of the plasmid in the genome was selected by growing the cells in the presence of 50 μg of ampicillin per ml. Homologous re-

```
ORF1 Met
<---- CATCGCCTTACCGCCGACATCGAAGTTCGCGTAACCGGTTTCGCCGACACCGCCTAAGACCTCGGGCAACGC  -134
           HindIII
      CAGGGCAAGCTTGGCCGCGCCTTCCATGTCCATACCACCCGCTCCCCGCATAAGACCCTGTTAAGCCTAAGT   -62
                                                                       +1
      CTTAAGGTTCGCGACACCTCTCCGAAAGCCGGTGTTTACCCGCGACCTGTATCACTCTGAACCAACAAGAGC    11

      CCTGCTCCACGTGGGCATGGCCGGTTCTGGTGGATGTCCA ATG ACC AAA ACG GTC CTT GTC GTC    75
                                                M   T   K   T   V   L   V   V     8
```

B

```
          -40        -30        -20        -10        +1
           •          •          •          •          •
tacA     CCGAAAGCCGGTGTTTACCCGCGACCTGTATCACTCTGAACC
rpoN     CGTTCGCGACCCGTTAACCAGAACCGCTCTTCAATGGCGT
fliF     TATAAACGCCTCGTTTACCTTGTACTGGGTAAATCCTGCCTA
fliQ     CCCTAACGCCCTGTTAACCATATTTCGTCCATCTTCGAGCC
fliL     GCAAAACACATCGTTAACCATGCTTCGTGCATGAGTACGGGTA
ccrM     GGTTAACGGCCCGCTAACCACGTCTCTCAACACCGGATTTAC
hemEPs   TCTTAATTTCTTATTAATATATGTTAGAAAGATCTTAA
cons.      TTAA  -N7-  TTAAC
```

FIG. 3. (A) Nucleotide sequence of the *tacA* upstream region. The ORF1 translational start codon in the opposite strand is indicated. The deduced amino acid sequence corresponding to the first eight amino acids of TacA is shown below each codon. A putative ribosome-binding site is doubly underlined. Inverted repeat sequences are indicated by arrows. The transcription start site of *tacA* determined by primer extension is indicated as +1. A DNA sequence similar to a conserved motif found in flagellar class II promoters is underlined. (B) Comparison of sequences upstream of the *tacA* gene with the promoter regions of the *rpoN* gene (3), the *fliF* gene (48), and several class II promoters as aligned by Quon et al. (40). Nucleotides corresponding to the consensus binding site for CtrA are boxed, and numbers indicate positions with respect to *tacA* transcription start site. The proposed consensus promoter sequence (cons.) for class II flagellar genes (40) is shown below.

combination is a relatively common event in *Caulobacter*, and of 22 clones analyzed by Southern hybridization, 18 had the plasmid integrated only in the *tacA* gene (not shown). Analysis of these clones by optical microscopy showed a similar phenotype (see below).

The new strain, named SP2016, was analyzed in search for a phenotype that could lead us to the pathways that are regulated by TacA. Optical microscopy analysis of this strain showed that the cells are motile but have a higher percentage of filamentous cells (around 30%) than the parental strain. Electron microscopy analysis showed that normal stalks are formed in SP2016 cells, even in the filamentous cells (not shown). A motility test was performed in 0.3% agar PYE plates, and the halo formed by the swimming cells of SP2016 was comparable in size to a halo formed by the LS176 parental strain (not shown). These results indicate that TacA is probably not involved in morphogenesis of polar structures. Strain SP2016 was able to grow in M2-glucose medium, and therefore TacA is not involved in any amino acid biosynthesis pathway. Several tests for metabolization of alternative carbon and nitrogen sources were performed, and SP2016 was able to utilize maltose, sucrose, lactose, and xylose as carbon sources and arginine, glutamic acid, aspartic acid, and histidine as nitrogen sources (not shown).

We performed a phage sensitivity test using the *Caulobacter* phages ϕCbK and ϕCb5 (1, 4) and verified that SP2016 shows lysis in a spot test when infected with either of these phages, indicating that this strain possesses the polar structures, such as pili, essential for infection. Infection of SP2016 by the filamentous phage ϕCb5 produced a clear lysis zone in a spot test, which contrasts with the turbid-plaque morphology observed when this phage infects the wild-type *Caulobacter* strain NA1000 (Fig. 6). In the parental strain LS176, ϕCb5 phage plaques are virtually undetectable, either because they are extremely turbid or because this strain is resistant to the phage. The clear-plaque phenotype of strain SP2016 is complemented when a copy of the *tacA* gene is provided in a plasmid, reverting to very turbid plaques upon infection by ϕCb5 (Fig. 6), indicating that this phenotype is not due to a polar effect in a downstream gene.

