Genetic Analysis of a Temporally Transcribed Chemotaxis Gene Cluster in *Caulobacter crescentus*

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ABSTRACT

Caulobacter crescentus performs chemotaxis by short intermittent reversals of rotation of its single polar flagellum. Tn*5* insertions causing a general chemotaxis phenotype, an inability to reverse swimming direction and to form large swarm colonies, have been mapped to an 8-kb region of the *C. crescentus* genome. These Tn*5* mutations had different effects on the methyl-accepting chemotaxis proteins (MCP), and the activities of methyltransferase and methylesterase. The Tn*5* insertion mutant SC1130 had no cross-reacting MCP and had reduced levels of activity of the methyltransferase and methylesterase. Other mutants bearing Tn*5* insertions retained cross-reacting MCP activity and were altered only in their methyltransferase and methylesterase activities. Using a cosmid library we isolated a clone that complemented SC1130. Complementation studies of the Tn*5* mutants using derivatives of the cosmid clone showed that all the Tn*5* insertions lie within a single operon that appears to encode many chemotaxis genes. The first gene in this operon was shown to encode an MCP by immuno-blot analysis of strains carrying β-galactosidase protein fusions to portions of the operon. The promoter of this operon was located by chromosomal integration of subclones of this region and by identifying DNA fragments that were capable of expressing *lacZ* transcriptional fusions. The transcription of the *che* operon occurred at a defined time in the cell cycle, prior to cell division.

THE stalked bacterium, *Caulobacter crescentus*, has a dimorphic cell cycle (see Figure 5). During a portion of its cell cycle it lacks a stalk and in its place has a single polar flagellum. The swarmer cell exists for about a quarter of the cell cycle and is chemotactically competent. Like other motile bacteria, *C. crescentus* can swim toward attractants and away from repellants. It preferentially rotates its polar flagellum in a clockwise direction (KOYASU and SHIRAKIHARA 1984). Wild-type *C. crescentus* exhibits short intermittent reversals in swimming direction caused by changes in the direction of rotation of its flagellum. These short intermittent reversals allow for a change in swimming direction of *C. crescentus*, which performs the same function as smooth and tumble swimming seen with *E. coli* (BERG and BROWN, 1972). Previously, a number of general chemotaxis mutants caused by Tn*5* insertions were shown to be unable to reverse direction of swimming (ELY *et. al.* 1986). These Tn*5* mutations mapped to a 19-kb *Dra*I fragment in the *C. crescentus* chromosome (ELY and GERARDOT 1988). The Tn*5* insertions in this cluster differentially affect three biochemical activities associated with chemotaxis; the methyl-accepting activity of the chemotaxis receptor protein (MCP), a methyltransferase and a methylesterase. In this study we provide evidence that the genes for these three activities are located in a large operon whose transcription is cell cycle regulated. It was previously shown that the synthesis of an MCP and methylesterase protein (GOMES and SHAPIRO 1984), and the appearance of the MCP and the methyltransferase and methylesterase activities (SHAW *et al.* 1983) were temporally regulated during the cell cycle. The newly synthesized MCP is preferentially localized to the pole of the predivisional cell that gives rise to the swarmer cell (NATHAN *et. al.* 1986; M. R. K. ALLEY, J. MADDOCK and L. SHAPIRO, unpublished results). Thus the MCP is segregated to the chemotactically competent swarmer cell progeny and not to the sessile stalk cell progeny (GOMES and SHAPIRO 1984).

To understand the signals that control the temporal expression and the positioning of the MCP in the predivisional cell, we have initiated experiments to define the organization and regulatory regions of the chemotaxis gene cluster. The aim of this study was to identify and clone the MCP gene and the regulatory region that controls its expression. This was done by initially complementing the general chemotaxis mutants and then further localizing the chemotaxis operon on the complementing cosmid. The MCP gene was found to be the first gene in a large operon that encoded several general chemotaxis functions.

TABLE 1

List of bacterial strains

Strain	Relevant properties	Reference or source
E. coli		
CC170	*CC118*::Tn*lacZ*	MANOIL (1990)
JBS134	CSH26::Tn*blaM*	J. K. BROÖME-SMITH and B. G. SPRATT (unpublished)
MT607	MM294 *rec*A56	E. R. SINGER (unpublished)
S17-1	*E. coli* 294::RP4-2 (Tc::Mu)(Km::Tn*7*)	SIMON, PRIEFFER and PUHLER (1983a)
C. crescentus		
CB15	Wild-type	POINDEXTER (1964)
CB15N	Synchronizable derivative of CB15	EVINGER and AGABIAN (1977)
SC1040	*cheB137*::Tn*5 str152*	ELY *et al.* (1986)
SC1057	*cheB144*::Tn*5 proA103 str140*	ELY *et al.* (1986)
SC1063	*cheR141*::Tn*5 proA103 str142*	ELY *et al.* (1986)
SC1064	*cheR142*::Tn*5 proA103 str140*	ELY *et al.* (1986)
SC1130	*cheR151*::Tn*5 cysB102 str142*	ELY *et al.* (1986)
SC1163	*cheB148*::Tn*5 str152*	B. ELY (unpublished)
CM5256	*rec-526*	O'NEILL, HYNES and BENDER (1985)

MATERIALS AND METHODS

Materials: DNA polymerase I, calf intestine alkaline phosphatase, polynucleotide kinase and Klenow fragment were obtained from Boehringer Mannheim Biochemicals. Restriction enzymes were from Boehringer Mannheim, New England Biolabs, or Bethesda Research Laboratories. [^{35}S] methionine was obtained as *trans*-label from ICN. All antibiotics were obtained from Sigma.

