Cell Cycle Control of a Holdfast Attachment Gene in *Caulobacter crescentus*

RAJI S. JANAKIRAMAN AND YVES V. BRUN*

Department of Biology, Indiana University, Bloomington, Indiana 47405-6801

Attachment to surfaces by the prosthecate bacterium *Caulobacter crescentus* is mediated by an adhesive organelle, the holdfast, found at the tip of the stalk. Indirect evidence suggested that the holdfast first appears at the swarmer pole of the predivisional cell. We used fluorescently labeled lectin and transmission electron microscopy to detect the holdfast in different cell types. While the holdfast was readily detectable in stalked cells and at the stalked poles of predivisional cells, we were unable to detect the holdfast in swarmer cells or at the flagellated poles of predivisional cells. This suggests that exposure of the holdfast to the outside of the cell occurs during the differentiation of swarmer to stalked cells. To investigate the timing of holdfast synthesis and exposure to the outside of the cell, we have examined the regulation of a holdfast attachment gene, *hfaA*. The *hfaA* gene is part of a cluster of four genes (*hfaABDC*), identified in strain CB2A and involved in attachment of the holdfast to the polar region of the cell. We have identified the *hfaA* gene in the synchronizable *C. crescentus* strain CB15. The sequence of the CB2A *hfaA* promoter suggested that it was regulated by σ^{54}. We show that the transcription of *hfaA* from either strain is not dependent on σ^{54}. Using a *hfaA-lacZ* fusion, we show that the transcription of *hfaA* is temporally regulated during the cell cycle, with maximal expression in late-predivisional cells. This increase in expression is largely due to the preferential transcription of *hfaA* in the swarmer pole of the predivisional cell.

Cell division of the gram-negative bacterium *Caulobacter crescentus* gives rise to two distinct progeny cells, a sessile cell (containing a stalk) and a motile cell (containing a single polar flagellum) (5). Stalked cells are often found attached to surfaces by means of an adhesive holdfast found at the tip of the stalk. Staining properties and enzyme sensitivity studies indicate that the holdfast is a complex polysaccharide (20), with acidic components such as uronic acids (33). It has been proposed that the appearance of the holdfast is temporally regulated during the cell cycle and that the holdfast first appears at the base of the flagellum in the swarmer pole of the predivisional cell (26). The presence of the holdfast at the tip of the stalk is thought to result from the growth of the stalk at the site previously occupied by the flagellum during the differentiation of swarmer to stalked cells. How the spatial and temporal regulation of holdfast expression is achieved is unclear.

A cluster of four genes (*hfaABDC*) involved in the attachment of the holdfast to the cell was previously identified by Tn5 insertion mutagenesis (16). The exact role of each of these genes is unknown. The C-terminal region of HfaA is similar to the C termini of pilus tip proteins, such as the PapG adhesin from *Escherichia coli* and the SmfG adhesin from *Serratia marcescens* (16). These adhesins interact with host cell polysaccharides and with a protein anchor in the pilus, thus mediating the attachment of these bacteria to host cell surfaces. It is possible that HfaA functions analogously in *C. crescentus* (16). HfaB is similar to proteins that function in transcriptional activation and may activate the transcription of *hfaC* (16). The sequence of HfaD contains three putative membrane-spanning regions, and it has been suggested that it acts as the membrane-associated protein anchor between HfaA and the cell

(17). HfaC is similar to ATP-binding transport-related proteins (17).

A sequence identical to the consensus for σ^{54}-dependent promoters was found upstream of the *hfaA* transcription start site, suggesting that its transcription may be subject to cell cycle regulation like other known σ^{54} promoters in *C. crescentus* (16). σ^{54} is present in both gram-negative and gram-positive members of the Eubacteria and is required for the expression of a wide variety of genes, including those involved in nitrogen fixation, pilus production, dicarboxylic acid transport, and xylene catabolism in these bacteria (21). In *C. crescentus*, σ^{54} does not appear to be required for general metabolic functions but is needed for the biosynthesis of two polar organelles, the flagellum and the stalk (6).

The *hfaA* gene was initially characterized in *C. crescentus* CB2A (16, 23). In this study, we extend this work to investigate the transcriptional regulation of *hfaA* in strain CB15. We show that *hfaA* is not transcribed by a σ^{54} promoter. We find that the transcription of *hfaA* is cell cycle regulated and that *hfaA* is maximally expressed in the swarmer compartment of the predivisional cell. The use of fluorescently labeled lectin to detect the holdfast on live cells indicates that neither the flagellar poles of predivisional cells nor swarmer cells possess a holdfast. However, the holdfast was readily detectable at the tips of short stalks. This is consistent with the hypothesis that the holdfast appears at the tips of nascent stalks during the differentiation of swarmer to stalked cells.

MATERIALS AND METHODS

Materials, bacterial strains, and growth conditions. Oligonucleotides hfaA Rev (5′GAACGAAGCCGAAAAGCTTGACATCGATTG3′), hfaA+135 (5′C CATTTTTTCGCTGCAGTGGGGCTACC3′), and hfaA+60 (5′GGGCTGGT CCCTGCAGTCTATCTAGGG3′) were obtained from either the Institute for Molecular and Cellular Biology at Indiana University or Operon Technologies, Inc. Ludox was obtained from Dupont, radionuclides were obtained from ICN Radiochemicals, and antibiotics were obtained from Sigma or Amresco. The strains and plasmids used in this study are described in Table 1. The strain YB1371 (NA1000 *hfaA*::pAA2) used in the analysis of the cell cycle expression of *hfaA* was constructed as follows. Plasmid pAA2 is a transcriptional fusion

