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Analysis of a Chemotaxis Operon from *Rhodospirillum centenum*

ZE-YU JIANG AND CARL E. BAUER*

Department of Biology, Indiana University, Bloomington, Indiana 47405

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A chemotaxis gene cluster from the photosynthetic bacterium *Rhodospirillum centenum* has been cloned, sequenced, and analyzed for the control of transcription during swimmer-to-swarm cell differentiation. The first gene of the operon (*cheAY*) codes for a large 108-kDa polypeptide with an amino-terminal domain that is homologous to CheA and a carboxyl terminus that is homologous to CheY. *cheAY* is followed by *cheW*, an additional homolog of *cheY*, *cheB*, and *cheR*. Sequence analysis indicated that all of the *che* genes are tightly compacted with the same transcriptional polarity, suggesting that they are organized in an operon. Cotranscription of the *che* genes was confirmed by demonstrating through Western blot analysis that insertion of a polar spectinomycin resistance gene in *cheAY* results in loss of *cheR* expression. The promoter for the *che* operon was mapped by primer extension analysis as well as by the construction of promoter reporter plasmids that include several deletion intervals. This analysis indicated that the *R. centenum che* operon utilizes two promoters; one exhibits a σ^{70}-like sequence motif, and the other exhibits a σ^{54}-like motif. Expression of the *che* operon is shown to be relatively constant for swimmer cells which contain a single flagellum and for swarm cells that contain multiple lateral flagella.

More than a century ago, Engelmann (7) observed that when purple sulfur photosynthetic bacteria of the genus *Chromatium* were illuminated with a broad spectrum of light, the cells accumulated at wavelengths that corresponded to the in vivo absorption peaks of the cells. A more accurate description of this phenomenon is that when smooth-swimming cells migrate into a dark region (or into regions of the spectrum where wavelengths are not absorbed by photopigments), they either tumble or reverse the direction of movement, depending on the species. The phenomenon has been termed a scotophobic (fear of darkness) response since the cells exhibit an aversion to darkness rather than a specific affinity for light (12, 29). Since a reduction in light intensity causes a tumbling/reversal response, the mechanism of moving through an increasing gradient of light intensity appears to involve a directed "random walk" such that the length of smooth swimming is longer when cells are going up a light gradient than when they are going down. Superficially, this process is not unlike the well-characterized bacterial "trial-and-error" walking up a chemical gradient that is mediated by chemoreceptors and the Che protein phosphorylation cascade (2, 40).

The mechanism whereby a reduction in light intensity causes a tumbling/reversal response is still unclear. However, recent work has revealed that a functioning photosynthetic apparatus is required for photosensory perception (3). In addition, chemical inhibition of electron carrier components such as the cytochrome bc_1 complex, are incapable of light perception (4). These results have led to the current dogma that the scotophobic response is mediated by the perception of a sudden decrease in the rate of photosynthesis rather than by light absorption through a specific photoreceptor. The mechanism of measuring a reduction in the efficiency of photosynthesis appears to involve monitoring alterations in the rate of photosynthesis-driven electron transport (10, 14).

The use of the bacterium *Rhodospirillum centenum* as a model organism for studying bacterial photoperception is particularly appealing since it is known to exhibit two distinctive photosensory processes (29, 30). When grown in liquid medium, *R. centenum* cells, which are motile by means of a single polar flagellum, exhibit a typical scotophobic response. However, when these cells are grown on agar solidified media, they differentiate into hyperflagellated swarmer cells that enable colonies to rapidly migrate (up to 75 mm/h) toward or away from light sources. Furthermore, an analysis of swarmer colony motility has shown that the colony migrates toward a light source irrespective of whether the cells are going up or down a gradient of light intensity (30). The ability of photosynthetic organisms to move toward a light source irrespective of intensity is a process distinct from scotophobic response and is correctly termed phototaxis (15). The ability of *R. centenum* to exhibit both scotophobic behavior in liquid medium and a phototactic response as a swarm colony makes it a particularly interesting organism for studies of eubacterial light perception.

One area that has not been addressed is whether the photosensory behavior of photosynthetic eubacteria involves the chemotaxis phosphorylation cascade. To investigate this possibility, we cloned, sequenced, and analyzed the transcription of the *che* operon from *R. centenum*. In a companion report (17), we describe detailed mutational analysis of individual genes in the *che* operon. The results of our analyses indicate that *R. centenum* contains a unique Che phosphotransfer cascade and that this cascade appears responsible for the transduction of signals that govern chemotaxis, phototaxis, and the scotophobic response.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type strain *R. centenum* SW (ATCC 51521) was grown either aerobically in PYVS or photosynthetically in CENS medium as described previously (25, 30).

All *Escherichia coli* strains were grown in Luria broth with appropriate antibiotics (33). *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.) and JM109 (Promega, Madison, Wis.) were used for routine DNA manipulations. S17-1 (36) was used to conjugally deliver plasmids into *R. centenum*. *E. coli* BL21(DE3)/pLysS (41) was used for overexpression of the *R. centenum* CheAY hybrid protein. Helper phage VCSM13 (Stratagene) was used for single-stranded DNA production from phagemids. For single-stranded DNA packaging, the cells were grown in 2×YT medium plus appropriate antibiotics.