Bacterial strains, plasmids and growth conditions: Bacterial strains used in this study are listed in Table 1 and all plasmid constructions are listed in Table 2. *C. crescentus* was grown at 32° in PYE medium (POINDEXTER 1964) or in minimal M2-glucose medium (JOHNSON and ELY 1977). Cultures were synchronized using a modification of the ludox centrifugation procedure of EVINGER and AGABIAN (1977). The synchrony was monitored by light microscopy.

Measurement of enzyme activities: Methylation of isolated membrane fractions by methyltransferase was carried out *in vitro* according to SPRINGER and KOSHLAND (1977) with modifications as described by SHAW *et al.* (1983). The methyltransferase activity was determined by counting the amount of radioactivity that was released upon hydrolysis of the carboxymethyl groups from the MCPs. The *in vitro* methylesterase activity was measured using *in vivo* methylated membranes (SHAW *et al.* 1983) as a substrate for the methylesterase from cell extracts prepared as described by GOMES and SHAPIRO (1984). The methylesterase activity, the loss of [^{3}H]methyl groups from the MCPs, was measured by densitometry of autoradiographs of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels.

Measurement of methyl-acceptor activity of isolated membranes: Methyl-acceptor activity in membrane preparations from several different Tn*5* insertion-bearing strains was assayed using soluble cell extracts as a source of methyltransferase. The membrane fraction from each strain was prepared as described previously (SHAW *et al.* 1983). A 100-μg aliquot of membrane fraction was incubated with 100 μg of wild-type soluble fraction and 400 pmol of *S*-adenosyl [^{3}H-methyl]methionine (4×10^{3} cpm/pmol), in a final volume of 80 μl at 30° for 30 min. Samples (20 μl) were removed and were precipitated with trichloroacetic acid on 1-cm squares of Whatman 3M filter paper. The [^{3}H]methanol released from the filters was measured as described by SHAW *et al.* (1983).

Construction of *rec* strains: To ensure that recombination did not occur during complementation studies, recombination deficient (*rec*$^{-}$) derivatives of the *che* Tn*5* mutants were constructed. To construct the *rec*$^{-}$ strains, the *rec*-526 mutation (O'NEILL, HYNES and BENDER 1985) was linked to a gentamycin/spectinomycin (Gm/Sp)-resistant, kanamycin-sensitive, Tn*5* derivative. This was performed by transposing Tn*5* Gm/Sp into CB15N*rec*, making φCR30 lysates on cells containing these transposon insertions, and transducing them into a CB15N *rec*$^{+}$ strain. This strain carried a Tn*5*-132 insertion (tetracycline resistant derivative of Tn*5*) closely linked to the *rec* gene (G. T. MARCZYNSKI, unpublished). Tn*5* Gm/Sp insertions linked to the *rec*-526 mutation would also be tetracycline sensitive. A Tn*5* Gm/Sp insertion was obtained that showed 20% linkage to the *rec*-526 mutation. The presence of the *rec*-526 mutation was tested by UV sensitivity and the inability to obtain transductants. This recombinant transposon allowed the *rec*-526 mutation to be introduced into kanamycin resistant *che* strains by transduction.

Construction of a cosmid library and isolation of a chemotaxis gene cluster: CB15N chromosomal DNA was partially digested with *Sau*3AI and size fractionated on a 10–40% sucrose gradient as described by SAMBROOK, FRITSCH and MANIATIS (1989). DNA fragments of 25–30 kb were ligated into the cosmid pLAFR5 (KEEN *et al.* 1988). The ligations were packaged using Gigapack λ packaging extracts (Stratagene) and the resulting phage particles were used to transduce the *Escherichia coli* strain S17-1. A cosmid, pR6 (see Figure 2), was obtained that complemented the general chemotaxis mutant strain SC1130 (Table 1). This cosmid also complemented the *che* phenotype of strains SC1040, SC1057, SC1063, SC1064 and SC1163.

Construction of Tn*lacZ* and Tn*blaM* insertions in pR6: Tn*lacZ* (Km^{r}) and Tn*blaM* (Sp^{r}) are derivaties of Tn*5* that can form translational fusions to *lacZ* and *blaM*, respectively. In this study they were used solely as transposition insertions in order to abolish complementation. Tn*lacZ* insertions into the cosmid pR6 were performed as follows. The *E. coli* strain CC170 carrying Tn*lacZ* and pR6 was grown overnight at 30° in superbroth (Stratagene) with tetracycline (10 μg/ml). The Tn*lacZ* transpositions onto pR6 were selected by conjugating pR6 into *C. crescentus* and selecting for kanamycin resistant transconjugants with the help of transfer functions from *E. coli* MT607 (pRK600). Conjugations were