* Corresponding author. Mailing address: Department of Biology, JH142, Indiana University, Bloomington, IN 47405-6801. Phone: (812) 855-8860. Fax: (812) 855-6705. E-mail: ybrun@bio.indiana.edu.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description or construction	Source or reference
E. coli		
S17-1	*E. coli* 294::RP4-2(Tc::Mu)(Km::Tn7)	29
DH5αF′	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17* (r⁻ m⁺) *deoR thi-1 supE44* λ⁻ *gyrA96 relA1*	18
C. crescentus		
CB15	Wild type	25
NA1000	*syn-1000*, previously called CB15N, a synchronizable derivative of CB15	10
CB2A	*rsaA* mutant of CB2	31
CB2AG9	*hfaAB*::Tn5 (Kmʳ)	23
SU213	CB15 *rpoN*::Tn5	6
SC1117	*flgH*::Tn5	9
YB1293	NA1000 ORF203::Ω Spʳ	14
YB1306	NA1000 ORF208::Ω Spʳ	14
YB1270	NA1000 ORF159::pRJ23	14
YB1369	CB15 *hfaA*::pAA2	This work
YB1370	CB15 *rpoN*::Tn5 *hfaA*::pAA2	This work
YB1371	NA1000 *hfaA*::pAA2	This work
Plasmids		
pBB31	1.7-kb *Bcl*I-*Bgl*II *hfaAB* fragment from CB2A cloned into pUC19	16
pIC-HfaA	325-bp *Pst*I-*Cla*I fragment from pBB31 containing the CB2A *hfaA* promoter cloned into *Pst*I-*Cla*I sites of pIC20H	This work
pRJ38	325-bp *Pst*I-*Hin*dIII fragment from pIC-HfaA cloned into *Pst*I-*Hin*dIII sites of pRKlac290	This work
pRJ41	Cosmid 4-1, a pLAFR5-derived cosmid containing *hfaA* from CB15	This work
pRJ39	3.0-kb *Pst*I-*Sma*I fragment from pRJ41 containing *hfaA* from CB15 cloned into *Pst*I-*Sma*I sites of pSKII⁻	This work
pRJ52	245-bp PCR product created by using hfaA+60 and hfaA Rev primers cut with *Pst*I and *Hin*dIII and cloned into *Pst*I-*Hin*dIII sites of pRKlac290	This work
pRJ54	*Pst*I-*Hin*dIII sites of pRKlac290 177-bp PCR product created by using hfaA+135 and hfaA Rev primers cut with *Pst*I and *Hin*dIII and cloned into *Pst*I-*Hin*dIII sites of pRKlac290	This work
pAA1	325-bp *Pst*I-*Cla*I fragment from pRJ39 cloned into *Pst*I-*Cla*I sites of pIC20R	This work
pAA2	325-bp *Bam*HI-*Eco*RI fragment from pAA1 containing the *hfaA* promoter cloned into *Bam*HI-*Eco*RI sites of pGSZ	This work
pUC19	Ampʳ, *lacZ*	35
pSKII⁻	Phagemid, Ampʳ, ColE1 ori, f1⁻ ori	2
pIC20R	2.7-kb phagemid, Ampʳ, derivative of pUC18	19
pIC20H	2.7-kb phagemid, Ampʳ, derivative of pUC18	19
pRKlac290	*lacZ* transcriptional fusion vector, Tetʳ, IncP-1 replicon, mob⁺	12
pGSZ	Speʳ, derivative of pGMTZ1	1

containing the CB15 *hfaA* promoter cloned upstream of the promoterless *lacZ* gene in the plasmid pGSZ. This plasmid was introduced into NA1000 by conjugation. As pAA2 does not replicate in *C. crescentus*, selection for gentamicin-resistant colonies selects for integrants that arise by homologous recombination.

E. coli strains were grown at 37°C in Luria-Bertani medium with one or more of the following antibiotics: ampicillin (100 μg/ml), gentamicin (15 μg/ml), or tetracycline (12 μg/ml). *C. crescentus* strains were routinely grown at 30°C in peptone-yeast extract (PYE) medium (25) supplemented with nalidixic acid (20 μg/ml) and either tetracycline (2 μg/ml) or gentamicin (2.5 μg/ml). M2 minimal glucose (M2G) medium (15) was used when cells were synchronized by Ludox density gradient centrifugation.

General DNA manipulations, cloning, and sequencing. General cloning procedures were done as described previously (3, 13). The *hfaA* gene from *C. crescentus* CB15 was identified by Southern blot hybridization as follows. A 400-bp *Sac*II fragment of CB2A *hfaA* from pBB31 was used as a probe to screen a *C. crescentus* cosmid library, and a cosmid containing *hfaA* (pRJ41) was isolated. A 3.0-kb *Pst*I-*Sma*I fragment from pRJ41 that hybridized to the CB2A *hfaA* probe was subcloned (pRJ39). Several overlapping subclones were generated from pRJ39 and used for sequencing CB15 *hfaA*. DNA sequencing was done by the dideoxynucleotide chain termination method (28) on double-stranded templates isolated with Qiagen Mini-Prep kits. Reactions were done by a modification of the Thermo Sequenase dye terminator cycle sequencing protocol (Amersham). The following program was used: 1 min at 96°C, followed by 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min in the presence of either M13 forward or reverse primers and labeled dideoxynucleoside triphosphates. Reactions were run in the Institute for Molecular and Cellular Biology at Indiana University on an ABI PRISM 377 DNA sequencer. The DNA sequence was analyzed with the package of the Genetics Computer Group of the University of Wisconsin (8) and Sequencher 3.0 (Gene Codes Corporation).

Detection of the holdfast with fluorescein-labeled lectin. A fluorescent lectin-binding assay was used to detect the holdfast (20). A volume (2 μl) of fluorescein-conjugated wheat germ agglutinin (FITC-WGA; Molecular Probes) (5 mg/ml of stock) was added to 200 μl of cells growing exponentially. The mixture was incubated at room temperature for 20 min, diluted with 1 ml of water, and centrifuged. The cell pellet was resuspended in 30 μl of Slowfade antifading reagent (Molecular Probes), and 1 μl was examined by fluorescence microscopy. Epifluorescence photomicroscopy was performed on a Nikon Eclipse E800 light microscope equipped with a Nikon B-2E fluorescein isothiocyanate (FITC) filter cube for FITC and a 100× Plan Apo oil objective. Images were captured by a Princeton Instruments cooled charge-coupled device camera (model 1317) and the Metamorph imaging software package (v. 3.0).

Electron microscopy. Cells were grown to mid-log phase in PYE medium, washed by centrifugation at 5,000 rpm for 5 min in an Eppendorf centrifuge, and resuspended in a one-fifth volume of phosphate-buffered saline. Cells (5 μl) were spotted onto carbon-coated grids and allowed to settle for 20 min. The grids were blotted dry and washed once with water. The cells were stained with 1% uranyl acetate for 30 s and washed four times with water. They were examined in a Philips model 300 electron microscope at 60 kV.