DNA techniques and PCR amplification. Standard methods were used for the isolation and manipulation of DNA (33). Restriction endonucleases and other

* Corresponding author. Mailing address: Department of Biology, Indiana University, Jordan Hall, Bloomington, IN 47405. Phone: (812) 855-6595. Fax: (812) 855-6705. E-mail: cbauer@bio.indiana.edu.

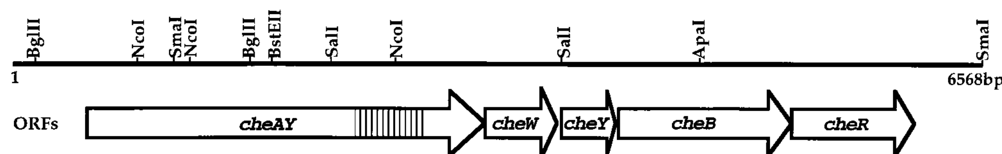

FIG. 1. Organization of the *R. centenum* chemotaxis gene cluster. The first ORF is the hybrid *cheA* and *cheY* gene. The linker region between the CheA and CheY portions of the hybrid is hatched.

DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.) or Amersham Life Science (Arlington Heights, Ill.) and were used as specified by the vendors. Oligonucleotide primers were purchased from GIBCO BRL (Gaithersburg, Md.).

PCR amplification of the portion of *cheA* was performed by designing oligonucleotide primers corresponding to the conserved N and G2 blocks of CheA protein (26). Degenerate primers to the CheA N block were 5′ (C/A)GNAA(T/C)TCNNTNGA(T/C)CA(T/C)GG 3′ and 5′ (C/A)GNAA(T/C)AG(T/C)NTNG A(T/C)CA(T/C)GG 3′. The primer designed to hybridize to the CheA G2 block was 5′ TTNACNAC(G/A)TCCATNCCNAC 3′. PCR was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.), using a Tricine-based buffer (27), 300 μM deoxynucleoside triphosphate, 20 μg of each primer, 1 μg of *R. centenum* chromosomal DNA, and 5 to 10 U of *Taq* DNA polymerase. *R. centenum* chromosomal DNA was isolated as described previously (33). After a denaturation step of 96°C for 5 min, PCR (30 cycles) was carried out as follows: 96°C for 45 s, 55°C for 2 min, and 72°C for 1 min. When three degenerate primers were combined, a DNA band of about 310 bp, which agreed with the expected size of a *cheA* fragment, was amplified. The PCR product was purified from an agarose gel and cloned into pT7Blue vector (Novagen Inc.). DNA sequence analysis of six clones indicated that all of them contained the same DNA sequence homologous to *cheA* from other bacteria.

The full-length *cheAY* was amplified by PCR using primers 5′ CACATATG GACGATCTGCTGAGCGA 3′ and 5′ CAGAGCTCATGCTGCACCTTTCGT 3′. To improve the yield and fidelity of the PCR product, a 30:1 (unit/unit) mixture of *Taq* and *Pfu* DNA polymerase (Stratagene) was used for amplification, using the following program (30 cycles): 96°C for 40 s, 58°C for 40 s, and 72°C for 4 min. The PCR primers incorporated an *Nde*I site covering the start codon of *cheAY* and a *Sac*I site following the stop codon. The PCR fragment was cloned in the *Sma*I site of pBSIISK$^+$ (Stratagene).

Amplification of the DNA fragment containing the promoter region upstream of *cheAY* gene was achieved by PCR using primers 5′ GGTAACCATCGAC TGGCAGGCCGAT 3′ and 5′ GGTAACCAGTGCGGCATTCGGATGTG 3′ (Pche3′). Notice that *Bst*EII sites flanked the PCR fragments to facilitate cloning into the reporter plasmid pZJD11 (see below for its construction). Also, a *Pfl*MI site was incorporated next to the *Bst*EII site in the first primer to allow deletion constructs to be made readily. A longer PCR fragment which extended ~500 bp further upstream from the promoter region was amplified by using primer 5′ GGTAACCTGGCCCAGGCCCCAGTCC 3′ and primer Pche3. The PCR fragments were cloned in the *Sma*I site of pUC19 (New England Biolabs), in which deletion constructs of the putative promoters were made.

Cloning of the chemotaxis operon and sequencing analysis. The 310-bp *cheA* fragment from *R. centenum* was radiolabeled with [^{32}P]dATP by using a random-primed labeling kit (Amersham Life Science). The probe was then used to screen an *R. centenum* genomic library (47) for cosmids that contained the *cheA* gene by colony hybridization (33). A total of eight positive colonies were obtained and found by restriction mapping to have overlapping inserts. One of the cosmids that contained an insert of about 30 kbp, pH$_2$, was chosen for further cloning and sequencing analysis. Restriction mapping and Southern hybridization analysis indicated that a ~5.5-kbp *Bst*EII and a ~5.5-kbp *Sma*I restriction fragment both hybridized to the *cheA* PCR fragment. The *Sma*I restriction fragment was subsequently cloned into *Sma*I-digested phagemids pBSIIKS$^+$ and pBSIISK$^+$, and the *Bst*EII fragment, blunted with T4 DNA polymerase and deoxynucleoside triphosphate, was cloned into the same vector digested with *Bam*HI and blunted. Restriction analysis of cloned *Bst*EII and *Sma*I fragments indicated that these two fragments overlapped about 700 bp with the former, covering the upstream sequence of *cheA*, and the latter, covering the downstream sequence of *cheA* (data not shown).

