New members of the *ctrA* regulon: the major chemotaxis operon in *Caulobacter* is CtrA dependent

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The *Caulobacter crescentus* che promoter region consists of two divergent promoters, directing expression of the major chemotaxis operon and a novel gene *cagA* (chemotaxis associated gene A). Analyses of start sites by primer extension and alignment of the divergent promoters revealed significant similarities between them at the –35 promoter region. Both *mcpA* and *cagA* are differentially expressed in the cell cycle, with maximal activation of transcription in predivisional cells. The main difference between the *mcpA* and *cagA* promoters is that, in common with the *fljK* flagellin, *cagA* is expressed in swarmer cells. A *cagA-lacZ* promoter fusion that contains 36 bases of untranslated mRNA has sufficient information to segregate the *lacZ* transcript to swarmer cells. Expression of *mcpA* and *cagA* was dependent on DNA replication. Transcriptional epistasis experiments were performed to identify potential regulators in the flagellar hierarchy. The sigma factor RpoN, which is required for flagellar biogenesis, is not required for *mcpA* and *cagA* expression. Mutations in the genes for the MS-ring and the switch complex (flagellar class II mutants) do not affect expression of *mcpA* and *cagA*. However, CtrA, an essential response regulator of flagellar gene transcription, is required.

Keywords: cell cycle, *Caulobacter crescentus*, transcriptional regulation, flagella

INTRODUCTION

Caulobacter crescentus undergoes obligate asymmetric cell division to yield two dissimilar cell types, a motile swarmer cell and a sessile stalked cell. The stalked cell is competent for DNA replication and can initiate cell growth to become a predivisional cell. However, the swarmer cell, which has a single polar flagellum, must differentiate into a non-motile stalked cell before cell growth and division can occur. Upon swarmer cell differentiation the single polar flagellum is ejected and a stalk synthesized at the site where the flagellum was previously located. This new stalked cell can initiate DNA replication and grow. Progression through the cell cycle is accompanied by synthesis of a new flagellum,

which is placed at the cell pole opposite the stalk in the predivisional cell.

Epistatic analyses of flagellar mutants in *C. crescentus* have grouped the flagellar genes into four classes (Wu & Newton, 1997). The essential response regulator *ctrA*, classified as a class I gene, controls class II genes, which include the genes for the switch complex, the MS-ring, the RpoN-dependent response regulator *flbD* (Ramakrishnan & Newton, 1990) and its cognate kinase *flbE* (Wingrove & Gober, 1996). Class III genes include the genes for the hook, the rod and the rings (L, P, E), which require *flbD* and the alternate sigma factor σ^{54} (*rpoN*) for transcription (Brun & Shapiro, 1992; Dingwall *et al.*, 1992a). The class III genes also require the completion of the MS-ring switch complex for expression (Newton *et al.*, 1989; Xu *et al.*, 1989). The major flagellins *fljK* and *fljL*, which are class IV genes, require in addition to *rpoN* and *flbD* (Brun & Shapiro, 1992; Mullin & Newton, 1993; Ninfa *et al.*, 1989) the completion of the hook–basal body complex for expression (Mangan *et al.*, 1999). At present it is unclear

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Abbreviation: HU, hydroxyurea.

Fig. 1. The major chemotaxis operon of *C. crescentus*. Above the restriction map, boxes denote coding sequences for the respective genes. The boxes for the *mcpA* and *cagA* genes are black. The black bars below the restriction map show the extent of the deletions. The *borA* gene encodes a putative homologue of a TonB-dependent outer membrane receptor protein. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; D, *Dra*I; H, *Hind*III; N, *Not*I; Sp, *Sph*I; S, *Sac*I. The bold letters E and B in the restriction map denote the 339 bp *Eco*RI–*Bam*HI fragment containing both *mcpA* and *cagA* promoters.

how the three flagellin genes in the beta cluster (*fljMNO*) are regulated as they do not have consensus σ^{54} -binding motifs in their promoters, although their expression has a partial requirement for *rpoN* (Ely *et al.*, 2000). Regulation of the remaining flagellin gene, *fljJ*, and the chemotaxis genes remains to be characterized.