Analysis of the promoter. The *hfaA* promoter and mutant versions of the *hfaA* promoter were cloned upstream of the *lacZ* gene in pRKlac290 or pGSZ and analyzed in wild-type and mutant backgrounds for promoter activity. Assays were done in duplicate on a minimum of two independent cultures in each case. β-Galactosidase activity conferred by these plasmids was measured at 30°C as described previously (22), except that cells were permeabilized with chloroform.

To assay the time of transcription of *hfaA*, late log-phase cultures grown in M2G medium containing tetracycline were synchronized by the Ludox density centrifugation method (10). Swarmer cells were collected and allowed to proceed through the cell cycle in fresh M2G medium at 30°C. At 15-min intervals, 1-ml
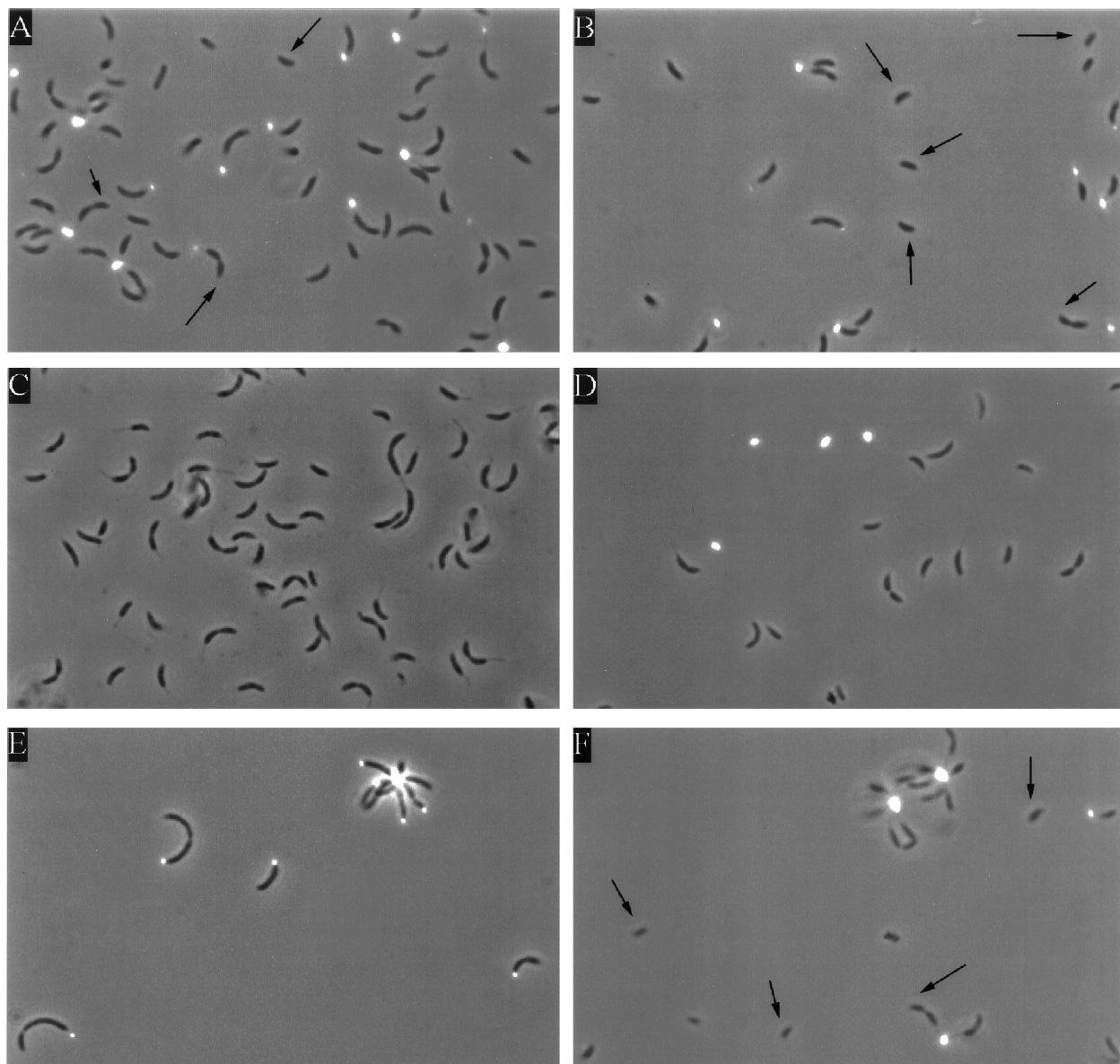
FIG. 1. Fluorescein-conjugated lectin labeling of the holdfast in various strains of *C. crescentus*. Micrographs were taken in combined fluorescence and phase-contrast modes of wild-type CB15 (A and B), NA1000 (lacking a holdfast) (C), holdfast-shedding mutant CB2AG9 (D), SU213 (*rpoN*::Tn5) (E), and SC1117 (*flgH*::Tn5) (F). The arrows indicate swarmer cells and the swarmer poles of predivisional cells.

culture samples were labeled with 15 μCi of [^{35}S]methionine (Trans-Label) for 5 min, collected by centrifugation, and frozen at −20°C. Cells were lysed in wash buffer (50 mM Tris, pH 8.3–450 mM NaCl–0.5% Triton X-100). A small volume of each sample was precipitated with 10% trichloroacetic acid, collected on glass fiber filters, and counted in a scintillation counter cocktail. Equivalent counts of radiolabeled protein were then immunoprecipitated with an antiflagellin antibody (an internal control for the cell cycle) and an anti-β-galactosidase antibody (Boehringer Mannheim). The samples were processed as described previously (14).

Pole-specific expression was measured as described previously (34). Synchronized swarmer cells were allowed to proceed to the predivisional stage and were pulse-labeled for 10 min with 30 μCi of [^{35}S]methionine. Unlabeled methionine (0.1 μM) was used to chase the label, and the cells were allowed to divide. The progeny swarmer and stalked cells were separated by Ludox density gradient centrifugation, and the transcription level from the *hfaA* promoter was determined as described above.

Nucleotide sequence accession number. The DNA sequence of the *hfaA* gene was submitted to GenBank and has been given the accession no. AF058792.