To expedite sequence analysis, a series of 450- to 500-bp nested deletions was generated on the *Sma*I and *Bst*EII clones, using exonuclease III and mung bean nuclease (33). Sequencing reactions were carried out by the dideoxy-chain termination procedure (34) with a Sequenase 2.0 kit (Amersham Life Science) and a supplement kit including IRD40-labeled dATP supplied by Li-Cor Inc. Reactions were run in a Li-Cor model 4000L automated DNA sequencer. DNA sequence was completed for both strands of the DNA. Sequence assembly and analysis were performed with the Genetics Computer Group Inc. (Madison, Wis.) sequence analysis package, using a SunOS 5.4 Microsystems Sparcstation.

Construction of an Ω-Sp polar insertion in *cheAY* gene. An Ω spectinomycin resistance (Ω-Sp) cassette, which is polar (28), was inserted into the CheA portion of the *cheAY* hybrid gene by first cloning a 1,413-bp *Nco*I fragment within *cheAY* into the vector pBSIIKS$^+$. The Ω-Sp cassette was then cloned into a unique *Bgl*II site at CheAY amino acid residue 375 (for details, see the accompanying report [17]). For creating chromosomal disruptions, the *cheAY*::Ω construct was cloned into the suicide vector pGmLacZ and recombined into the chromosome by a double-recombination event as described in detail in reference 17.

Heterologous expression of *cheAY*. The putative *R. centenum cheAY* gene was cloned in the T7 DNA polymerase expression vector pET-28a(+) (Novagen Inc.) digested with *Nde*I and *Sac*I. The construct was transformed in *E. coli* BL21 (DE3)/pLysS for inducible expression by isopropyl thiogalactopyranoside (IPTG; final concentration, 1.5 mM). Selective labeling of protein(s) expressed from the T7 promoter with ^{35}S was achieved by treating cells with rifampin prior to addition of [^{35}S]methionine as described previously (41, 42). The CheAY polypeptide was visualized by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and standard autoradiography (33).

Primer extension. Total RNA from *R. centenum* was purified and quantitated spectrophotometrically as previously described (19). The primer 5′ GGTTATC GGCAGTGCGGCAT 3′, which is complementary to nucleotides −18 to −37 relative to the putative *cheAY* translational start codon, was end labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (33). The unincorporated ATP was separated from the primer by using a G-50 Nick column (Pharmacia Biotech Inc., Piscataway, N.J.). The primer extension reaction was performed by mixing 12 μg of total RNA, 20 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.), and reaction buffer in the presence of 20 μM actinomycin D. The primer extension reaction, in a total volume of 50 μl, was carried out at 37°C for 1 h, after which cDNA was precipitated with ethanol and resuspended in loading buffer. For the sequencing ladder, the ^{32}P-labeled primer was used in sequencing with a ΔTAQ cycle sequencing kit (Amersham) with the *cheA Bst*EII fragment clone as the template. The ladder and the RNA primer-extended product were electrophoresed side by side on a 6% polyacrylamide-urea gel. The result was visualized by autoradiography.

Construction of β-galactosidase reporter plasmid and β-galactosidase assay. pZM400 (21) is an RK2-derived plasmid that was shown to replicate and be stably maintained in *R. centenum* (18). However, since *R. centenum* is naturally resistant to kanamycin, the kanamycin cassette in pZM400 was replaced by a gentamicin resistance cassette (45), generating plasmid pZJD11. The 536-bp PCR fragment with the putative promoter sequence upstream of the *cheAY* gene was cloned into the unique *Bst*EII site of pZJD11, giving rise to reporter plasmid pZJD11-Pche. Similarly, the longer PCR product extending ~500 bp further upstream of the promoter region was cloned in pZJD11, which was called pZJD11-Pche′. Two deletion constructs from pZJD11-Pche were made: pZJD11-PcheΔ1, with a deletion from the *Pfl*MI site to the unique *Bgl*II site, and pZJD11-PcheΔ2, with a deletion from the *Bsp*EI site to the *Bsm*I site. Also, one deletion construct from pZJD11-Pche′ was made: pZJD11-PcheΔ3, with the same deletion as in pZJD11-PcheΔ2. Orientation of the insertions was confirmed by restriction digestion. Then all constructs were conjugated from *E. coli* S17-1 in wild-type *R. centenum*.

β-Galactosidase (*lacZ*) assays were performed as described previously (48). Cell extracts were obtained from cells carrying the reporter plasmids grown aerobically in CENS medium either in liquid medium or on 0.8% agar plates. Cells on plates were incubated for 4 to 5 h at 42°C and collected by rinsing off the surface with ice-cold phosphate-buffered saline.