The clear-plaque phenotype of SP2016 with ϕCb5 could be the result of cells being either more sensitive to infection or completely lysed by phage multiplication. To address these questions, we determined the plating efficiencies of ϕCb5 in *C. crescentus* SP2016, NA1000, and bNY30. Since ϕCb5 plaques are not detectable in the parental strain LS176, we used the wild-type strain NA1000 as an internal control. Strain bNY30 is a derivative of CB13B1a, which is nonflagellated and hypersensitive to phage ϕCb5, and this increased sensitivity is probably due to the fact that this strain is hyperpiliated (38a). Plating of phage ϕCbK in either of the three strains resulted in the same number and morphology of plaques (Table 2). ϕCbK phage receptor was shown to be associated with the flagellum structure (4), and although strain bNY30 is nonflagellated, it still is sensitive to ϕCbK infection. However, when strain bNY30 is infected with phage ϕCb5, it shows a 10-fold increase in the number of plaques compared to NA1000 and SP2016 (Table 2), and the individual phage plaques produced in bNY30 are about 50 to 100 times larger in diameter than plaques in strains NA1000 and SP2016 (not shown). The difference in plating efficiency and size of the ϕCb5 plaques among these strains suggests that SP2016 probably has normal piliation and is not hypersensitive to infection as is strain bNY30.

The phenotypes observed for these clones were also obtained when a second construction, containing a fragment cor-

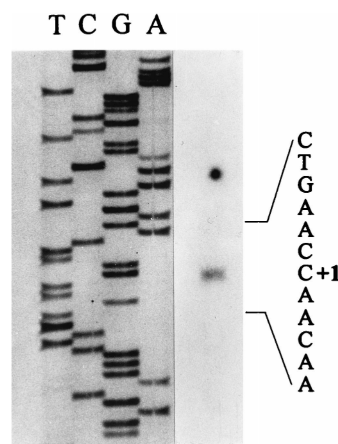

FIG. 4. Determination of the transcriptional start site of *tacA*. Fifty micrograms of *Caulobacter* RNA was hybridized to a ^{32}P-labeled 18-mer, and the hybrid was extended with 25 U of avian myeloblastosis virus reverse transcriptase. The extended product was analyzed by electrophoresis in a urea-polyacrylamide sequencing gel. A sequence reaction of an M13 recombinant phage containing the *tacA* promoter region was performed with the same oligonucleotide and run in the same gel. The sequence of the complementary strand is shown on the right to indicate the exact nucleotide where transcription initiates (+1).

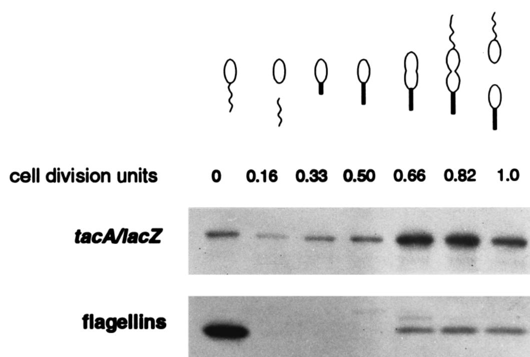

FIG. 5. Temporal pattern of *tacA* expression. A synchronized population of *Caulobacter* NA1000 carrying plasmid pMV101 was pulse-labeled with [^{35}S]methionine at regular intervals during the cell cycle. Labeled proteins (10^6 cpm) isolated from several time points during the cycle were immunoprecipitated with anti-β-galactosidase antibody. The precipitated proteins were resolved by SDS-polyacrylamide electrophoresis and visualized by fluorography. The upper autoradiogram shows the pattern of expression of the *tacA-lacZ* transcription fusion. As a control of the synchrony, proteins were also precipitated with an antiflagellin antibody (bottom). The drawing at the top is a representation of the cell types found at each time point of the *Caulobacter* cell cycle, as judged by light microscopy. One cell division unit is equivalent to a generation time of 180 min.

responding to the amino-terminal region of the protein, was used. In this case, a 387-bp *Xho*I/*Nco*I fragment (Fig. 1) was subcloned into pUC19 and used to disrupt the *tacA* gene as described before (not shown). Integration of the plasmid with this latter construct causes a truncation of the protein up to amino acid 153, which generates a nonfunctional protein. These results confirm that the *tacA* gene has been inactivated by insertion of the plasmid in strain SP2016.