RESULTS

σ^{54} is not required for holdfast synthesis or attachment. Since the CB2A *hfaA* gene contains sequences at nucleotides −24 and −12 (numbered in relation to the transcription initiation site) that are identical to those recognized by the σ^{54}-RNA polymerase holoenzyme (16), we expected a strain that lacks σ^{54} (such as an *rpoN*::Tn5 mutant) to display the holdfast-shedding phenotype seen in the *hfaAB*::Tn5 mutant. To determine if this was the case, we labeled the holdfast in an *rpoN*::Tn5 mutant, SU213, with FITC-lectin. The wheat germ agglutinin lectin binds specifically to the holdfast, and its conjugation to FITC allows its visualization by epifluorescence microscopy (20). We assayed for the presence of the holdfast and quantitated this labeling in the wild-type strains CB15 and CB2A. Wild-type CB15, which forms rosettes and contains a

TABLE 2. Quantitation of holdfast labeling in different strains with
fluorescein-labeled lectin

C. crescentus strain	% of cells labeled[a]	
	Predivisional	Swarmer
CB15	81	0
CB2A	87	0
SU213 (*rpoN*::Tn5)	75	ND
SC1117 (*flgH*::Tn5)	79	0
CB2AG9 (*hfaAB*::Tn5)	10	ND

[a] A minimum of 112 predivisional cells and 45 swarmer cells were examined in
each case. ND, not determined.

normal holdfast, showed spots of FITC-lectin attached to the
stalks in stalked cells and predivisional cells (Fig. 1A and B).
No fluorescent labeling was seen in NA1000, a strain that lacks
a holdfast, indicating that the background level of fluorescence
from this technique is very low (Fig. 1C). Figure 1D shows
labeling of the holdfast material in CB2AG9, an *hfaAB*::Tn5
mutant, which synthesizes a normal holdfast but sheds it into
the medium (23). This shed holdfast was labeled, as indicated
by the FITC-conjugated lectin spots which are not associated
with cells. Only 10% of the predivisional cells in CB2AG9 were
labeled, indicating that most of the predivisional cells had shed
their holdfasts (Table 2). In the *rpoN*::Tn5 mutant, 75% of the
predivisional cells were labeled with fluorescent lectin (Fig. 1E;
Table 2). This is comparable to the percentages of predivi-
sional cells labeled by FITC-lectin in the *C. crescentus* wild-
type strains CB15 and CB2A (Table 2). In addition, shed
holdfasts were not detected in the culture medium of the
rpoN::Tn5 mutant. This suggests that σ[54] is not required for
hfaA expression. It is also possible that two promoters drive the
expression of *hfaA* and only one of them is σ[54] dependent or
that CB15 *hfaA* is regulated differently than CB2A *hfaA*. Al-
ternatively, shedding may not occur efficiently in the stalkless
rpoN mutant, because the holdfast is not subject to the same
shearing force as when it is at the tips of stalks in wild-type
cells.

Identification of *hfaA* from *C. crescentus* CB15. To determine
whether *hfaA* from CB15 had regulatory sequences similar to
those of the CB2A *hfaA* gene, we cloned *hfaA* from CB15 (see
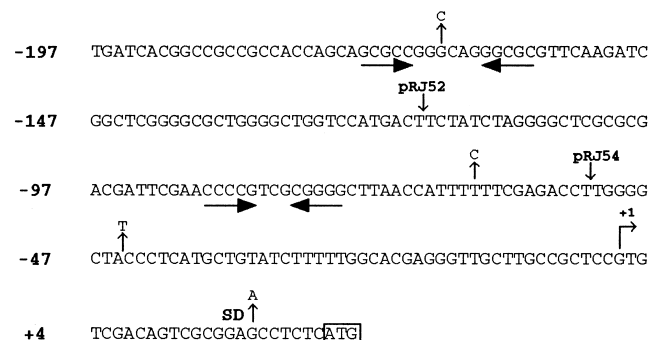Materials and Methods). Analysis of the nucleotide sequence

FIG. 2. Sequence of the *hfaA* promoter region in CB2A and CB15. Positions
where the CB15 *hfaA* promoter sequence differs are shown above the sequence.
Inverted repeat sequences are shown by inverted horizontal arrows. The 5′
endpoints of plasmids pRJ52 and pRJ54, used for deletion analysis, are indi-
cated. The transcription initiation site (+1), as mapped previously (16), is indi-
cated by the bent arrow; the translation initiation codon is boxed, and the
nucleotides are numbered relative to the transcription start site. The putative
Shine-Dalgarno sequence is underlined.

TABLE 3. Expression of the *hfaA* promoter in different
mutant backgrounds

C. crescentus strain	Genotype	β-Galactosidase activity[a]
NA1000	*syn-1000*	2,560
CB15	Wild type	2,350
CB2A	Wild type	2,580
SU213	*rpoN*::Tn5	3,400
YB1270	ORF159 mutant	1,730
YB1293	ORF203 mutant	2,480
YB1306	ORF208 mutant	2,360
YB1369	CB15 *hfaA*::pAA2	1,230
YB1370	*rpoN*::Tn5 *hfaA*::pAA2	2,350

[a] All results are expressed in Miller units and have a standard deviation of less
than 10%. Activities of pRJ38 were measured in each case, except in YB1369 and
YB1370, for which the activity of the chromosomal fusion pAA2 was measured.

of the CB15 *hfaA* gene indicated that it was 98% identical to
the CB2A *hfaA* gene, while the predicted product of CB15
hfaA (GenBank accession no. AF058792) is 95% identical to
that of CB2A HfaA. Only 3 nucleotides differed between CB15
and CB2A in the 200 bp upstream of the transcription start site
(Fig. 2). This is consistent with previous evidence which re-
vealed that the freshwater *C. crescentus* strains CB15 and
CB2A are highly similar (32). The high degree of identity
between the CB15 and CB2A *hfaA* regulatory sequences
makes it unlikely that the CB15 and CB2A *hfaA* genes are
regulated differently; therefore, we studied the regulation of
the CB2A *hfaA* promoter in CB15.