Nucleotide sequence accession number. The nucleotide sequence of the *R. centenum che* gene cluster has been deposited in GenBank under accession no. U64519.

RESULTS

Cloning and sequence analysis of the *che* operon. A portion of *cheA* from *R. centenum* was isolated by PCR amplification using degenerate oligonucleotide primers deduced from the highly conserved N and G2 blocks (26) in CheA sequences from *E. coli*, *Salmonella typhimurium*, *Bacillus subtilis*, *Myxococcus xanthus*, and *Enterobacter aerogenes* (6, 9, 20, 24, 39). PCR amplification yielded a ca. 310-bp DNA fragment which

A.

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                                                                                *
rcent.ay   MD------DLLSEFLTETNENLAVLDVELVRLER---NPNDPGLLSNIFRLVHTIKGTCGFLGLPRLESVAHAAENVLGRFRDGELSVTPYAVSLILESL
mxant.ay   MDTEALKKSLLKKFQEVTADRLQKIQLGVLDLEKETADQAAED----VARELHTMKGEARMLGLAAIGQLAHAAEDVLRAERFGK-TATEVATDVLLRAC
ecoli.a    MSMDI--SDFYQTFFDEADELLADMEQHLLVLQP---EAPDAEQLNAIFRAAHSIKGGAGTFGFSVLQETTHLMENLLDEARRGEMQLNTDIINLFLETK
bsubt.a    MDMN----QYLDVFIDESKEHLQTCNEKLLLLEK---DPTDLQLVHDIFRAAHTLKGMSATMGYTDLAHLTHLLENVLDAIRNGDMEVTSDWLDILFEAL
rspha.a    --MDQ--SDIRSAFFVECEDLMEALNEGLDRIEDTLDDGHDDETVNAVFRAVHSIKGGAGAFKLDALVRFAHQFETTLDALRAGRVSADPPLLALLHKAA

rcent.ay   DTIKGIL-SVLEQTEAEPAGSDEDLINRLNAMAEGRELPKAGAAPAAEAPAAA--------------------------------------------------
mxant.ay   DVLSDLNEDLSGANTGNPA--SEEMVRMLAEVSGQTPPAIAGARPVAPPPAPPPAPVAAPVVTPAAVAAPP-APVQAPVAPPPTQ---------------
ecoli.a    DIMQEQLDAYKQSQEPD-AASFDYICQALRQLAL--EAKGETPSAVT------RLSV-VAKSEPQDEQSRSQSPRRIILSPLKA-----GEVDLLEEELG
bsubt.a    DHLETMVQSIIDGGDGKRDISE--VSAKLDVNGAHAESAASAEPAEAQSSASDWEYDEFERTVIQEAEEQGFKRYEIKISLNEN---CMLKAVRVYMVFE
rspha.a    DRLSDLLQAARTGSETA-TIDPDDLVAQLAQAAG--EEEAGEADAEDLGFVPMRLDLDLPAAAPDEGQSGCYS---IDFSPTRALYACGNDTSILFRVLS

rcent.ay   --------PAATPAAPAEQPDEHG---------FVPVPASKEV--LAAATAAVPAPAGARA--VATAAEPPPPAEPAPPAARVEAVDDTRGEP-------
mxant.ay   --APVAEPGAHAAAAAPHPAAAHGRD----------EEAPSAAKSAVADRSIRVNVEVLDALGLLAGDLLVESARGRLRSSETEALFERFSRLGDRFLRL
ecoli.a    HLTTLTDVVKGADSLSAI----------------LPGDIAEDDITAVLCFVIEADQIT-FETVEV-SPKISTPPVLKLAAEQAPTGRVEREKTTRSNE---
bsubt.a    KLNEVGEVAKTIPSAEVLETEDFGT---DFQVCFLTHQSAEDIEQLINGVSEIEHVEVIQGAPLTSAEKPEESKQEDSPAAAVPANEEKQKQPAKNDEQA
rspha.a    DLGRM-EVRLDRSRLPPDFDALDWQESWLDWHLTLETDEPEHQIDEVFEFVEDLCRLE-IRPMAMPAPRTDPDEPDPEPDPPASGPAAAKKSPAEDH---

rcent.ay   KESAVAQQSIRVHVDLLENLMTMVSELVLTRNQLLQILRSQK---ESEFAAPLQRLNHVTSELQEGVMKTRMQPIGNAWAKLPRLVRDLSHELHKKIELQ
mxant.ay   AEEIDISNEVREQLDRVESDLHMLRD------------DAFRFVRRND--DGINTLHGNLAKMADHVAEARLVPLSTVFDAFPRAVREMSRTQGKEVDLV
ecoli.a    -STS-----IRVAVEKVDQLINLVGELVITQSMLAQRSSELDPVNHGDLITSMGQLQRNARDLQESVMSIRMMPMEYVFSRYPRLVRDLAGKLGKQVELT
bsubt.a    KHSAGGSKTIRVNIDRLDSLMNLFEELVIDRGRLEQIAKELE---HNELTETVERMTRISGDLQSIILNMRMVPVETVFNRFPRMIRQLQKELNKKIELS
rspha.a    -RTSSPRATVRVELDRVDRLINIVGELVINQAMLSQCVQDEGVPPRSPVRNRLDDFRNLARELQESVMAIRAQAIKPLFQRMSRIARESASEISQKQIRLV