TABLE 2

List of plasmids

Plasmid	Relevant characteristics or construction	Reference or source
pBR322	Ap^r, Tc^r, ColE1 replicon	BOLIVAR *et al.* (1977)
pBR325	Ap^r, Cm^r, Tc^r, ColE1 replicon	BOLIVAR (1978)
pIC20H	Ap^r, pUC derivative with polycloning site flanked by *Hin*dIII sites	MARSH, ERFLE and WYKES (1984)
pACYC184	Cm^r, Tc^r, P15A replicon	CHANG and COHEN (1978)
pLVC9	Cm^r, *mob*$^+$, P15A replicon, for mobilization of ColE1 derived vectors with a *bom* site	G. WARREN
pSUP5011	Ap^r, Cm^r, Tc^r::Tn*5* *mob*, source of *neo* for pCHN1	SIMON, PRIEFFER and PUHLER (1983b)
pJBS633	Km^r, Tc^r, ColE1 replicon *bom*$^+$ for protein fusions to mature β-lactamase (*bla*M)	BROOME-SMITH and SPRATT (1986)
pDAH274	Ap^r, Km^r, P15A replicon *lacZ* transcriptional fusion vector	D. A. HODGSON
pNM280, 281, 282	Ap^r, pUC derivative. Three reading frames *lacZ* translation fusion vectors	MINTON (1984)
pJBZ 280, 281, 282	Km^r, ColE1 replicon, *bom*$^+$, *lacZ* translation fusion vectors, derivatives of pJBS633 and pNM280, 281 and 282	This study
PGMTZ1	Gm^r derivative of pACYC184 with the *lacZ* gene from pDAH274; IncP-1 *oriT*	This study
pRK600	pRK2013*npt*::Tn*9*, Cm^r, ColE1 replicon, *tra*$^+$	E. R. SIGNER
pRK2501	Km^r, Tc^r, IncP-1 replicon, *mob*$^-$	KAHN *et al.* (1979)
pRK290	Tc^r, IncP-1 replicon, *mob*$^+$	DITTA *et al.* (1985)
pRK290KS2	Sp^r, Tc^r, IncP-1 replicon, *mob*$^+$; pBluescript KS polylinker	This study
pLAFR5	Tc^r, IncP-1 replicon, cosmid	KEEN *et al.* (1988)
pWA10	The *Eco*RI fragment containing the Tn*5* insertion from SC1130 inserted into pBR325	This study
pWA12	The wild-type 2.3-kb *Eco*RI fragment inserted into pBR325	This study
pWA14	A 5.6-kb *Bam*HI fragment inserted into pBR322	This study
pWA100	The 5.6-kb *Bam*HI fragment inserted into pRK290	This study
pR6	A 26-kb insert in pLAFR5	This study
pR6ΔH	A *Hin*dIII deletion derivative of pR6 with a 19-kb insert in pLAFR5	This study
pCHZ1&6	A 2.3-kb *Eco*RI insert in pGMTZ1 in either orientation	This study
pCHZ1B	A *Bam*HI deletion derivative of pCHZ1	This study
pCHZ1P	A *Pst*I deletion derivative of pCHZ1	This study
pCHZ1S	A *Sal*I deletion derivative of pCHZ1	This study
pCHZ6B	A *Bam*HI deletion derivative of pCHZ6	This study
pCHZ6S	A *Sal*I deletion derivative of pCHZ6	This study
pRCZ1	pCHZ1 inserted into the *Hin*dIII site of pRK2501	This study
pRCZ1B	A *Bam*HI deletion derivative of pRCZ1	This study
pRCZ1P	A *Pst*I deletion derivative of pRCZ1	This study
pCH04	The 0.4-kb *Bam*HI/*Eco*RI fragment containing the *che* promoter inserted in pIC20H	This study
pCHN2	The 1.1-kb *Bgl*II/*Sal*I fragment from pSUP5011 containing the promoterless *neo* gene inserted into pCHO4 downstream of the *che* promoter	This study
pRCHN1	The *Hin*dIII fragment from pCHN2 ligated into pRK290KS2	This study
pCH8	The 5.6-kb *Bam*HI fragment from pWA14 inserted into pCHO4 in the correct orientation	This study
pRCH8	The 6.0-kb *Hin*dIII fragment from pCH8 ligated into pLAFR5	This study
pCEE281	The 2.3-kb *Eco*RI fragment from pWA12 inserted into pJBZ281	This study

performed as described by ELY (1979). Plasmid DNA was isolated from the transconjugants by a modified alkaline lysis method (BRUN, BRETON and LAPOINTE 1991). The locations of the Tn*lacZ* insertions were determined by analysis of restriction enzyme digests of the plasmid DNA. The insertions Ω21, Ω22, Ω17, Ω10, Ω14, Ω31, and Ω40 are all Tn*lacZ* insertions in pR6. Tn*blaM* insertions in pR6, Ω1, Ω3 and Ω7, were obtained in a similar manner except that pR6 was placed initially in the *E. coli* strain JBS134 and transconjugants were selected for spectinomycin resistance. Plasmid DNA was introduced into the *E. coli* strain S17-1 by electroporation (SMITH *et al.* 1990).

Immunoprecipitations: Immunoprecipitations were performed as described by GOMES and SHAPIRO (1984) except that cells were labeled in 1.5-ml aliquots with 15 μCi of [^{35}S] methionine for 5 min at 32°. Immunoprecipitated proteins were resolved on a 12% SDS-polyacrylamide gel and then fixed and fluorographed with Amplify from Amersham as described by the manufacturer. Antibody to the product of the *neo* gene, neomycin phosphotransferase II (NPT II) was

obtained from 5′→3′ (West Chester, Pennsylvania), and antibody to flagellin was from our laboratory.

Immuno-blot analysis. An extract of *C. crescentus* obtained by boiling 150 μl of overnight culture in SDS sample buffer was applied to a SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose (0.2 μm from S&S) by an electroblotter (Bio-Rad), as described by TOWBIN, STAEHELIN and GORDON (1979). The nitrocellulose filter was blocked with 5% non-fat dry milk (Carnation) in TTBS buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.05% Tween 20). Then the filter was incubated with the primary antibody for 2 hr at room temperature. Anti-Tar antiserum (a gift from D. KOSHLAND) was used at a 1/10,000 dilution and the anti-β-galactosidase antibody, purchased from 5′→3′, was used at the same dilution. The filter was washed and a secondary antibody, goat-anti-rabbit horseradish peroxidase (BMB), was used at a 1/40,000 dilution for 1 hr at room temperature. After washing extensively with TTBS buffer, the signal was detected by chemiluminescence using the Amersham ECL detection system.