The *hfaA* gene is not transcribed by a σ[54] promoter. The
presence of a promoter proximal to *hfaA* was previously sug-
gested by complementation and S1 mapping studies (16). To
confirm the presence of a promoter for *hfaA* in this region, a
325-bp fragment containing the putative CB2A *hfaA* promoter
and the first 30 codons of *hfaA* was cloned upstream of a
promoterless *lacZ* gene to generate a transcriptional fusion.
This fusion (pRJ38 [Table 3]) yielded approximately 2,500
Miller units of β-galactosidase activity in both CB15 (wild
type) and NA1000 (a synchronizable derivative of CB15). De-
leting sequences upstream of nucleotide −117 (pRJ52) re-
duced transcriptional activity to 1,090 Miller units. Removing
bases upstream of nucleotide −52 (pRJ54) completely abol-
ished transcription (Table 4). Thus, essential promoter ele-
ments are present upstream of nucleotide −52, and sequences
sufficient for promoter activity are present downstream of nu-
cleotide −117.

To determine if the transcription of the *hfaA* gene is con-
trolled by a σ[54] promoter, we studied its expression in an *rpoN*
null mutant. When the pRJ38 *hfaA-lacZ* fusion was introduced
into SU213, a strain that lacks σ[54], it produced 3,400 Miller

TABLE 4. Expression of mutant *hfaA* promoters in wild-type and
rpoN::Tn5 backgrounds

Plasmid	Nucleotide range[a]	β-Galactosidase activity[b] in:	
		Wild type	*rpoN*::Tn5
pRJ38	−197 to +125	2,500	3,220
pRJ52	−117 to +125	1,090	1,540
pRJ54	−52 to +125	90	110

[a] The numbers represent the endpoints of the fragments cloned upstream of
lacZ, relative to the *hfaA* transcription start site, which has been designated +1.
[b] All results are expressed in Miller units and have a standard deviation of less
than 10%.

A

pRJ38 (■)

YB1371 (△)

plasmid

B

C

minutes   0 15 30 45 60 75 90 105 120 135 150
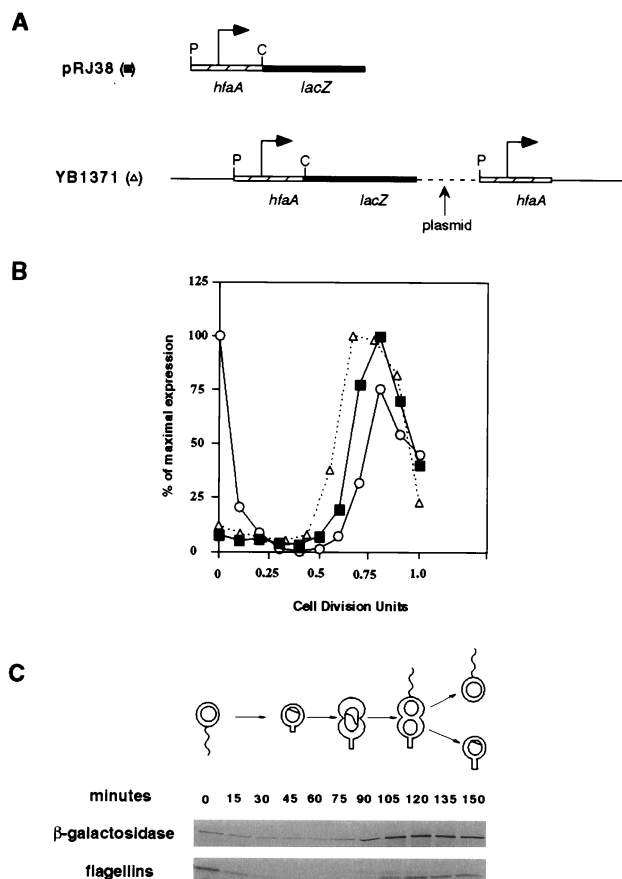
β-galactosidase

flagellins

FIG. 3. Cell cycle expression of the *hfaA* promoter. (A) The construct pRJ38, containing the *hfaA* promoter fused to a promoterless *lacZ* gene, is shown, and the relevant restriction sites are indicated (P, *Pst*I; C, *Cla*I). The bent arrow indicates the position of the promoter. A diagram of the integrated *hfaA-lacZ* transcriptional fusion in YB1371 is also shown. The hatched box represents *hfaA*, and the black box represents *lacZ*. The plasmid sequences are represented by dashed lines, while the bent arrow indicates the location of the promoter driving *hfaA*. Integration of the fusion plasmid restores a wild-type *hfaA* gene downstream of the promoter. (B) Graph of the cell cycle expression of plasmid-borne *hfaA* (■) and the 25-kDa flagellin protein (○), compared to the temporal transcription of chromosomal *hfaA* (△). The gel corresponding to the autoradiographs shown in panel C was quantitated by phosphorimaging, and the intensities of the bands were plotted as the percentages of the maximal intensity for the band corresponding to each protein. The time of division was 150 min, which is represented as 1 cell division unit. Each synchrony experiment was repeated twice with similar results. (C) Immunoprecipitation of β-galactosidase and flagellins from the strain containing pRJ38 throughout the cell cycle. The progression through the cell cycle is shown above the autoradiograph.

units of β-galactosidase activity (Table 3). Similarly, transcription of the pRJ52 fusion was approximately 1.5-fold higher in the *rpoN* mutant. These results demonstrate that σ⁵⁴ is not required for promoter activity in the 325-bp fragment of *hfaA*. The increase in β-galactosidase activity of the *hfaA-lacZ* fusion in the *rpoN*::Tn*5* mutant suggests that σ⁵⁴ or a σ⁵⁴-dependent gene has a negative effect on *hfaA* transcription. The same effect of an *rpoN* mutation on *hfaA* transcription was observed with an *hfaA-lacZ* transcriptional fusion integrated at the *hfaA* locus. The structure of the integrations is shown in Fig. 3A. The chromosomal *hfaA-lacZ* fusion produced 1,230 Miller units of β-galactosidase activity in CB15 (YB1369 [Table 3]) and 2,350 Miller units in the *rpoN*::Tn*5* mutant (YB1370 [Table 3]).