The major chemotaxis operon of *C. crescentus*, shown schematically in Fig. 1, was identified using classical bacterial genetics; *che* mutants were isolated by transposon mutagenesis (Ely *et al.*, 1986) and gene fusions were constructed to identify promoter elements (Alley *et al.*, 1991). We have shown that this operon contains one chemoreceptor gene designated *mcpA* (Alley *et al.*, 1992) and 11 other chemotaxis genes (Tsai & Alley, 2000). The genome sequence of *C. crescentus* (Niernan *et al.*, 2001) is complete and the 4 Mb genome accommodates numerous chemotaxis genes including 18 putative chemoreceptor homologues, seven of which have recently been shown to be cell-cycle regulated (Laub *et al.*, 2000). The large number of chemotaxis genes in *C. crescentus* makes their regulation an attractive system to study, and by investigating chemotaxis gene regulation we hope to gain an insight into regulatory networks that integrate cell-cycle cues with chemotactic signals.

METHODS

Bacterial strains, plasmids and media. The strains and plasmids that were used in this study are listed in Table 1. *C. crescentus* was routinely grown in PYE complex medium (Ely, 1991) or M2G minimal medium (Ely, 1991) supplemented where appropriate with sucrose (3%), tetracycline (1 µg ml⁻¹), naladixic acid (20 µg ml⁻¹), kanamycin (5 µg ml⁻¹) and xylose (0.2% w/v). *Escherichia coli* strains were routinely grown in TB (Sambrook *et al.*, 1989) for preparation of plasmids, or SOB (Sambrook *et al.*, 1989) for preparation of electro-competent cells. Plasmids were mobilized into *C. crescentus* from *E. coli* S17-1 by biparental conjugation (Ely, 1991). The Tn5 insertions in strains SC1032, SC1042, SC1055, SC1107, SC1117, SC1131, SC1132, SC1133, SC1138 (Ely & Ely, 1989), PC5001 and PC5506 (Ohta *et al.*, 1984) were transduced (Ely, 1991) into strain MRKA208.

Primer extension. Two primers were utilized to identify the transcriptional start site for *cagA*, CAGA1 (5'-GATCGGA-TATTTTCATCCCGCT-3'), and CAGA2 (5'-TGCTCCTAG TAATGAATTCAGG-3') from Pharmacia Biotech. For *mcpA* a single primer was utilized, MCPA1 (5'-GGATCCGTTTC-

ATCGCAAGG-3') from Pharmacia Biotech. Total RNA was isolated from late-exponential-phase wild-type cells (MRKA208) by hot phenol/SDS lysis (Salser *et al.*, 1967). Primers were end-labelled with T4 polynucleotide kinase (Boehringer Mannheim) according to the manufacturers' instructions. Primer annealing to RNA (10 µg total RNA, 200 fmol labelled primer) was carried out in hybridization buffer (40 mM PIPES, 1 mM EDTA, 0.4 M NaCl, 80% formamide) at 30 °C overnight. Annealed primer/template mix was recovered by precipitation with ethanol. Extension with AMV reverse transcriptase (Promega) was performed as specified by the manufacturers at 42 °C for 1 h. Reaction products were analysed by denaturing polyacrylamide electrophoresis on 6% polyacrylamide gels. Extension products were run alongside sequencing reactions carried out with the same primers.

Assays of promoter activity. The β -galactosidase activity of the promoter *lacZ* fusion constructs was assayed (Miller, 1972) in various genetic backgrounds from cultures grown at 28 °C to a mean OD₆₀₀ of 0.8. Five independent experiments were performed for each promoter fusion in every genetic background shown. In the CtrA depletion experiments, the activity of the promoter fusions was examined in three independent experiments. All β -galactosidase assays were carried out in duplicate.

Synchronization and immunoprecipitation. Pure populations of swarmer cells were isolated by Percoll (Pharmacia) density centrifugation from the strain MRKA779, which has the β -lactamase gene disrupted by Tn5. Cells were concentrated 10-fold by centrifugation at 2500 g for 10 min and resuspended in 10 mM phosphate buffer (6.1 mM Na₂HPO₄, 3.9 mM KH₂PO₄ pH 7.0) mixed with an equal volume of Percoll (Pharmacia) and centrifuged (10000 g, 20 min, 23 °C). Swarmer cells were washed twice with 10 mM phosphate buffer, then resuspended to OD₆₀₀ 0.4 in M2G and allowed to proceed synchronously through the cell cycle. Samples were taken every 20 min, with a mean cell cycle length of 180 min. Where progeny cells were examined, synchronous predivisional cells were allowed to divide. Following cell division, progeny swarmer and stalked cells were separated by Percoll gradient centrifugation as described before. Cell samples were lysed and prepared for immunoprecipitation essentially as described by Quon *et al.* (1996).