Transcriptional activities of the *tacA* promoter and the divergent *orf1* promoter were determined by using transcriptional fusions of these promoter regions to a promoterless *lacZ* gene (plasmids pMV101 and pMV102, respectively [Fig. 1B]). A shorter version of the *tacA* promoter was also constructed, by cloning a 0.5-kb *Hin*dIII/*Sal*I fragment that does not contain the *orf1* promoter (pMV05). Determination of promoter activity was done by measuring the β-galactosidase activity of the three constructs in several background strains (Table 3). The activity of the *tacA* promoter in pMV101 generates about 4,000 Miller units of β-galactosidase activity in both NA1000 and LS176 strains. A significant promoter activity was still seen in pMV05, suggesting that the promoter region comprised between the *Hin*dIII site and the transcription start site is sufficient to provide approximately normal levels of expression. When this activity was measured in the *tacA* mutant strain SP2016, a 1.6- to 2.0-fold increase in activity was observed for both constructs. This effect is probably due to elimination of a negative autoregulation in the disrupted strain, as described for some promoters of this kind (30, 36, 60). We cannot determine whether this increased level of expression is due to a loss of temporal regulation, leading to higher constitutive transcription, because the mutant strain SP2016 has lost the ability to be synchronized. We obtained a similar result when analyzing the activity of the *tacA* promoter in the *rpoN* mutant strain SC1055, where we observed a consistent increase in transcription from 35 to 50%. When the activity of the reporter plasmid pMV101 was assayed in several class II flagellar mutant strains, no difference in the levels of transcription was found (not shown). Plasmid pMV102, which contains an *orf1-lacZ* promoter fusion, showed lower but still significant levels of transcription on both SP2016 and SC1055 (Table 3), indicating that this promoter activity is not dependent on either σ^{54} or TacA.

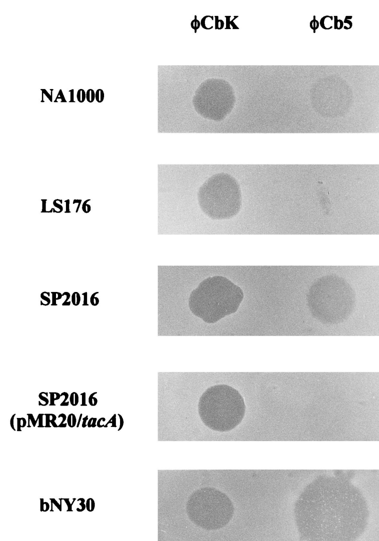

FIG. 6. Phenotypic analysis of *tacA* gene disruption. Overnight cultures of different *Caulobacter* strains were pour plated in PYE medium, and 3 μl of phage lysate (>10^{10} PFU/ml) of either phage φCbK or φCb5 was spotted over each culture.

TABLE 2. Efficiencies of plating of φCb5 and φCbK in different *Caulobacter* strains

Strain	Relative efficiency of plating[a]	
	φCbK	φCb5
NA1000	1.0	1.0
LS176	0.4	0[b]
SP2016	1.1	1.0
bNY30	1.3	10.6

[a] The efficiency of plating of each phage was defined as 1.0 in strain NA1000.
[b] Plaques were not detectable.

DISCUSSION

Control of gene expression in response to either external stimuli or intracellular signals involves a complex network that transmits information from receptor molecules to a transcriptional regulator. Signal transduction in bacteria involves a phosphorylation cascade in which a histidine kinase autophosphorylates in response to a signal and then transfers its phosphate group to a response regulator, in what is called a two-component regulatory system (50). This cascade of phosphorylation controls several metabolic as well as developmental processes in bacteria, including the regulation of sporu-

TABLE 3. Transcriptional activities of *lacZ* fusions in several *Caulobacter* host strains

Caulobacter strains	β-Galactosidase activity[a]		
	pMV101	pMV05	pMV102
NA1000	4,021	2,991	2,033
LS176	3,970	2,770	2,170
SP2016	6,372	5,460	1,433
SC1055	5,357	4,132	1,419

[a] Transcriptional activity was measured as β-galactosidase activity in Miller units. Each value is the average of at least three independent experiments.

lation in *Bacillus subtilis* (16) and fruiting body formation in *Myxococcus xanthus* (32). Several response regulators which control gene expression through direct binding to the promoter region of target genes belong to the family of σ^{54} activator proteins, the most prominent example being protein NtrC (34, 37). These activator proteins, when phosphorylated, activate the σ^{54} RNA polymerase to promote the formation of an open complex, therefore initiating transcription (39).