                           ┌──N-Block──┐                               ┌G1-Block┐
rcent.ay   MLGAETELDRQVIELIKDPLIHMVRNSGDHGLELPAERAAKGKSETGRITLNAFHEGGHITIEISDDGRGLAIEKIKAKAIQNGLATEAELAAMSDQQIQ
mxant.ay   IENADIGVDRSMLGDVRDALVHLLRNSVDHGVESPDTRQQLGKPLNGRIRIRVRVDGDMLHIEVEDDGRGIDPERLRQAAISKRLINAVQAAALSEREAI
ecoli.a    LVGSSTELDKSLIERIIDPLTHLVRNSLDHGIELPEKRLAAGKNSVGNLILSAEHQGGNICIEVTDDGAGLNRERILAKAASQGLTVSE---NMSDDEVA
bsubt.a    IIGAETELDRTVIDEIGDPLVHLIRNSIDHGIEAPETRLQKGKPESGKVVLKAYHSGNHVFIEVEDDGAGLNRKKILEKPLER-VITEKEAETLEDNQIY
rspha.a    TEGESTEVDKTVIERLADPLIHMIRNAVDHGIEPADRRLHLGKPPVGLITLTAPHRSGRVLIEIKDDGAGINRPRVLEIAQGKGLVAQD--AQLTEEEID

 ┌─F-Block─┐   ┌─G2-Block─┐
rcent.ay   SFIMKPGFSTAAAVTSVSGRGVGMDVVKTNIERIGGTIEMKSVEGKGTTFTIKIPLTLAIVSALIVECATERFAIPQISVIELVRAATDSEHKIERIHGT
mxant.ay   ELIFRPGFSTRDQVSELSGRGVGMDVVKRKVETLGGSVGVSSRIGRGSTITLRLPQSLALMKVLLVRLGDDVYGMPAADVEAVMRVKPDDRLEIF---GT
ecoli.a    MLIFAPGFSTAEQVTDVSGRGVGMDVVKRNIQKMGGHVEIQSKQGTGTTIRILLPLTLAILDGMSVRVADEVFILPLNAVMESLQPREADLHPL--AGGE
bsubt.a    ELIFAPGFSTADQISDISGRGVGLDVVKNKLESLGGSVSVKSAEGQGSLFSIQLPLTLSIISVLLIKLEEETFAIPISSIIE---TAVIDRKDILQTHDR
rspha.a    GLLFMPGFSTASVVSDLSGRGVGMDVVKSAIESLGGRITIASDPGVGTTFTISLPLTLAVLDGMVVDVGGQTMVVPVSAIVETLRPRPADIHVL--GSGD

rcent.ay   PVLRLRNRLLPLVSLQRLLKLGADDEDKRETEIVVTQVGN-YVFGIIVDKVFDTEEIVVKP-VAPILRHIELFSGNTILGDGSVLMILDPNGIAAVTGEM
mxant.ay   LAVRHRGKPTALVALGPLLGLNGGNRFDKPPAVVVRHGEDHA--ALVVDGFVDEREVAVKPCGGEFLKAAPFIAGTALEDGRIAVLLHVPDI--------
ecoli.a    RVLEVRGEYLPIVELWKVFNVAGAKTEATQGIVVILQSGGRR-YALLVDQLIGQHQVVVKN-LESNYRKVPGISAATILGDGSVALIVDVSALQAINREQ
bsubt.a    EVIDFRGHIVPVVYLKEEFKIEDTRKDAEQLHIIVVKKGD-KPTAFVVDSFIGQQEVVLKS-LGDYLTNVFAISGATILGDGEVALIIDCNALII*
rspha.a    QVVAIRGSLVPIVDCGSIFGFRAPVRSYEESVLLVVETARQKLCALVVDTIHDQRQVVIKG-LENGYGRIPGVAAATILGDGRIALIIAPEEAVDIGTSG

rcent.ay   AVQDSAAATDITSKQSRREDDKLAMLLFTAGSGGPKAVPLSLVARLEDIDLNQVELSNGSPVVQYRGKLMPLVPIDPAWQIVRDKRQPVVVFADGERSMG
mxant.ay   -----------------------------------------MAEVRRM------------ARPVTQAPA----------------------------------
ecoli.a    RMANTAA*
bsubt.a
rspha.a    GTFSMEF*

rcent.ay   LVVDEIVDIVEDRLNVELGTERPGFLGSAIIAGKATDVIDAGFYLTQAFKDWFGSSRSENFEDVRAHRVLLVDDSPFFRNLITPTLTVHGYDVVS-VESA
mxant.ay   ----------AKRL------------------------------------------------------RVLLVDDSPIARATEGALVKALG-HSVEEAQDG
ecoli.y                                                                 MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDG
bsubt.y                                                                    MAHRILIVDDAAFMRMMIKDILVKNGFEVVAEAENG
rspha.y1                                                                   MPLTVLAIDDSRTIRELLREALVQAGFE-VHLAIDG
rcent.y                                                                     MKSCLVVDDSRVVRKVARKILEELGFT-CTEAEDG