Inhibition of DNA synthesis. MRKA779 cultures harbouring plasmids reporting *mcpA* and *cagA* expression were treated with hydroxyurea at 3 mg ml⁻¹ (Osley *et al.*, 1977). Samples were withdrawn at 10 min intervals and immunoprecipitations carried out as described before, using an antibody specific for β -galactosidase (CP laboratories).

Table 1. Strains and plasmids

Strain	Genotype, relevant characteristics	Reference
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr</i> – <i>hsdRMS</i> – <i>mcrBC</i>) Δ <i>lacX74</i> ϕ80 <i>dlacZ</i> Δ <i>M15</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ139 <i>rpsL</i>	GibcoBRL
S17-1	M294::RP4 2 (Tc::Mu) (Km::Tn7)	Simon <i>et al.</i> (1983)
<i>C. crescentus</i>		
MRKA208	CB15N Δ <i>bla6</i> , synchronizable derivative of CB15	M. R. K. Alley
MRKA348	CB15N Δ <i>bla6</i> <i>fliM</i> 196::Tn5	This study
MRKA413	CB15N Δ <i>bla6</i> <i>flbD</i> 198::Tn5	This study
MRKA414	CB15N Δ <i>bla6</i> <i>fliF</i> 601::Tn5	This study
MRKA442	CB15N Δ <i>bla6</i> <i>flgH</i> 194::Tn5	This study
MRKA446	CB15N Δ <i>bla6</i> <i>flhA</i> 608::Tn5	This study
MRKA520	CB15N <i>ctrA</i> Ω <i>aadA1</i> <i>xylXp</i> :: <i>ctrA</i>	Quon <i>et al.</i> (1996)
MRKA590	CB15N Δ <i>bla6</i> <i>rpoN</i> 610::Tn5	This study
MRKA642	CB15N Δ <i>bla6</i> <i>flgK</i> 311::Tn5	This study
MRKA779	CB15N <i>bla</i> 101::Tn5	This study
MRKA667	CB15N Δ <i>bla6</i> <i>flbG</i> 602::Tn5, first gene in <i>flgE</i> operon	This study
MRKA674	CB15N Δ <i>bla6</i> Δ(<i>mcpA</i> – <i>cheD</i>) <i>che10</i>	This study
MRKA773	CB15N Δ <i>bla6</i> Δ(<i>cheE</i> – <i>borA</i>) <i>che49</i> 1ΩCm ^r	This study
Plasmids		
pRSZ6	Broad-host-range bidirectional <i>gusA</i> , <i>lacZ</i> promoter fusion vector	M. R. K. Alley
pCH04	339 bp <i>EcoRI</i> – <i>Bam</i> HI containing the <i>che</i> promoter in pIC20H	This study
pRCH04	339 bp <i>EcoRI</i> – <i>Bam</i> HI fragment from pCH04 into the <i>EcoRI</i> site of pRSZ6 (<i>cagA</i> – <i>lacZ</i>)	This study
pCS141	<i>mcpA</i> – <i>lacZ</i> in pLAC290	C. Stephens* (unpublished)
pCS44	<i>fliL</i> – <i>lacZ</i> in pLAC290	Stephens & Shapiro (1993)
pLAC290	pRK290 containing a promoterless <i>lacZ</i> gene	Gober & Shapiro (1992)
pCS139	<i>tacp</i> – <i>lacZ</i> in pLAC290	C. Stephens* (unpublished)
pWZ162	<i>fliQ</i> – <i>lacZ</i> in pLAC290	Zhuang & Shapiro (1995)
pBMSK6	5.8 kb <i>Bam</i> HI fragment containing <i>cagA</i> into pBluescript SKII+	This study

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RESULTS

Transcriptional start sites of *mcpA* and *cagA*

The transcriptional start site for *mcpA* was determined by primer extension using a primer (MCPA1) which was complementary to the coding strand 12 bp downstream of the ATG translational start codon (Fig. 2a). Three major extension products were observed, which is in strong agreement with the data obtained from S1-nuclease mapping (data not shown). The –10 region (Fig. 2c) was unlike that defined for *C. crescentus* biosynthetic promoters, GCTANAWC, which has been shown to be used by the RpoD RNA polymerase holoenzyme (Malakooti *et al.*, 1995). The *mcpA* –10 region bore some similarity (4/8 matches) to the *FliA* (σ²⁸) –10 consensus sequence (Helmman & Chamberlin, 1988) but the –35 region was even more divergent (Fig. 2c). No similarity to the consensus –35 sequence for biosynthetic promoters was observed (Malakooti *et al.*, 1995). A putative CtrA-binding site was detected, but

only 5 out of 9 residues matched the proposed consensus sequence, TTAA-N₇-TTAAC (Marczynski *et al.*, 1995; Quon *et al.*, 1996). Neither half-site was completely conserved with the consensus motif.