Whereas in many bacteria σ^{54} regulates metabolic pathways in response to environmental signals, in *Caulobacter* it seems to be used in regulating differentiation processes in response to cell cycle cues. The gene encoding *C. crescentus* σ^{54} (*rpoN*) was isolated, and analysis of a Tn*5* insertional mutation of this gene showed that besides being necessary for the expression of late flagellar genes, σ^{54} is involved in stalk biogenesis and coordination of cell division (7). How these events are regulated simultaneously by σ^{54} and what cell cycle cues initiate each of the different morphogenetic pathways remain unknown. A likely possibility would be the use of distinct activator proteins to regulate different sets of promoters. This would allow an independent control of each pathway while maintaining a common regulation coordinated by the same sigma factor.

Flagellum biogenesis in *Caulobacter* is subject to a genetic hierarchy in which the expression of genes encoding early structures is required for the expression of late flagellar genes. Class III and IV flagellar genes are transcribed by the σ^{54} RNA polymerase, and their expression also depends on other class II genes. One of the class II genes, *flbD*, encodes a transcriptional regulator that activates the expression of late flagellar genes through binding to *ftr* enhancer sequences (6, 35, 55). FlbD is responsible for the activation of class III and IV flagellar genes in the swarmer pole of the predivisional cell and also for the repression of its own transcription (*fliF* operon) in this same pole (6, 56). The role of FlbD in pole-specific regulation of flagellar gene expression suggests that it is the main, if not the sole, σ^{54} activator involved in flagellar biogenesis in *Caulobacter*.

In an effort to identify new metabolic pathways that are regulated by σ^{54} in *C. crescentus*, we started by trying to clone other activator proteins through PCR amplification of their conserved central domain. Analysis of many amplification fragments by DNA sequencing showed only two different sequences that possessed the conserved amino acid residues present in all σ^{54} transcriptional activators. One of them corresponded to the *flbD* gene, and the other corresponded to a new gene, called *tacA*. This result was quite unexpected, since several different metabolic processes are regulated by σ^{54} in other bacteria. In *Pseudomonas* species, σ^{54} regulates expression of the pilin genes, metabolism of aromatic compounds, pathogenicity, and alginate synthesis, each process depending on a different transcriptional activator (26, 28, 29, 58). In *Rhizobium* spp., σ^{54} controls nitrogen fixation and dicarboxylate transport (9, 42). In *Caulobacter*, regulation of flagellar biogenesis is directly related to the cell cycle, and expression of both the *rpoN* and *flbD* genes is developmentally regulated (7, 53). The fact that only one activator distinct from *flbD* was isolated does not exclude the possibility that other σ^{54} activators exist in *Caulobacter*. However, it could be indicative that σ^{54} is used to regulate a small number of important processes in *C. crescentus*, probably by triggering the expression of a specific subset of genes responsible for a metabolic pathway in response to a cell cycle signal.

The complete *tacA* gene was isolated from a *Caulobacter* cosmid library, and the deduced amino acid sequence showed that the TacA protein contains all of the conserved residues present in σ^{54} activators. TacA possesses in its amino-terminal domain the two aspartic acids (Asp-11 and Asp-54) and a lysine (Lys-104) corresponding to those that comprise the acid pocket which is the site of aspartyl phosphorylation in CheY (50). This finding suggests that TacA activity may be regulated by phosphorylation, probably by a histidine kinase, following the pattern found in other two-component regulatory systems. The activity of FlbD is regulated by differential phosphorylation during the cell cycle, although FlbD does not possess the aspartic acid residues corresponding to positions 9 to 11 in TacA (Fig. 2) (56, 57). There is no evidence for a putative kinase gene adjacent to *tacA*, since no sequences resembling histidine kinases are found 630 bp downstream of *tacA*. The two open reading frames upstream of *tacA*, ORF1 and ORF2, do not show similarity with any histidine kinases sequences and therefore are unlikely candidates for the TacA kinase.