RESULTS

Analysis of Tn*5* insertion mutants with a defect in general chemotaxis functions: Six clustered Tn*5* insertion mutations caused production of small colonies in swarm agar plates and an inability to reverse direction of swimming as seen under the light microscope (ELY *et al.* 1986). The level of the MCP, and the *in vitro* activities of the methyltransferase, and methylesterase were determined for each mutant strain and compared to wild-type levels (Figure 1B). The Tn*5* insertion in SC1130 reduced all three activities (Figure 1B). Tn*5* insertions in SC1063 and SC1064 did not affect the level of the MCP but they did reduce the activity of the methyltransferase and methylesterase. The Tn*5* insertion in SS1057 only affected the methylesterase activity, while SC1040 had low levels of methylesterase activity and also showed lowered methyltransterase activity. The Tn*5* insertion in SC1163 had wild-type levels of MCP and methyltransferase activity, although methylesterase activity was reduced. In *E. coli* (SLOCUM and PARKINSON 1983; STOCK and KOSHLAND 1978) and *Salmonella typhimurium* (DEFRANCO and KOSHLAND 1981), the genes encoding these three biochemical activities are organized in an operon. In both these bacteria, the MCP gene is located upstream of the methyltransferase and methylesterase genes. Our biochemical assays suggested that the Tn*5* insertion in SC1130 could reside in a MCP gene. We previously demonstrated that antiserum raised against one of the Salmonella MCPs, Tar, cross-reacts with a *C. crescentus* methyl-accepting chemotaxis protein (GOMES and SHAPIRO 1984). To determine if the Tn*5* insertion in SC1130 interrupted an MCP gene, immuno-blots were performed on extracts from wild-type (CB15), SC1130 and SC1063 strains using the anti-Tar antiserum (Figure 1C). These immuno-blots showed that the cross-reacting band was missing only in strain SC1130,

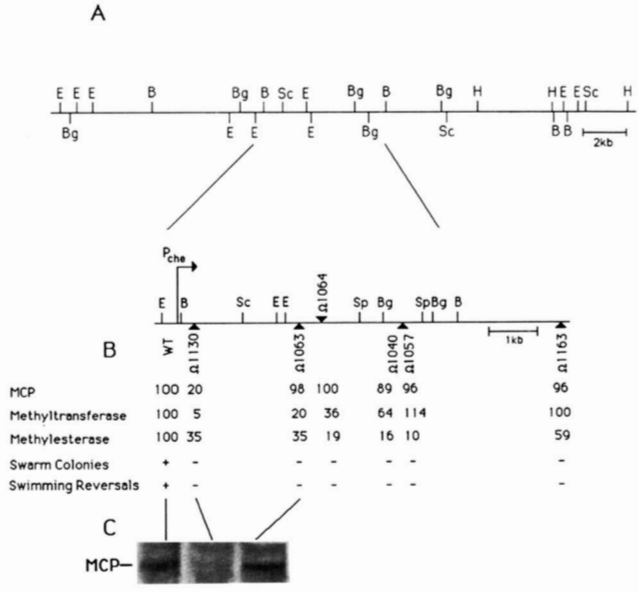

FIGURE 1.—Chemotaxis phenotype of Tn*5* insertions in an 8-kb region of the *C. crescentus* chromosome. (A) A restriction map of the chemotaxis gene cluster and the surrounding region, with an expanded restriction map of 8 kb of DNA showing the position of the Tn*5* insertions (black triangles) that yield a *che* phenotype. The position of the Tn*5* insertions were mapped by DNA blot analysis. The Tn*5* insertions are numbered by inserting Ω in front of the respective strain number. E = *Eco*RI, B = *Bam*HI, Bg = *Bgl*II, Sa = *Sal*I, Sc = *Sac*I, SP = *Sph*I and P = *Pst*I. (B) Chemotaxis phenotypes and biochemical activities of wild-type and Tn*5* insertion mutant strains. The *in vitro* activities of the MCP, methyltransferase and methyesterase were measured as described in MATERIALS AND METHODS, and are reported as a percentage of the CB15 wild-type strain. The MCP activity was measured using wild-type soluble extracts to methylate membranes isolated from each of the mutant strains. The *che* phenotype is defined by small swarm colonies in semisolid agar (0.3%) and the inability to reverse the direction of swimming, as determined by light microscopy. (C) An equal number of cells of CB15, SC1130 and SC1063 were used in an immuno-blot assay with anti-Tar antiserum, as described in MATERIALS AND METHODS. The size of the *C. crescentus* MCP is 72 kD, as previously described (GOMES and SHAPIRO 1984).

suggesting that the Tn*5* insertion in SC1130 may lie within the gene for this protein or a gene controlling its synthesis. The Tn*5* insertion in SC1063 lies outside the gene for the MCP.

Complementation analysis using wild-type and mutant clones of the chemotaxis gene cluster: To help define the organization of these genes and the extent of the complementing fragment, the cluster was cloned and complementation studies were performed with the Tn*5* mutant strains.

The *Eco*RI DNA fragment containing the Tn*5* insertion SC1130 was cloned (pWA10) by selecting for kanamycin resistance in an *E. coli* host strain. This cloned fragment was then used as a probe to isolate the corresponding wild-type 2.3-kb *Eco*RI fragment, pWA12, and the linking 5.6-kb *Bam*HI fragment, pWA14 (Figure 2). When the 5.6-kb *Bam*HI fragment was placed in a vector that was capable of replicating