In the process of constructing *lacZ* fusions to the *mcpA* promoter we identified an additional promoter. Transcription was divergent from the *mcpA* promoter and the transcribed gene encoded a predicted ORF of 311 bp (Fig. 1). We have designated this ORF *cagA*, for chemotaxis associated gene *A*, because it is located between two chemoreceptors (Fig. 1). No homologues of *CagA* were identified using BLAST, PSI-BLAST or FASTA3 analyses and no observable phenotype was associated with a knock-out mutant. However, the proximity of *cagA* to the major chemotaxis operon might indicate some level of co-regulation. In order to test this hypothesis we began by determining the transcriptional start site for *cagA*. Two separate experiments utilizing different primers (CAGA1, CAGA2) confirmed the start site of *cagA* to be 57 bp upstream of the deduced

Fig. 2. (a, b) Primer extension to determine transcriptional start sites. Start sites were determined by primer extension and products were analysed by denaturing PAGE alongside a sequencing ladder derived from the same primer. The arrows indicate the start sites for (a) *mcpA* and (b) *cagA*. (c) DNA sequence of the promoter region (GenBank accession no. X66502). Start sites for *cagA* and *mcpA* are boxed; the black boxes indicate putative CtrA-binding motifs. Dots indicate residues identical to the CtrA consensus motif. Underlined sequences are putative FliA (σ^{28})-binding motifs. Dotted arrows represent a direct repeat. Numbers along the DNA sequence indicate spacing from the respective start sites. (d) Alignment of the *cagA* and *mcpA* promoters. The numbers indicate the spacing from the start of transcription. Residues in bold are conserved between the *mcpA* and *cagA* promoters.

translation initiation codon (Fig. 2b, c). Analysis of the upstream sequence (Fig. 2c) indicated a poor match to the -10 consensus sequence for the housekeeping sigma factor (Malakooti *et al.*, 1995). A good match to the consensus binding motif for CtrA was identified, with 7

out of 9 residues conserved with the established consensus (Fig. 2c). In this instance the downstream half-site (nearest to the transcriptional start site) is completely conserved. When the *mcpA* and *cagA* promoters are aligned according to their transcriptional start sites

Fig. 3. Cell cycle transcription of *cagA*. Strain MRKA779 harbouring a plasmid-borne *cagA-lacZ* transcriptional fusion was synchronized and allowed to proceed through the cell cycle. After SDS-PAGE, immunoprecipitated labelled proteins were visualized by autoradiography. The same labelled cell extracts were immunoprecipitated with antisera to flagellin, McpA and CtrA as appropriate controls. Time points (min) correlate with stages of the cell cycle as illustrated by the drawings above the panels. Cell cycle stages were monitored by light microscopy.

(Fig. 2d) we noticed that there was a stretch of identical residues (10/13 residues) around the -35 promoter region, which might indicate a shared regulator. If *cagA* and *mcpA* share a common regulator then the *cagAp* should be cell cycle regulated.

Transcription of *mcpA* and *cagA* is temporally regulated

To test this hypothesis we investigated the timing of transcription of the *cagA* gene using a promoterless *lacZ* gene fused to the *cagA* promoter (pRCH04). Cells harbouring pRCH04 were synchronized as described in Methods. Swarmer cells were allowed to proceed synchronously through the cell cycle and were pulse-labelled with [35 S]methionine at various times during the cell cycle. As controls we used antisera to flagellin and the global regulator CtrA. The cell cycle expression of the flagellins showed the same pattern as seen by Osley *et al.* (1977), with maximal flagellin gene transcription in late predivisional cells. The global regulator CtrA was transcribed before *cagA* and *mcpA* (Fig. 3). Expression of *mcpA* and the *cagA-lacZ* transcriptional fusion was maximal at 120 min (Fig. 3). The main difference between *mcpA* and *cagA* expression was that in swarmer cells there was significant synthesis of LacZ from the *cagA-lacZ* promoter fusion. Synthesis of the 25 kDa flagellin (FljK) also occurred in the swarmer cell population. Synthesis of the 25 kDa flagellin in the swarmer cell is due to segregation of the *flgK* mRNA to the swarmer cell following cell division (Milhausen & Agabian, 1983). To determine if a similar mechanism applied to *cagA*, we tested the *cagA-lacZ* promoter fusion, which has 36 bases of untranslated *cagA* mRNA, in a segregation experiment.