We determined that the effect of the *rpoN*::Tn*5* mutation on *hfaA* transcription was not due to polar effects on downstream

genes. The transcription of the *hfaA-lacZ* fusion was assayed in ORF208, ORF203, and ORF159 mutants (14). In both the ORF208 and ORF203 mutant backgrounds, the expression of *hfaA* was comparable to that in wild-type cells, whereas in the ORF159 mutant, *hfaA* expression decreased by 30% (Table 3). This result is similar to what is observed with the expression of the σ⁵⁴-dependent flagellar gene *fljK* in these mutant backgrounds (14). Since the expression of *hfaA* is not increased in any of these mutant backgrounds, the open reading frames downstream of *rpoN* do not play a role in the σ⁵⁴-mediated repression of the *hfaA* promoter. These results indicate that *rpoN* acts genetically like a negative regulator of *hfaA* transcription.

Cell cycle and compartment-specific expression of *hfaA*. We used a transcriptional fusion of the *hfaA* promoter to *lacZ* to analyze the cell cycle transcription of *hfaA* (Fig. 3). Swarmer cells were isolated by Ludox density gradient centrifugation from a mixed culture of NA1000 cells harboring pRJ38. These swarmer cells were allowed to proceed synchronously through the cell cycle. At 15-min intervals, samples of the culture were pulse-labeled with [³⁵S]methionine and cell extracts were immunoprecipitated with antiflagellin and anti-β-galactosidase antibodies. Figure 3C shows the results of this immunoprecipitation after the samples had been subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The synthesis of β-galactosidase was at a low level for the first half of the cell cycle, after which it increased approximately 10-fold (Fig. 3B). The peak level of expression from the *hfaA* promoter occurred at the predivisional cell stage coincident with the increase in flagellin expression. The expression from this promoter then decreased as the cells divided. This demonstrates that the transcription of *hfaA* is temporally controlled in *C. crescentus*, with the maximal level of expression occurring in the predivisional cells.

We also analyzed the temporal control of *hfaA* transcription in the NA1000::*hfaA-lacZ* integration strain, YB1371. In this strain, the *lacZ* gene is transcribed from the *hfaA* promoter in the 325-bp fragment and any upstream promoter(s) (Fig. 3A). The transcription of *hfaA* was assayed in synchronized cells as described above. As indicated in Fig. 3B, the cell cycle expression of *hfaA* is similar whether it is measured by using a plasmid-borne or a chromosomal fusion.

The swarmer and stalked compartments of the predivisional cell differ not only in morphology but also in their programs of gene expression. Because a holdfast is already present at the stalked pole, *hfaA* expression may not be required in the stalked compartment of the predivisional cell. To determine in which pole of the predivisional cell *hfaA* is transcribed, NA1000 cells containing the *hfaA-lacZ* fusion were synchronized, and the swarmer cells were allowed to proceed to the late-predivisional cell stage. At this stage, proteins were pulse-labeled for 10 min with [³⁵S]methionine and then chased with an excess of nonradioactive methionine (Fig. 4). The cells were allowed to divide, and progeny cells were isolated. Cell extracts were immunoprecipitated with antiflagellin and anti-β-galactosidase antibodies and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. In this experiment, the amounts of labeled β-galactosidase in the swarmer and stalked cell fractions reflect the rate of transcription of *hfaA* from its promoter in the swarmer or stalked compartment of the predivisional cell (11). As seen in Fig. 4, the transcription of *hfaA* occurred preferentially in the swarmer pole of the predivisional cell.

The holdfast is not detectable in swarmer cells. The ability of the swarmer poles of predivisional cells to attach to surfaces has suggested that the holdfast first appears at the base of
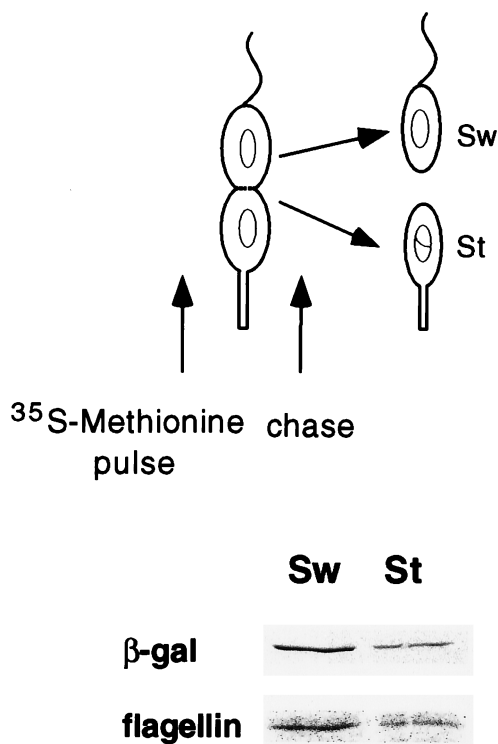
FIG. 4. Cell type-specific expression of *hfaA*. The transcriptional fusion containing the *hfaA* promoter region (pRJ38) is shown in Fig. 3A. Cells containing pRJ38 were synchronized, and the swarmer cells were allowed to proceed to the late-predivisional stage (135 min). Proteins were labeled with [^{35}S]methionine for 10 min and then chased with unlabeled methionine as shown. After division, the stalked (St) and swarmer (Sw) cells were separated by density gradient centrifugation and then processed as shown in Fig. 3. The autoradiograph of immunoprecipitated β-galactosidase and flagellin proteins is shown. This experiment was repeated twice with reproducible results.

the flagellum at the swarmer pole of the predivisional cell (25, 26). To determine if the holdfast is present at the swarmer pole, we used FITC-lectin to label the holdfast in various strains. Approximately 80% of the predivisional cells were labeled in the *C. crescentus* strains CB15 (Fig. 1A and B) and CB2A (data not shown), with labeling always occurring at the stalked pole (Table 2). Thus, lectin binds very efficiently to the stalked pole of the predivisional cell. In hundreds of predivisional cells examined in many cell cultures, we never observed FITC-lectin labeling at the swarmer pole of the predivisional cell (Fig. 1). In addition, we never observed FITC-lectin labeling of swarmer cells (Fig. 1) but readily observed labeling at the tips of stalked cells possessing short stalks. To eliminate the possibility that the fluorescein-conjugated lectin could not bind a putative holdfast at the flagellated pole of a predivisional cell because of the flagellum, we labeled the holdfast in a flagellar mutant (*flgH*::Tn5 [Table 2 and Fig. 1F]). A total of 79% of the predivisional cells were labeled in the *flgH*::Tn5 strain, and the FITC-conjugated lectin spots were always associated with the stalked poles of these predivisional cells. No labeling was observed in swarmer cells or at the flagellated poles of predivisional cells (Fig. 1), but labeling was readily observed at the tips of short stalks. All these observations are consistent with electron micrographs, in which the holdfast is clearly visible as an amorphous material at the tips of stalks but not at the base of the flagellum in predivisional or swarmer cells (Fig. 5). This suggests that the holdfast appears at the tips of stalks at the time of or shortly after the initiation of stalk synthesis.