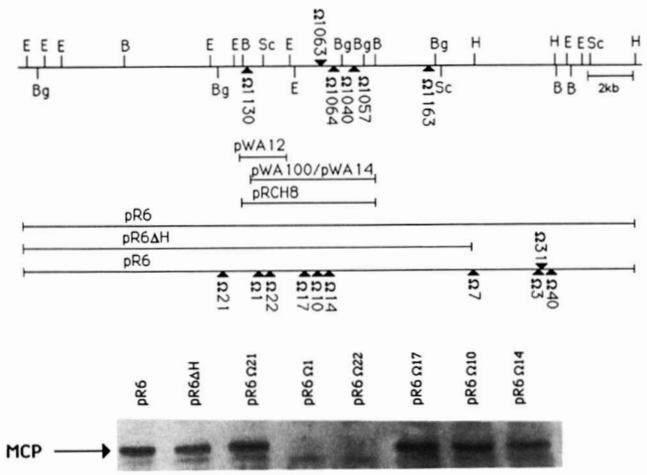

FIGURE 2.—Complementation of Tn*5* insertion *che* mutations in a CB15N *rec*⁻ background. Subclones of the gene cluster, shown below the restriction map, were isolated as described in MATERIALS AND METHODS. A complementing cosmid (pR6) was isolated and mutagenized with Tn*lacZ* and Tn*blaM*. The resulting plasmids were tested for their ability to complement the six chromosomal insertion mutations indicated by black triangles on the restriction map at the top of the figure. Black triangles shown on the pR6 insert indicate the site of Tn*lacZ* and Tn*blaM* insertions. The insertions Ω1, Ω3 and Ω7 are Tn*blaM*, and the rest are Tn*lacZ*. Complementation was tested by the swarm plate assay. Immuno-blots using anti-Tar antiserum were performed on strain SC1130 containing the cosmid pR6 and derivative Tn*lacZ* and Tn*blaM* insertions as shown in the above restriction map; MCP (72 kD).

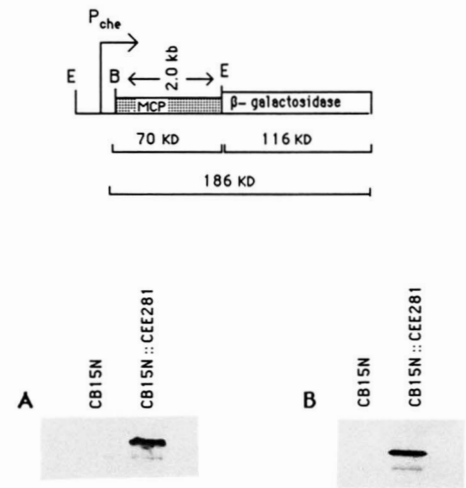

FIGURE 3.—Western blot of a 186-kD MCP/β-galactosidase fusion protein using anti-Tar antiserum (A) or anti-β-galactosidase antibody (B). The CB15N lane is the wild-type control without the fusion present. In both lanes equal numbers of cells, as determined by $A_{660\,nm}$, were boiled in SDS sample buffer and loaded on a 7.5% SDS-polyacrylamide gel. The immuno-blot procedure was carried out as described in MATERIALS AND METHODS.

in *C. crescentus* (pWA100), it was unable to complement SC1130 (Figure 2). A cosmid library was, therefore, constructed for the purpose of obtaining larger clones capable of complementating the *che* mutants, as described in MATERIALS AND METHODS. A cosmid with an insert of 26 kb was obtained that was capable of complementing all six Tn*5* *che* mutants; it was designated pR6 (Figure 2). It was possible to reduce the size of the insert in the cosmid to 19 kb, pR6ΔH, and to retain complementation. An attempt was made to construct a smaller complementing clone by joining the 5.6-kb *Bam*HI fragment, encompassing most of the Tn*5* insertions, and the neighboring 0.4-kb *Eco*RI/*Bam*HI fragment. The resulting plasmid, pRCH8, was not able to complement SC1130 (Figure 2) suggesting that the *che* genes are clustered in an operon that is larger than the 6-kb insert in pRCH8.

To obtain evidence that the Tn*5* insertions lie in an operon, and to define the extent of the operon, Tn*lacZ* and Tn*blaM* insertions in the cosmid pR6 were obtained as described in MATERIALS AND METHODS. The locations of the Tn*lacZ* and Tn*blaM* insertions in the pR6 cosmid are shown in Figure 2. Complementation tests using the swarm plate assay with each of the six chromosomal Tn*5* insertions were performed in *rec*⁻ genetic backgrounds. The insertions Ω21 and Ω7 complemented all the *che* mutants, while the insertions Ω1, Ω22, Ω17, Ω10 and Ω14 were unable to complement any of the *che* mutants (Figure 2). Therefore, the Tn*lacZ* insertion Ω21 and the Tn*blaM* insertion Ω7 denote the maximal borders of the complementing region, the chemotaxis operon. Insertions Ω3, Ω31 and Ω40 are outside the operon and, like Ω7, complement all six Tn*5* insertions. These results suggest that all the original Tn*5* insertions that gave rise to a *che* phenotype lie in a single operon.

Identification of the promoter proximal gene as a MCP gene: The data in Figure 1C suggested that a gene proximal to the promoter encoded a protein that cross-reacted with anti-Tar antiserum. To map the location of this gene more precisely, certain Tn*lacZ* insertions in pR6 were selected for immuno-blot analysis. These Tn*lacZ* insertions in pR6 were conjugated into the *che* strain SC1130 prior to immuno-blot analysis. In this experiment, the presence of a cross reacting band with anti-Tar antiserum will be assignable to a gene in the cosmid insert because SC1130 does not have this protein (Figure 1C).