The *cagA* transcript is targeted to the swarmer cell

Cells harbouring the plasmid pRCH04 were synchronized as described previously, and swarmer cells were allowed to proceed through the cell cycle. At the late predivisional stage, cells were pulse-labelled with

Fig. 4. Segregation of *cagA* transcript following cell division. MRKA779 harbouring pRCH04 (*cagA-lacZ*) was synchronized, allowed to proceed through the cell cycle, and after pulse-labelling for 10 min with [35 S]methionine at the predivisional stage (140 min), cells were allowed to divide. Swarmer and stalked cell populations were separated by Percoll density centrifugation. After SDS-PAGE, labelled immunoprecipitated proteins were visualized by phosphorimager analysis. Flagellin and NptII were included as positive and negative controls respectively. ST, stalked cell fraction; SW, swarmer cell fraction.

[35 S]methionine for 10 min, then chased with an excess of non-radioactive methionine. Following cell division, swarmer and stalked cells were separated by Percoll density-gradient centrifugation. Labelled proteins were immunoprecipitated and localization of proteins was determined. Fig. 4 shows that most of the labelled *cagAp*-directed β -galactosidase was specifically targeted to the swarmer cells, suggesting that the presence of *cagA* mRNA in swarmer cells is due to specific targeting of the transcript. NptII was used as a non-segregated control and as expected was equally distributed between swarmer and stalked cell fractions (Fig. 4).

DNA replication is required for *mcpA* and *cagA* transcription

Cell division and flagellum biogenesis are dependent on completion of chromosome replication (Osley & Newton, 1980; Osley *et al.*, 1977). Additionally class II flagellar gene promoters *fliQp*, *fliFp* and *fliLp* are

Table 2. Effect of inhibition of DNA synthesis on promoter activity

Exponentially growing cultures of MRKA779 harbouring pRCH04 (*cagA-lacZ*) or pCS141 (*mcpA-lacZ*) were treated with HU at time zero. Equivalent counts from each sample were immunoprecipitated with antibodies to β -galactosidase, NptII or flagellin. Immunoprecipitated samples from each time-point after HU addition were analysed by SDS-PAGE and labelled proteins were visualized by phosphorimager analysis. Quantitation was with IPLab Gel software. Expression levels for each time point are shown as a percentage relative to the maximum intensity observed at time zero (HU addition).

Time (min)	<i>mcpA</i>	<i>cagA</i>	<i>nptII</i>	25 kDa flagellin
0	100	100	100	100
10	24.3	51	82	50.8
20	14.49	28	76	47
30	13.6	33	82	35
40	5	25.8	70	37
50	7.6	26	77	35
60	3.5	16	70	18
70	2.3	7.6	68	5.7

Table 3. Effect of class II and class III flagellar mutants on the promoter activity of *mcpAp* and *cagAp*

Plasmids pCS141 and pRCH04, which report the transcriptional activity of *mcpA* and *cagA*, were introduced into various class II and class III flagellar mutants and β -galactosidase activity was measured according to Miller (1972). Experiments were carried out five times, with duplicate assays each time. These data are mean values and are presented as a percentage compared to wild-type. The standard deviations ranged from 5 to 15%.

	Flagellar mutant	<i>mcpA-lacZ</i> (%)	<i>cagA-lacZ</i> (%)
Class II	Wild-type	100	100
	<i>flhF</i>	205	142
	<i>flhM</i>	206	140
	<i>flhA</i>	197	129
	<i>flhD</i>	169	155
	<i>rpoN</i>	226	154
Class III	<i>flbGflgDE</i>	158	141
	<i>flgH</i>	150	130
	<i>flgKL</i>	159	111
Chemotaxis	$\Delta che10$	153	161
	$\Delta che491$	153	129

sensitive to an interruption in DNA replication (Dingwall *et al.*, 1992b). Like the class II flagellar genes, *mcpA* and *cagA* are expressed differentially during the cell cycle; therefore it is important to ascertain whether replication of the chromosome is a prerequisite for transcription. Transcription of *mcpA* and *cagA* was