DISCUSSION

The holdfast is important for the attachment of *C. crescentus* cells to surfaces; however, little is known about the regulation of holdfast synthesis and holdfast attachment genes during the cell cycle. Furthermore, the time of holdfast appearance at the pole of the cell is not known. In this paper, we investigate two aspects of holdfast synthesis: the transcription of a holdfast attachment gene, *hfaA*, and the appearance of the holdfast at the pole of the cell. We demonstrate that *hfaA* transcription is temporally controlled during the cell cycle, with maximal transcription in the swarmer pole of the predivisional cell. We show that the holdfast is not present in swarmer cells or at the swarmer poles of predivisional cells. Our results suggest that the holdfast appears during the differentiation of swarmer to stalked cells.

The *hfaA* gene possesses a σ^{54} promoter sequence at the requisite distance from the transcription start site (16). However, our results indicate that *hfaA* is not transcribed by a σ^{54}-dependent promoter. *hfaA* is still transcribed in a σ^{54} null mutant, and lectin labeling of the holdfast in an *rpoN*::Tn5 mutant demonstrates that σ^{54} is not required for holdfast attachment. It is possible that *hfaA* is transcribed by the σ^{54}-RNA polymerase holoenzyme under certain conditions that are as yet unknown. Surprisingly, our data indicates that σ^{54} or a σ^{54}-dependent event has a negative effect on the transcription of *hfaA*. The ability of σ^{54} to bind to certain promoters in the absence of core polymerase (7) raises the possibility that σ^{54} itself could bind to the sequences at nucleotides -24 and -12 of the *hfaA* promoter and repress the transcription of *hfaA*. A similar case that has recently been found is the *Bradyrhizobium japonicum fixRnifA* promoter that contains two overlapping promoters: one that is σ^{54} dependent and one that is dependent on a second unidentified form of RNA polymerase (4). Alternatively, the increase in *hfaA* transcription in the *rpoN* mutant could be an indirect consequence of the pleiotropic phenotype of *rpoN* mutants. The fact that some of the *rpoN* mutant cells have a holdfast at both poles suggests that *rpoN* mutants have an increased level of holdfast synthesis (Fig. 1E).

Using an *hfaA-lacZ* fusion integrated at the *hfaA* locus, we were able to show that the transcription of *hfaA* is temporally regulated during the cell cycle, with maximal levels of transcription occurring in predivisional cells. Because the holdfast does not seem to appear until the differentiation of the swarmer cell during the next cell cycle (see below), the reason for the preferential transcription of *hfaA* in the swarmer compartment of the predivisional cell is unclear. This burst in *hfaA* transcription may serve to load *hfaA* mRNA or HfaA itself in the swarmer compartment prior to the beginning of the next cell cycle. This would ensure that the holdfast attachment protein is present in the swarmer cell, ready to be used when the swarmer cell differentiates into a stalked cell.

Previous observations that the swarmer poles of predivisional cells can attach to surfaces suggested that the holdfast first appears at the flagellated poles of predivisional cells (25, 26). Electron micrographs fail to reveal the presence of a holdfast at the base of the flagellum, whereas it is clearly visible at the tips of short stalks. In our studies, we were unable to detect any binding of fluorescent lectin to swarmer cells or to the flagellated poles of predivisional cells, whereas the binding of lectin to stalked cells and to the stalked poles of predivisional cells was very efficient. Our failure to detect any binding
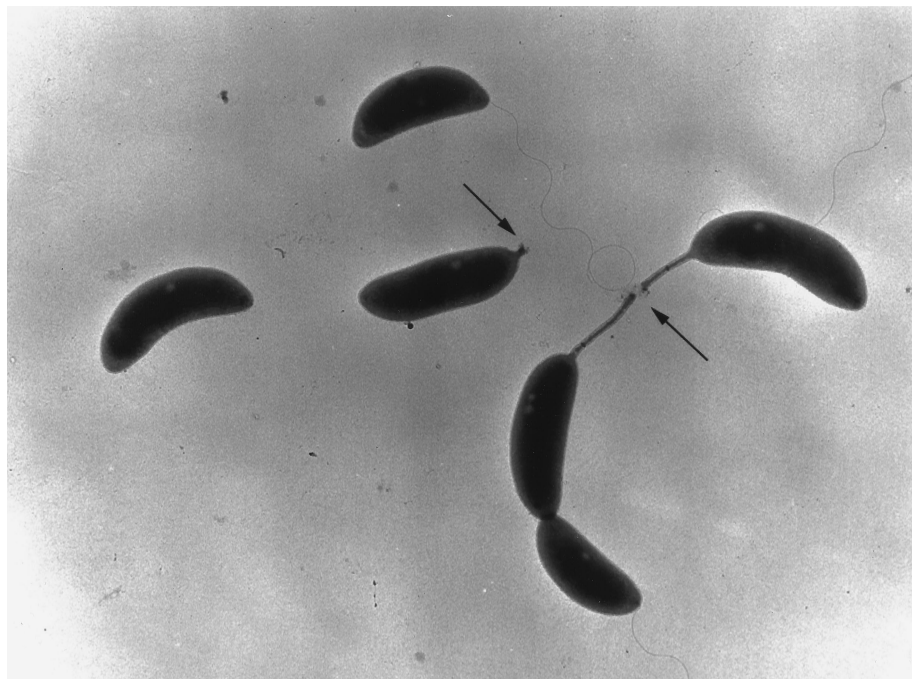
FIG. 5. The holdfast is visible after the initiation of stalk synthesis in this transmission electron micrograph of wild-type *C. crescentus* (CB15) grown in PYE medium. The holdfast material is indicated by arrows and is visible as amorphous material at the tips of stalks.

of fluorescent lectin at the swarmer pole is not due to the presence of the flagellum, because we could not detect any binding to the swarmer pole in a flagellar mutant. These results suggest that the exposure of the holdfast to the outside of the cell occurs during the differentiation of swarmer to stalked cells. In addition to a single flagellum, the swarmer pole contains pili (30). Pili are involved in mediating attachment to surfaces in many bacteria (13) and have also been implicated in promoting the primary adhesion event in *Hyphomonas*, another prosthecate bacterium (27). Thus, perhaps the attachment of the swarmer pole of *C. crescentus* to surfaces is mediated by the pilus and not the holdfast. This is consistent with the observation that swarmer cells collide and stick more frequently to glass surfaces than nonmotile stalked and dividing cells (24). Based on our findings, we suggest that the holdfast is either not present or not accessible at the swarmer poles of predivisional cells and that other adhesive components of that pole, perhaps pili, facilitate its attachment to surfaces.