The strains bearing cosmid pR6 or its deletion derivative, pR6ΔH (see Figure 2), showed a cross-reacting band, as did those bearing the Tn*lacZ* insertion plasmid pR6Ω21 (Figure 2). This result confirms that a gene that encodes a MCP homolog resides on the pR6 cosmid, somewhere between the start of transcription and the *Hin*dIII site beyond the Tn*5* insertion in SC1163 (see Figure 2). As demonstrated in Figures 4 and 5, the *che* promoter is located downstream from the Tn*lacZ* insertion pR6Ω21. Strains carrying plasmids pR6Ω1 and pR6Ω22 lack cross-reacting protein (Figure 2), while those bearing pR6Ω17, pR6Ω10 or pR6Ω14, downstream of the

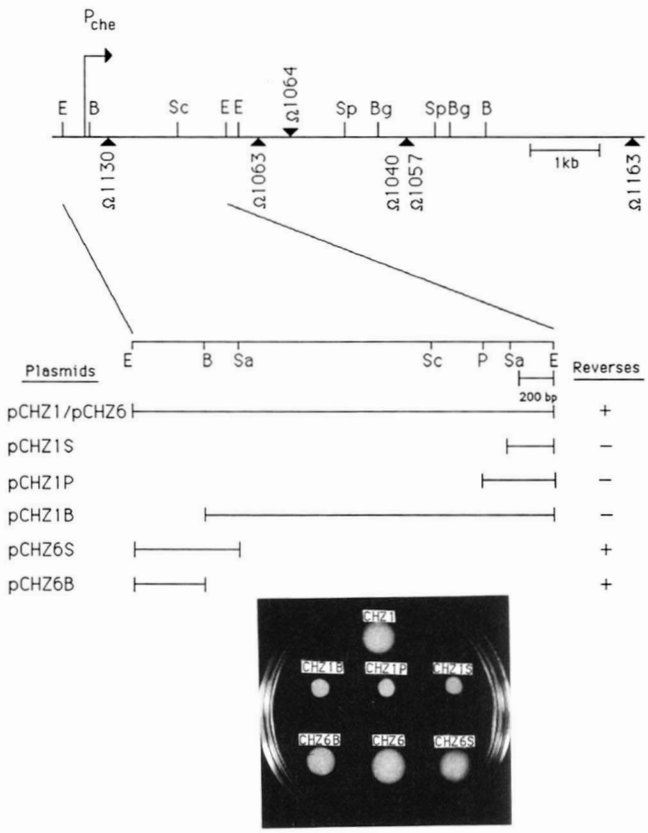

FIGURE 4.—Localization of the promoter of the *che* operon by homologous recombination. Plasmids that were incapable of replication in *C. crescentus* were integrated into the *C. crescentus* chromosome of CB15N via homologous recombination. All insertions were confirmed by DNA blot analysis. The extent of the *C. crescentus* DNA insert in each plasmid is shown as is the phenotype of the derivative strain after integration of the plasmid; swimming reversals (+ or −) and swarm size on semisolid agar plates.

insertions Ω1 and Ω22, all have cross-reacting protein. These results suggest that an MCP gene is located between the *Bam*HI site at the start of the operon and the Tn*lacZ* insertion Ω17 (see Figure 2).

To further prove that the promoter proximal gene encodes a MCP, a protein fusion to β-galactosidase was constructed using the 2.3-kb *Eco*RI fragment that encompasses the putative MCP gene (Figure 3). The *Eco*RI fragment was placed in three vectors, pJBZ280, pJBZ281 and pJBZ282, which allow fusions in each of three reading frames to β-galactosidase that lack the first 8 amino acids. Only one of these plasmid constructs, pCEE281, when integrated into the *C. crescentus* chromosome showed β-galactosidase activity. Immuno-blot analysis was performed on this strain and on a CB15N wild-type control, using anti-Tar antiserum and anti-β-galactosidase antibodies to identify fusion products (Figure 3, A and B). A 186-kD anti-Tar cross-reacting band was seen in the CB15N::CEE281 lane but not in the wild-type lane (Figure 3A). This 186-kD protein also cross-reacted with the anti-β-galactosidase antibody, providing evidence that this cross-reacting band is an MCP/β-galactosidase fusion protein. Since native β-galactosidase is 116 kD, the fusion appears to have approximately 70 kD of the *C. crescentus* MCP, which is almost all of the native MCP considering its size is 72 kD (GOMES and SHAPIRO, 1984). Thus, an MCP gene appears to be the first gene in this large *C. crescentus che* operon.

Localization of a single promoter region for the chemotaxis gene cluster: The polarity of the Tn*5* insertions presented in Figure 1B suggest that transcription goes from the insertion in SC1130 to the insertion in SC1163. To localize the *che* promoter, we used homologous recombination to map the promoter to a 400-bp *Eco*RI/*Bam*HI fragment. The rationale for this experiment is that when the inserted DNA contains only sequences internal to the operon, a single homologous recombination event will disrupt the operon by the insertion of plasmid sequences. However, if the DNA fragment contains 5′ or 3′ sequences, then the operon is not disrupted and will re-form upon integration. The 2.3-kb wild-type *Eco*RI fragment from pWA12 and deletion derivatives were used for the recombination assay (Figure 4). We chose to analyze this region of the gene cluster because it spans the site of the Tn*5* insertion in SC1130, that gave rise to the most pleiotropic biochemical phenotype (Figure 1B). The 2.3-kb *Eco*RI fragment was placed in the vector pGMTZ1 (which is incapable of replicating in *C. crescentus*) in both orientations to construct pCHZ1 and pCHZ6. These plasmids were transfered to *C. crescentus* by conjugation using the helper plasmid pRK600. Since these plasmids are incapable of replicating in *C. crescentus*, gentamycin resistant colonies that arise from the mating are recombinants. This was confirmed by DNA blot analysis. When either of these plasmids were integrated into the wild-type chromosome, the cultures maintained normal reversal frequency and formed wild-type swarms on soft agar plates (Figure 4). Deletion derivatives of the inserts in both pCHZ1 and pCHZ6 were constructed (Table 2). These were integrated into the *C. crescentus* chromosome and their phenotypes were observed (Figure 4). In all derivatives that retained the 400-bp *Eco*RI/*Bam*HI fragment, namely pCHZ6S and pCHZ6B, a wild-type chemotaxis phenotype was observed. However, in all transconjugants that no longer retained the 400-bp *Eco*RI/*Bam*HI fragment, namely CHZ1S, CHZ1P and CHZ1B, a *che* mutant phenotype was observed. These data suggest that a promoter necessary for chemotaxis function resides in the 400-bp *Eco*RI/*Bam*HI fragment.