TacA shares with the other σ^{54} activators a domain (GSFTGA) that has been proposed to be the site of interaction with σ^{54} (54). In fact, the NtrC protein from *Rhodobacter capsulatus* has a natural deletion in this region and activates transcription of two σ^{54}-independent promoters, *nifA1* and *nifA2*, being unable to complement a *ntrC* defect in *E. coli* (19). The fact that TacA contains this domain, together with all other features of the protein, suggests that TacA could be an activator of σ^{54}-dependent promoters. Experiments to identify these promoters are now being carried out.

To investigate the processes regulated by TacA, we have isolated a *tacA* null mutant (SP2016) where the *tacA* gene was disrupted by a pUC19 plasmid. The *tacA* mutant strain is fully motile, as determined by swarm agar plate assays, and promoter fusions of class III and IV flagellar genes are expressed in SP2016 (not shown). Although strain SP2016 presents a higher percentage of filamentous cells, which could indicate a cell division defect, the normal phenotype is still the predominant.

The *tacA* mutant strain shows a clear-plaque phenotype when infected with the RNA phage φCb5, and this phenotype can be complemented when a copy of the *tacA* gene in a plasmid is reintroduced into the mutant cells. Pili have been shown to be the receptor sites for phage φCb5, and adsorption of bacteriophage φCb5 prevents the loss of pili at the proper time during development (47). Analysis of the infection pattern of strain SP2016 compared to the φCb5-hypersensitive *Caulobacter* strain bNY30 suggests that the *tacA* mutation does not increase sensitivity to the phage by causing hyperpiliation, although a putative role of TacA in regulating pili expression is still to be verified.

Strain SP2016, like its parental strain LS176, is defective in the formation of the crystalline surface array (S layer). This phenotype, combined with the *tacA* mutation, could cause the cells to be more sensitive to lysis when infected with phage φCb5. One attractive possibility is that TacA is necessary for the expression of cell wall components, which could explain both the higher percentage of filamentous cells and the clear-plaque phenotype observed in the *tacA* mutant strain.

The promoter region of *tacA* possesses a sequence similar to the conserved sequences found in class II flagellar promoters. All class II flagellar genes sequenced so far were shown to contain this conserved element in their promoter regions and display a temporal pattern of expression similar to that shown by *tacA* (7, 30, 48, 49, 53, 60). This coordinate expression of the *rpoN* gene and its activators (*flbD* and *tacA*) could ensure maximal levels of expression of the target promoters. The genes that share this sequence were postulated to be transcribed by a common regulatory protein (48). Recently, Quon et al. identified a two-component signal transduction protein (CtrA) that binds to this sequence (40). These authors propose

that CtrA acts directly on promoters containing this consensus sequence, coordinating the expression of several cell cycle-dependent genes. The possible role of CtrA in the regulation of *tacA* expression is under investigation.

The fact that *tacA* has the same regulatory elements as the class II genes could indicate that the metabolic pathway controlled by TacA is somehow connected to flagellar biogenesis and the other processes regulated by CtrA. Its activity is necessarily related to RpoN, since TacA acts together with RpoN in the transcription of a subset of σ^{54}-dependent promoters. In *Caulobacter*, RpoN is necessary for the morphogenesis of polar structures, like the flagellum and the stalk, and is also involved in cell division. TacA is probably not the mediator of σ^{54} in polar morphogenesis, since *tacA* mutant cells show normal flagella and stalks. Whether it has a role in cell division is yet to be determined.

Although we cannot rule out the possibility of TacA regulating a metabolic process in response to external stimuli, the fact that *tacA* expression is regulated during the cell cycle suggests that it might be involved in some specific process triggered by a cell cycle cue. We still have to determine whether the TacA protein levels also parallel its pattern of gene expression. Other class II genes are differentially transcribed during the *Caulobacter* cell cycle, but their protein levels do not change significantly (30, 55). The FlbD protein is not asymmetrically distributed in the predivisional cell, but its action is restricted to the swarmer pole due to its localized phosphorylation (55, 57). The TacA protein has all of the conserved residues that compose the phosphorylation site of other σ^{54} activators, suggesting that its activity could also be regulated by phosphorylation. We are presently investigating whether *tacA* gene expression is restricted to one pole of the predivisional cell and what the spatial distribution of the TacA protein is.

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