ACKNOWLEDGMENTS

REFERENCES

1. **Alley, M. R. K., S. L. Gomes, W. Alexander, and L. Shapiro.** 1991. Genetic analysis of a temporally transcribed chemotaxis gene cluster in *Caulobacter crescentus*. Genetics **129:**333–342.
2. **Altin-Mees, M. A., and J. M. Short.** 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. **17:**9494.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley/Greene, New York, N.Y.
4. **Barrios, H., R. Grande, L. Olvera, and E. Morett.** 1998. In vivo footprinting analysis reveals that the complex *Bradyrhizobium japonicum fixRnifA* promoter region is differently occupied by two distinct RNA polymerase holoenzymes. Proc. Natl. Acad. Sci. USA **95:**1014–1019.
5. **Brun, Y., G. Marczynski, and L. Shapiro.** 1994. The expression of asymmetry during cell differentiation. Annu. Rev. Biochem. **63:**419–450.
6. **Brun, Y. V., and L. Shapiro.** 1992. A temporally controlled sigma factor is required for cell-cycle dependent polar morphogenesis in *Caulobacter*. Genes Dev. **6:**2395–2408.
7. **Buck, M., and W. Cannon.** 1992. Specific binding of the transcription factor sigma-54 to promoter DNA. Nature **358:**422–424.
8. **Devereux, D., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Ely, B., and T. W. Ely.** 1989. Use of pulsed field gel electrophoresis and transposon mutagenesis to estimate the minimal number of genes required for motility in *Caulobacter crescentus*. Genetics **123:**649–654.
10. **Evinger, M., and N. Agabian.** 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. J. Bacteriol. **132:**294–301.
11. **Gober, J. W., R. Champer, S. Reuter, and L. Shapiro.** 1991. Expression of positional information during cell differentiation in *Caulobacter*. Cell **64:**381–391.
12. **Gober, J. W., and L. Shapiro.** 1992. A developmentally regulated *Caulobacter* flagellar promoter is activated by 3′ enhancer and IHF binding elements. Mol. Biol. Cell **3:**913–926.
13. **Hultgren, S. J., S. Abraham, M. Caparon, P. Falk, J. St. Geme III, and S. Normark.** 1993. Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. Cell **73:**887–901.
14. **Janakiraman, R. S., and Y. V. Brun.** 1997. Transcriptional and mutational analyses of the *rpoN* operon in *Caulobacter crescentus*. J. Bacteriol. **179:**5138–5147.
15. **Johnson, R. C., and B. Ely.** 1977. Isolation of spontaneously derived mutants of *Caulobacter crescentus*. Genetics **86:**25–32.
16. **Kurtz, H. D., Jr., and J. Smit.** 1992. Analysis of a *Caulobacter crescentus* gene cluster involved in attachment of the holdfast to the cell. J. Bacteriol. **174:**687–694.
17. **Kurtz, H. D., Jr., and J. Smit.** 1994. The *Caulobacter crescentus* holdfast: identification of holdfast attachment complex genes. FEMS Microbiol. Lett. **116:**175–182.
18. **Liss, L. R.** 1987. New M13 host: DH5αF′ competent cells. Focus **9:**3–13.
19. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32:**481–485.
20. **Merker, R. I., and J. Smit.** 1988. Characterization of the adhesive holdfast of marine and freshwater caulobacters. Appl. Environ. Microbiol. **54:**2078–2085.
21. **Merrick, M. J.** 1993. In a class of its own—the RNA polymerase sigma factor

sigma 54 (sigma N). Mol. Microbiol. **10:**903–909.
22. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Mitchell, D., and J. Smit.** 1990. Identification of genes affecting production of the adhesion organelle of *Caulobacter crescentus* CB2. J. Bacteriol. **172:** 5425–5431.
24. **Newton, A.** 1972. Role of transcription in the temporal control of development in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **69:**447–451.
25. **Poindexter, J. S.** 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28:**231–295.
26. **Poindexter, J. S.** 1981. The caulobacters: ubiquitous unusual bacteria. Microbiol. Rev. **45:**123–179.
27. **Quintero, E. J., K. Busch, and R. M. Weiner.** 1998. Spatial and temporal deposition of adhesive extracellular polysaccharide capsule and fimbriae by *Hyphomonas* strain MHS-3. Appl. Environ. Microbiol. **64:**1246–1255.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Simon, R., U. Prieffer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1:**784–790.
30. **Smit, J.** 1987. Localizing the subunit pool for the temporally regulated polar pili of *Caulobacter crescentus*. J. Cell Biol. **105:**1821–1828.
31. **Smit, J., and N. Agabian.** 1984. Cloning of the major protein of the *Caulobacter crescentus* periodic surface layer: detection and characterization of the cloned peptide by protein expression assays. J. Bacteriol. **160:**1137–1145.
32. **Stahl, D. A., R. Key, B. Flesher, and J. Smit.** 1992. The phylogeny of marine and freshwater caulobacters reflects their habitat. J. Bacteriol. **174:**2193–2198.
33. **Umbreit, T. H., and J. L. Pate.** 1978. Characterization of the holdfast region of wild-type cells and holdfast mutants of *Asticcacaulis biprosthecum*. Arch. Microbiol. **118:**157–168.
34. **Wingrove, J. A., E. K. Mangan, and J. W. Gober.** 1993. Spatial and temporal phosphorylation of a transcriptional activator regulates pole-specific gene expression in *Caulobacter*. Genes Dev. **7:**1979–1992.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.