                         #
rcent.ay   DDALALCEAGEEFDVIVSDIEMPGMNGLDFAQAVRASGRWAQVPMVALSSHASPRDLDRGRQAGFNDYVAK--FDRDALIFTLQQTISETKGAA
mxant.ay   EEAYVKVQN-NTYDLILTDVQMPKLDGFSLARRLKSTPAVARIPVIILSSLASPEDKRRGLDAGADAYLVKGELGVEVLAQAIDR-------LT
ecoli.y    VDALNKLQAGG-YGFVISDWNMPNMDGLELLKTIRADGAMSALPVLMVTAEAKKENIIAAAQAGASGYVVKP-FTAATLEEKLNKIFE--KLGM
bsubt.y    AQAVEKYKEHS-PDLVTMDITMPEMDGITALKEIKQIDAQA--RIIMCSAMGQQSMVIDAIQAGAKDFIVKP-FQADRVLEAI----N--KTLN
rspha.y1   LDGLEKLEAAK-PHAVITDINMPRMDGFGFIRAVREQPQHSALPIIVLTTESAAELKAKAREAGATAWIVKP-FDEAKLVSALRRV-----AVA
rcent.y    KQAMEKCAEAM-PDAVLLDWNMPVMTGIEFLRRLRKMTGGQAPKVVFCTTENDLAHIQEALSAGANEYIMKP-FDSDIIQTKFQQV-----GLL
                                                                    ●        ●    ●    ● ●  ●●●  ●  ●
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B.

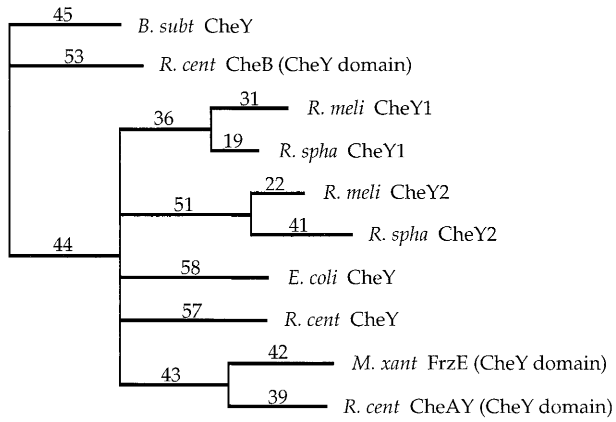

FIG. 2. Sequence comparison of CheA and CheY proteins. (A) Sequence alignments of CheA (.a), CheAY hybrid (FrzE) (.ay), and CheY (.y) proteins from *E. coli* (ecoli), *B. subtilis* (bsubt), *M. xanthus* (mxant), *R. sphaeroides* (rspha), and *R. centenum* (rcent). N, G1, F, and G2 blocks in CheA are depicted. *, autophosporylated histidine in CheAY; #, aspartate residue in CheY that is phosphorylated by the kinase CheA. Conserved residues are highlighted in black boxes, and other homologous residues are shaded gray. Dots under the CheY sequences are putative residues involved in signalling to the flagellar motor switch complex. Sequences were first aligned with Clustal W and manually adjusted to minimize gaps in the alignment. (B) Unrooted phylogenetic tree, constructed by using the PAUP program, showing the relationship among various CheY polypeptides. Numbers above horizontal bars represent arbitrary phylogenetic distances, and lengths of bars are proportional to evolutionary distances. *B. subt*, *B. subtilis*; *R. meli*, *R. meliloti*; *R. spha*, *R. sphaeroides*. The *R. centenum* (*R. cent*) CheB CheY homologous region is from amino acid residues 21 to 153 of CheB, the *M. xanthus* (*M. xant*) FrzE CheY domain is from residues 660 to 778, and the *R. centenum* CheAY CheY domain is from residues 776 to 901.

fits the predicted size based on the conserved distance between the blocks. Sequence analysis of the PCR fragment confirmed that it arose from an *R. centenum cheA* homolog, as indicated by the presence of the additional conserved G1 block and F block. The PCR product was subsequently used as a hybridization probe to screen an *R. centenum* genomic library (47) for clones that contained the entire *cheA* gene. One of the positive cosmids, pH_2, was chosen for further sequencing analysis.

A codon preference plot of a ca. 6.5-kbp region, using a codon preference table generated from previously sequenced *R. centenum* genes, revealed the existence of five closely linked open reading frames (ORFs) (Fig. 1). A search of protein databases indicated that each of the identified ORFs exhibited sequence similarity to known *che* genes from other organisms. The first ORF encodes a large 901-amino-acid-residue polypeptide that exhibits sequence similarity to both CheA and CheY (Fig. 2). This ORF has thus been given the gene designation *cheAY*.