measured using plasmid-borne transcriptional fusions of *mcpA* and *cagA* to a promoterless β -galactosidase gene (pCS141, pRCH04). The DNA replication inhibitor hydroxyurea (HU) was added to exponentially growing cultures of wild-type cells harbouring pCS141 and pRCH04. Cells were pulse-labelled at 10 min intervals after addition of HU, followed by immunoprecipitation with anti- β -galactosidase antibody. Immunoprecipitation of NptII and flagellin was employed as a control. A significant decrease of 75% for *mcpA* and 50% for *cagA* was observed by 10 min post addition of HU (Table 2). In comparison, transcription of the *nptII* gene was relatively unaffected, since 70% expression levels remained 70 min post-HU addition. An 80% decrease in transcription of flagellin gene promoters was observed 60 min post addition of HU, in accordance with previous observations (Osley & Newton, 1980; Osley *et al.*, 1977). The *mcpA* and *cagA* promoters are sensitive to a block in DNA synthesis. Since the effect of inhibiting DNA replication could be mediated by the class II flagellar genes, it was essential to determine the dependence of *mcpA* and *cagA* transcription on class II gene expression. In the *E. coli* flagellar hierarchy chemotaxis genes are classified as class III genes along with flagellin gene *fliC*.

Where are the *mcpA* and *cagA* genes in the *Caulobacter* transcriptional hierarchy?

We tested the expression of the *mcpA* and *cagA* promoters fused to *lacZ* in various class II and class III flagellar mutants. Both promoters showed an increase in expression in all class II mutants tested (Table 3), which is a common feature of class II promoters. This indicated that the major chemotaxis operon has a class II promoter. This situation is very different from that found in *E. coli*, where chemotaxis gene expression requires the expression of class II genes. The *mcpA* and *cagA* promoters show elevated levels of expression in class III flagellar mutants (Table 3). This is not usually observed with the archetypal class II promoters, but it is seen with the *flhA* promoter (Newton *et al.*, 1989). To test whether any genes in the major chemotaxis operon play a part in regulation of *mcpAp* or *cagAp* we determined their levels of expression in two chemotaxis deletions (Table 3). Both chemotaxis deletions caused an increase in expression from both promoters, suggesting that there may be some level of auto-regulation.

CtrA positively regulates transcription of *mcpA* and *cagA*

CtrA is the master regulator of the flagellar gene regulon in *C. crescentus* (Quon *et al.*, 1996). This two-component response regulator acts in either a positive or negative regulatory manner to activate or repress expression of several genes involved in flagellar biogenesis, chromosome replication (Marczynski *et al.*, 1995) and lipopolysaccharide production (Leclerc *et al.*, 1998). Since *cagA* and *mcpA* expression is not abolished in class II flagellar mutants we determined whether *ctrA*

Table 4. CtrA regulation of *mcpA* and *cagA* promoters

The *ctrA* xylose-conditional strain (MRKA520) harbouring reporter plasmids pCS141 (*mcpA-lacZ*), pRCH04 (*cagA-lacZ*), pCS44 (*flhL-lacZ*), pWZ162 (*flhQ-lacZ*) or pCS139 (*tacp-lacZ*) was grown to OD₆₀₀ 0.8 in PYE plus xylose and washed to remove the inducer xylose as described in Methods. At hourly time points, samples were withdrawn and the activity of the β -galactosidase reporter gene assessed (Miller, 1972). Figures are given as percentage activity compared to the activity of the promoter at time zero. Experiments were carried out three times and the numbers are means $\pm$ SD.

Time (h)	<i>mcpA-lacZ</i>	<i>cagA-lacZ</i>	<i>tacp-lacZ</i> *	<i>flhQ-lacZ</i> †	<i>flhL-lacZ</i> †
0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
1	105 $\pm$ 7	73 $\pm$ 6	108 $\pm$ 9	97 $\pm$ 12	89 $\pm$ 4
2	90 $\pm$ 7	58 $\pm$ 4	112 $\pm$ 19	99 $\pm$ 13	99 $\pm$ 2
3	69 $\pm$ 2	42 $\pm$ 2	140 $\pm$ 12	102 $\pm$ 19	56 $\pm$ 9
4	51 $\pm$ 8	31 $\pm$ 3	88 $\pm$ 8	81 $\pm$ 5	64 $\pm$ 5
5	47 $\pm$ 1	26 $\pm$ 2	108 $\pm$ 21	66 $\pm$ 2	51 $\pm$ 2

* The *tac* promoter fused to *lacZ* was used as a control promoter that is not dependent on *ctrA*.