Additional evidence that the promoter is in the 400-bp *Eco*RI/*Bam*HI fragment was obtained by placing the 2.3-kb *Eco*RI fragment in front of a promoterless *lacZ* gene (Figure 5). Stop codons in all three reading-frames 5′ to the ATG for *lacZ* ensured that the

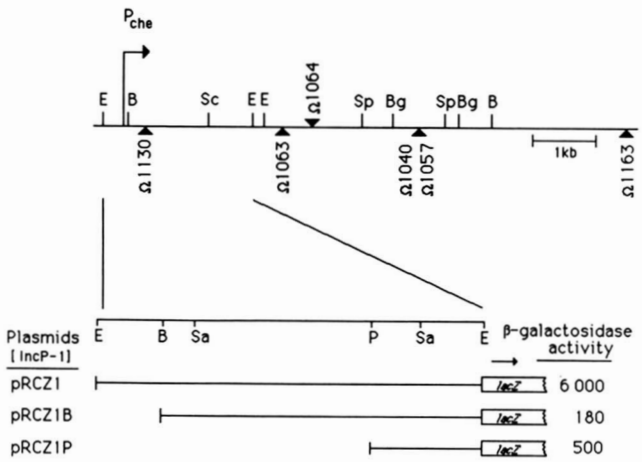

FIGURE 5.—Analysis of *lacZ* transcription fusions to 5′ deletions of the *che* operon. The two plasmids, pRCZ1B and pRCZ1P, were both derived from pRCZ1 by restriction digestion and religation (Table 2). These plasmids have Inc P-1 replicons and hence are able to replicate in *C. crescentus*. ONPG assays were performed on CB15N *rec*⁻ strains containing these plasmids, as described by MILLER (1972), except that cells were permeabilized with chloroform. The β-galactosidase activity is given in Miller units (MILLER 1972).

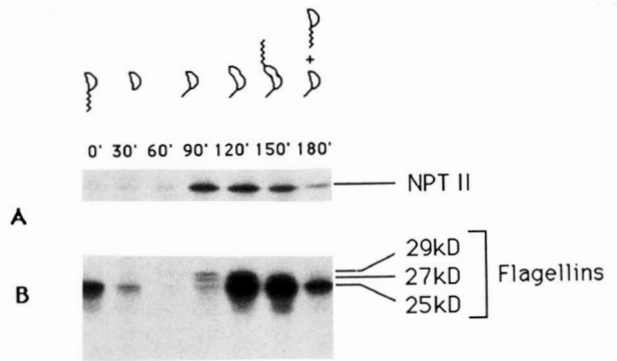

FIGURE 6.—Expression of a *che::neo* transcription fusion as a function of the cell cycle. The 0.4-kb *Eco*RI/*Bam*HI fragment containing the *che* promoter was placed in front of a promoter-less *neo* gene in pRK290. Swarmer cells containing this plasmid (pRCHN1) were isolated by Ludox centrifugation (EVINGER and AGABIAN 1977), and were then allowed to proceed through the cell cycle. At the indicated time points, samples were pulse-labeled for 5 min with [^{35}S]methionine. Cell extracts were prepared and equal amounts of counts were immunoprecipitated, as described by GOMES and SHAPIRO (1984), with (A) anti-NPT II antibody and (B) anti-flagellin antibody. Immunoprecipitated protein was resolved on a 12% SDS-polyacrylamide gel. The temporal expression of the flagellins was used as an internal control for the synchrony of the cell cycle (180-min cell cycle). The synchrony was also monitored by observing cells under the light microscope throughout the cell cycle. The three flagellin species are indicated. The 25-kD NPT II protein in panel A is indicated by an arrow.

construction would be a transcription fusion. This construction was placed in a plasmid capable of replicating in *C. crescentus* and was designated pRCZ1 (Table 2). Deletion derivatives of pRCZ1 were constructed by digestion and ligation to yield pRCZ1B and pRCZ1P, shown diagramatically in Figure 5. When these three plasmids were transferred via conjugation to CB15N *rec*⁻, pRCZ1 gave significantly higher β-galactosidase activity than either of the two deletion derivatives (Figure 5). The slight rise in activity of pRCZ1P relative to pRCZ1B was probably due to low level readthrough of transcripts from the kanamycin resistance gene in the vector as these sequences are brought closer to the *lacZ* gene in pRCZ1P. These results demonstrate that the 2.3-kb *Eco*RI fragment contained the 5′ and not the 3′ end of the operon and provide additional evidence that the *che* promoter is located in the 400-bp *Eco*RI/*Bam*HI fragment.