Since a CheAY hybrid polypeptide has been found only in *Myxococcus* (24), which utilizes gliding motility, we decided to confirm that the *R. centenum cheAY* gene indeed encodes a single polypeptide. For this analysis, we overexpressed the *R. centenum cheAY* gene in *E. coli*, using a T7 RNA polymerase-based expression system (see Materials and Methods). Coomassie blue staining of the gel after denaturing SDS-PAGE separation of polypeptides from uninduced and induced cell extracts showed the presence of a unique polypeptide in the induced extracts (data not shown) with a electrophoretic molecular mass of about 108 kDa (the calculated size for CheAY fused to the histidine tag is 99.3 kDa). To verify that this polypeptide was synthesized from the T7 promoter, we selectively labeled polypeptides derived from T7 transcripts by first inhibiting host cell transcription with rifampin and then adding [^{35}S]methionine. Since T7 polymerase is resistant to rifampin, prolonged incubation in the presence of this drug will generate cells that contain a single T7 polymerase-derived transcript that can be used for selective labeling of T7-expressed polypeptides (41, 42). An autoradiograph of the SDS-PAGE-separated polypeptides indicated the selective labeling of a single 108-kDa polypeptide superimposable with the unique polypeptide visualized by Coomassie blue staining (Fig. 3). These results confirm our sequence data showing that *R. centenum* synthesizes a hybrid CheAY polypeptide.

Alignment of CheAY with CheA from other species (Fig. 2A) indicates that the amino-terminal region of CheAY (amino acids residues 1 to 616) exhibits 35% identity and 56% similarity to CheA from *E. coli*. Notable areas of sequence conservation include those of the previously mentioned N block (amino acid residues 330 to 345), the G1 block (amino

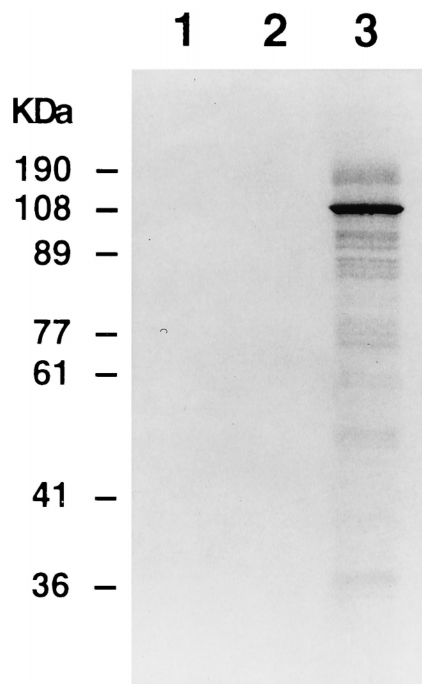

FIG. 3. Selective labeling of *R. centenum* CheAY hybrid protein expressed in *E. coli* with [^{35}S]methionine, using an inducible T7 RNA polymerase expression system. Lane 1, sample from uninduced *E. coli* BL21(DE3)/pLysS harboring expression vector pET28^{+} and *cheAY*; lane 2, sample from IPTG-induced *E. coli* containing vector pET28^{+} only; lane 3, sample from IPTG-induced *E. coli* with pET28^{+} and *cheAY*. The positions of prestained protein molecular markers are shown on the left.

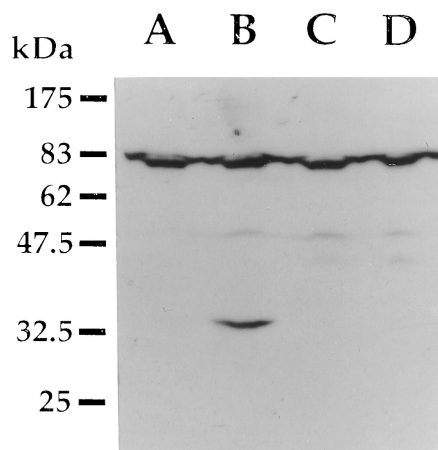

FIG. 4. The chemotaxis gene cluster in *R. centenum* is organized in an operon. Western blot analysis of whole *R. centenum* cell samples from wild-type cells (lane A), wild-type cells with the *cheR*::Flag tag chromosomal insertion (lane B), the *cheAY* mutant with insertion of an Ω element (lane C), and the *cheAY* Ω insertion mutant with the *cheR*::Flag tag (lane D), using monoclonal antibody M2. Approximately 50 μl of cells at 300 Klett photometric units was collected and mixed with sample buffer to load on each lane. Only lane B shows the presence of the CheR::Flag protein (ca. 35 kDa). The higher-molecular-mass band is an endogenous protein that cross-reacted with the M2 antibody. Positions of prestained protein molecular markers are shown on the left.

acid residues 374 to 383), the F block (amino acid residues 414 to 423), and the G2 block (amino acid residues 429 to 440). The CheA homologous region is then followed by a 159-amino-acid linker region (amino acid residues 617 to 775) which exhibits no strong similarity to any protein in the databases. The CheA-CheY hybrid of *M. xanthus* (termed FrzE) also exhibits a linker region between the CheA and CheY domains, although it is smaller and exhibits no sequence similarity with the linker of *R. centenum* CheA-CheY hybrid. The CheY domain (amino acid residues 776 to 901) from the hybrid protein exhibits 29% identity and 52% similarity to CheY from *E. coli*. All of the previously identified critical residues in *E. coli* CheY that are involved in forming the acid pocket for acceptance of the phosphate from CheA (Asp 12, Asp 13, Asp 57, and Lys 109, using *E. coli* CheY coordinates) (38, 43) are present in the CheY domain of CheAY. However, a highly conserved proline at position 110 (using *E. coli* numerical nomenclature) is absent in the CheY domain.