† The *flhQ-lacZ* and *flhL-lacZ* promoter fusions are controls for *ctrA*-dependent genes (Quon *et al.*, 1996).

was required for the expression of these genes. Because the *ctrA* gene is essential we used a strain that has *ctrA* under the control of an inducible xylose promoter (Quon *et al.*, 1996). Therefore, we can determine if CtrA regulates a promoter by removing xylose from the growth medium and measuring the change in β -galactosidase reporter gene activity. In these experiments, CtrA is not detectable in a Western blot after 2 h of xylose depletion (data not shown). Since β -galactosidase is a stable protein we are observing dilution of β -galactosidase activity in the cells. The *flhQ* and *flhL* promoters fused to a β -galactosidase reporter gene were used as controls for CtrA-dependent expression (Quon *et al.*, 1996; Wu *et al.*, 1998) and as expected the levels of β -galactosidase activity dropped when xylose was depleted (Table 4). The *tac* promoter was used as a non-*ctrA* regulated promoter (Table 4). The β -galactosidase activity expressed from the *mcpA* and *cagA* promoters started to drop within 2 h of xylose depletion (Table 4), which suggests that like *flhQp* and *flhLp*, *mcpAp* and *cagAp* are regulated by *ctrA*.

DISCUSSION

Targeting of *cagA* mRNA

The structural components of the flagellar filament, FljKMNO, are synthesized in swarmer cells. However, most of the flagellar genes, including those for the components of the basal body, switch and hook, are not expressed in swarmer cells. In the case of the FljK flagellin, mRNA is specifically targeted to the swarmer cell. The *fljK* gene is not transcribed in swarmer cells and it is the stability of the *fljK* mRNA that ensures its presence in this cell type (Milhausen & Agabian, 1983). Therefore it is unusual that *cagA* is expressed in swarmer cells. The kinetics of β -galactosidase synthesis from the *cagA-lacZ* promoter fusion after the addition of HU are

similar to those seen with the FljK flagellin (Table 3). Perhaps the most likely explanation for the apparent synthesis of CagA in swarmer cells is that it is due to mRNA stability, as demonstrated for *fljK* mRNA (Milhausen & Agabian, 1983). Like *fljK*, *cagA* transcripts are preferentially targeted to the swarmer cell upon division of the predivisional cell. Deletion analyses of the *fljK* mRNA showed that sequences 5' to the translation start codon are sufficient to impose regulation (Anderson & Newton, 1997). The *fljK* mRNA has a dramatically shorter half-life in the absence of flagellar assembly and it is proposed that FlbT mediates this by binding to the untranslated mRNA region (UTR) of *fljK* (Anderson & Gober, 2000; Mangan *et al.*, 1999). In our *cagA-lacZ* construct there are 36 bases of untranslated *cagA* mRNA. However, there is no significant homology between the UTR of *cagA* and *fljK* to suggest that *cagA* is regulated by a similar mechanism to *fljK*.

How does CtrA regulate *mcpAp* and *cagAp*?

The putative CtrA-binding motif in the *mcpA* promoter is very poor, since only 5 out of 9 residues match. The *cagA* promoter has a putative motif which matches 7 out of 9 residues found in the CtrA consensus. However, the putative CtrA-binding motif in *cagAp* is slightly closer to the start of transcription when compared to CtrA-binding sites found in most class II flagellar promoters (Fig. 5). The absence of a well-conserved CtrA motif centred around the -35 region in both promoters may suggest that the effect of CtrA on these promoters is indirect. Furthermore, alignment of the *cagA* and *mcpA* promoters (Fig. 2d) revealed significant identity in the -35 region, perhaps indicative of a binding site for a shared regulator. The primary region where there is most identity coincides with the region where the upstream consensus CtrA half-site would be positioned (compare Fig. 2d and Fig. 5). Further evidence to suggest

Fig. 5. Alignment of class II flagellar promoters with *mcpA*p and *cagA*p. Numbers indicate the spacing from the transcription start site of the promoter. Residues in bold are the consensus motif for CtrA-binding sites. The boxed nucleotides are the transcriptional start sites as published for *fliF*p (Van Way *et al.*, 1993), *fliQ*p (Zhuang & Shapiro, 1995), *fliA*p (Ramakrishnan *et al.*, 1991; Sanders *et al.*, 1992), *fliX*p (Mohr *et al.*, 1998) and *fliL*p (Yu & Shapiro, 1992). + and - signify positive and negative *ctrA* regulation respectively.