Temporal expression of the *che* operon: The synthesis and activity of three chemotaxis proteins, MCP, methyltransferase, and methylesterase, has been previously shown to be regulated in a cell cycle dependent manner (SHAW *et al.* 1983; GOMES and SHAPIRO 1984). In order to determine if the *che* promoter is temporally regulated, a *neo* transcriptional fusion was constructed that contained only the 400-bp *Eco*RI/*Bam*HI promoter-fragment in front of a promoterless *neo* gene in a plasmid that was capable of replication in *C. crescentus* (Figure 6). This plasmid, pRCHN1, was transferred to CB15N, and swarmer cells were isolated as described in MATERIALS AND METHODS. These swarmer cells were allowed to proceed through the cell cycle and at the time points shown in Figure 6, samples were pulse labeled with [^{35}S]methionine. Half the sample was immunoprecipitated with antibody to the product of the *neo* gene, NPT II, and half with antibody to the flagellins. Immunoprecipitation with anti-flagillin antibody was used as a control of the fidelity of the synchrony and as a reference marker for determining the time of initiation of *che* promoter transcription. Figure 6 shows that the *che::neo* fusion in pRCHN1 initiates transcription at 90 min into a 180-min cell cycle, coincident with the transcription of the 29-kD flagellin and somewhat before the maximal expression of hook and the 27-kD and 25-kD flagellins. Therefore, the *che* promoter is transcribed in a cell cycle dependent manner. Transcription ceases prior to cell division, as is the case for the synthesis of the three chemotaxis proteins described previously (GOMES and SHAPIRO 1984).

DISCUSSION

The phenotype caused by the Tn*5* insertion mutation SC1130 suggested that the MCP, methyltransferase and methylesterase genes were organized in an operon, as is the case for *E. coli* and *S. typhimurium* (SLOCUM and PARKINSON 1983; STOCK and KOSHLAND 1978; DEFRANCO and KOSHLAND 1981). Evidence that six Tn*5* insertions in this chromosomal cluster lie in the same operon was obtained by complementation of

mutant strains with clones of this region containing Tn*lacZ* and Tn*blaM* insertions. A minimum size for this operon was calculated to be 8.0 kb.

A 72-kD MCP was previously identified in *C. crescentus* cell membranes based on its ability to accept [^{3}H]methyl groups in a carboxymethyl bond and by immunoprecipitation of the methyl-labeled protein with anti-Tar antiserum (GOMES and SHAPIRO 1984). Immuno-blot analysis performed on extracts of SC1130 using anti-Tar antiserum failed to detect this 72-kD cross-reacting protein, suggesting that this operon encodes an MCP. Immuno-blots of strains containing Tn*5* insertions in complementing clones and protein fusions to β-galactosidase confirmed the presence of a gene encoding an MCP in the promoter proximal region of the operon. However, we cannot rule out the possibility that *C. crescentus* has other MCPs, either much less abundant or serologically distinct from the *S. typhimurium* Tar MCP.

General chemotaxis mutants so far mapped to this region of the chromosome were previously designated *cheR*, *cheB* and *cheT* (ELY *et al.* 1986). Initially, the Tn*5* insertions in SC1040, SC1057 and SC1163 were designated *cheB*, and the Tn*5* insertions SC1063, SC1064 and SC1130 were designated *cheR*. However, the gene designations must now be revised because experiments reported here indicate that the Tn*5* insertion in SC1130 is in an MCP gene (now designated *mcpA*). Furthermore, DNA sequences obtained for the next gene in the operon (W. ALEXANDER, unpublished) show 40% identity to the *E. coli cheA* protein. The Tn*5* insertions SC1063 and SC1064 are probably in this gene. The following open reading frame encodes a protein with 37.9% identity to the *E. coli cheW* protein. These observations suggest that the first three genes in the *C. crescentus che* operon are *mcpA*, *cheA* and *cheW*. The polar effects of insertions in these genes on methyltransferase and methylesterase activity are probably due to their location downstream in this large operon. The final genetic designations of the genes in this operon should await sequence analysis. Because the entire complemented region encompasses at least 8 kb of DNA, there is enough room for several general chemotaxis functions.

The activities of the MCP, the methyltransferase, and the methylesterase were previously shown to be expressed in a cell cycle-dependent manner (SHAW *et al.* 1983), and the synthesis of the MCP and the methylesterase protein was shown to occur in the predivisional cell (GOMES and SHAPIRO 1984). We have shown here that the gene cluster allowing the expression of these activities is transcribed in a cell cycle-dependent manner. The promoter region was localized by homologous recombination with 5′ fragments of the cluster and by the expression of transcriptional fusions. Primer extension and sequence analysis of the 5′ regulatory region revealed a promoter that is similar to that recognized by *E. coli* σ^F (M. R. K. ALLEY and P. FREDERIKSE, unpublished). We have previously shown that when the *E. coli tsr* gene is inserted into the *C. crescentus* chromosome, or when it is carried on a plasmid in *C. crescentus* strains, the gene is transcribed from its own σ^F promoter in a cell cycle dependent manner (FREDERIKSE and SHAPIRO 1989). The pattern of transcription was analogous to that observed with the endogenous *mcpA* gene. These observations suggest that the *E. coli* and the *C. crescentus* MCP gene share some signals for the cell cycle control of transcription.

The MCP that is synthesized in the predivisional cell specifically segregates to the swarmer cell upon division (GOMES and SHAPIRO 1984). This segregation appears to be the result of localization of the newly synthesized MCP to the nascent swarmer portion of the predivisional cell, as observed in polar vesicles of the predivisional cell (NATHAN *et al.* 1986) and by *in situ* immunogold-electron microscopy (M. R. K. ALLEY, J. MADDOCK and L. SHAPIRO, unpublished). In addition, the MCP is turned over in a cell cycle-specific manner during the swarmer to stalk cell transition (M. R. K. ALLEY and P. FREDERIKSE, unpublished). Now that an MCP gene is identified and isolated we can proceed to define the amino acid sequences that mediate polar segregation and cell cycle turnover.

Two soluble chemotaxis proteins, the methyltransferase and the methylesterase are similarly segregated to the progeny swarmer cell upon division and are apparently turned over during the swarmer cell transition (GOMES and SHAPIRO 1984). Our preliminary characterization of the *C. crescentus* chemotaxis genes will allow us to address how soluble proteins are targeted and turned over during the development and asymmetric division of this bacterium.

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