Fig. 6. Flagellar and chemotaxis hierarchy in *C. crescentus*. The figure on the left depicts the structure of the *C. crescentus* flagellum, with the flagellar gene products patterned according to their place in the transcriptional hierarchy: cross-hatched (class II), white (class III) and black (class IV). CM, cytoplasmic membrane; PL, peptidoglycan layer; OM, outer membrane; HAP, hook-associated protein. The figure on the right shows the hierarchy as a traditional flow diagram with the dashed bars showing the separation between the different subclasses of the class II genes. Each black box denotes separate transcriptional units.

that the *mcpA* promoter is different from other CtrA-dependent class II promoters is provided by the finding that transcription of *mcpA* is inhibited when membrane synthesis is blocked (Brassinga *et al.*, 2000), whilst other flagellar class II promoters are not affected. Finally, although we have been able to obtain a DNase I footprint of the *fliQ* promoter with CtrA we have been unsuccessful in binding CtrA to the *mcpA* promoter (S. E. Jones, unpublished). As demonstrated in this study and elsewhere, expression of an increasing number of genes is dependent on CtrA. However, many of these promoters reveal a complex pattern of regulation; for example *flm* genes (Leclerc *et al.*, 1998) are subject to positive regulation by CtrA but their putative promoters do not possess any good sites for CtrA binding. No transcription of flagellar or chemotaxis genes has been detected in swarmer cells despite the presence of CtrA (Domian *et al.*, 1997). This implicates accessory factors,

in addition to CtrA, which may influence the temporal expression of flagellar and chemotaxis genes (Reisenauer *et al.*, 1999).

Reclassification of flagellar and chemotaxis class II promoters

Results of transcriptional epistasis taken together with analysis of CtrA-dependence of the promoters analysed in this study prompted a re-examination of the flagellar hierarchy. The class II genes have been previously classified in the *C. crescentus* flagellar hierarchy as being required for the expression of class III and IV genes, and more recently as being positively regulated by CtrA binding to their promoters. We propose that class II promoters should comprise three distinct subclasses: A, B and C (Fig. 6). The *flhA* promoter should be classified as IIA since it is expressed early in the cell cycle

(Ramakrishnan *et al.*, 1991; Sanders *et al.*, 1992) and is negatively regulated by CtrA (data not shown). The archetypal class II promoters *fliQp*, *fliLp* and *fliFp* should be classified as IIB. FljJ, the earliest synthesized flagellin (Loewy *et al.*, 1987), is still expressed in a hook mutant, and is positively regulated by CtrA (data not shown). Since *fliJp* does not require σ^{54} or other class II genes for expression *fliJ* can be placed in class IIB. Further evidence that FljJ should be placed in a different class from the other flagellins is that blocking membrane synthesis abolishes synthesis of FljK and FljL flagellins but not that of the FljJ flagellin (Brassinga *et al.*, 2000). Inspection of the putative promoter of *fliJ* identified a good candidate CtrA-binding motif (Fig. 5), which would suggest that CtrA directly regulates *fliJ* like the other class IIB genes. Expression of the *mcpA* operon is not dependent on any of the class II genes, including the sigma factor gene *rpoN*. As mentioned previously the *mcpA* promoter does not have a CtrA consensus at the conserved position relative to the transcriptional start site. Thus, we propose that *mcpAp* should be classified as class IIC.

This study has probed regulation of several flagellar and chemotaxis genes, and has revealed new subclasses within the flagellar and chemotaxis hierarchy. Interruption of DNA synthesis prevented expression of *cagA* and *mcpA*, indicating that transcription of these genes was triggered by DNA replication, in common with other members of the flagellar regulon. The closest homologue to FlhA in *C. crescentus*, SigX, does not impact on chemotaxis gene regulation (S. E. Jones & M. R. K. Alley, unpublished). Instead chemotaxis gene regulation is mediated by CtrA, but other factors probably interact with CtrA-mediated regulation in order to facilitate cell-cycle control of the chemotaxis proteins. Chemotaxis gene regulation in *C. crescentus* is therefore significantly different from the *E. coli* paradigm.

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