From Fig. 1 and the CheAY alignment in Fig. 2, we observe that the third ORF in the *R. centenum che* gene cluster (*cheY*) encodes a polypeptide that is also homologous to CheY. Thus, *R. centenum* has two CheY homologs, one fused to CheA and the other free existing. More than one CheY homolog has been reported to exist in the photosynthetic bacterium *Rhodobacter sphaeroides* (44) as well as in *Rhizobium meliloti* (13). A dendograph of the various CheY proteins indicates that the *R. sphaeroides* and *R. meliloti* CheY1 proteins form a separate lineage from the CheY2 proteins of these species (Fig. 2B). This finding suggests that the duplication of *cheY* genes from *R. sphaeroides* and *R. meliloti* likely predates the divergence of these species. Interestingly, the CheY domain of CheAY from *R. centenum* groups with that of CheY domain of *Myxococcus* FrzE. Since *R. centenum* is a member of the α1 subdivision (8) and *Myxococcus* is a member of the more distant δ1 subdivision of purple bacteria (46), it seems that the CheAY hybrid may not be recently derived; instead, it might be a more ancient sequence that has been retained in these species. Analysis of *R. centenum* CheY protein indicates that it is more closely related to CheY from *E. coli*, followed by the CheY1 from *R. sphaeroides* and *R. meliloti*.

Analysis of the remaining ORFs in the *che* cluster indicates that the second ORF encodes a CheW homolog with 28% identity and 59% similarity to CheW from *E. coli*. The fourth ORF encodes a CheB homolog with 38% identity and 63% similarity to CheB from *E. coli*, and the fifth ORF encodes a CheR homolog with 31% identity and 54% similarity to CheR from *E. coli*. The *cheR* gene is followed by a inverted repeat sequence (ca. 180 nucleotides downstream of the stop codon) that resembles a prokaryotic *rho*-independent transcriptional terminator.

***R. centenum che* genes are cotranscribed.** As demonstrated by sequence analysis, all of the *che* genes are tightly clustered with the same transcriptional polarity, indicating that they are most likely cotranscribed as in an operon. To ascertain whether the *che* genes are indeed cotranscribed, we set up an analysis of *cheR* expression (the last gene in the operon) in the presence or absence of an upstream transcription termination site, Ω-Sp. To measure CheR expression, we constructed an epitopically tagged version of CheR (CheR::Flag) which contains a eight-amino-acid extension at the carboxyl terminus that can be monitored by Western blot analysis using commercial monoclonal antibodies generated to the epitope tag. The CheR::Flag construct was recombined into the chromosomal copy of CheR by homologous recombination using a suicide plasmid. As observed in Fig. 4, lane B, CheR::Flag can readily be observed by Western blot analysis in this construct. To test for cotranscription, we also constructed a version of the CheR::Flag strain that contained a polar spectinomycin resistance gene that was recombined into *cheAY*. As demonstrated in lane C, insertion of the Ω-Sp cassette into *cheAY* results in loss of a CheR::Flag signal, thereby demonstrating that transcription of the last gene of the operon, *cheR*, is dependent on transcription that is initiated upstream of the Ω-Sp termination site inserted within *cheAY*.

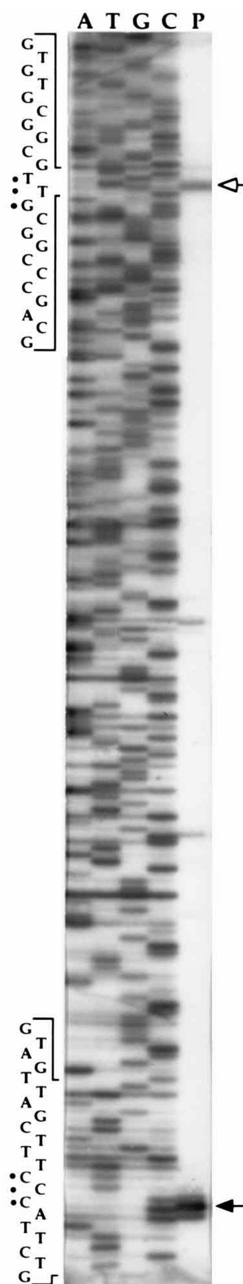

FIG. 5. Mapping of the transcriptional start sites of the chemotaxis gene operon by primer extension. Lanes A, T, G, and C, DNA sequencing reaction using the same primer for performing the primer extension; lane P, primer extension reaction. The two strong signals are indicated by arrows on the right; the positions of three weak signals are depicted in the complete sequence of the putative promoter region in Fig. 6A. The nucleotide sequences surrounding the two arrows are given on the left; transcriptional start sites are indicated by dots.

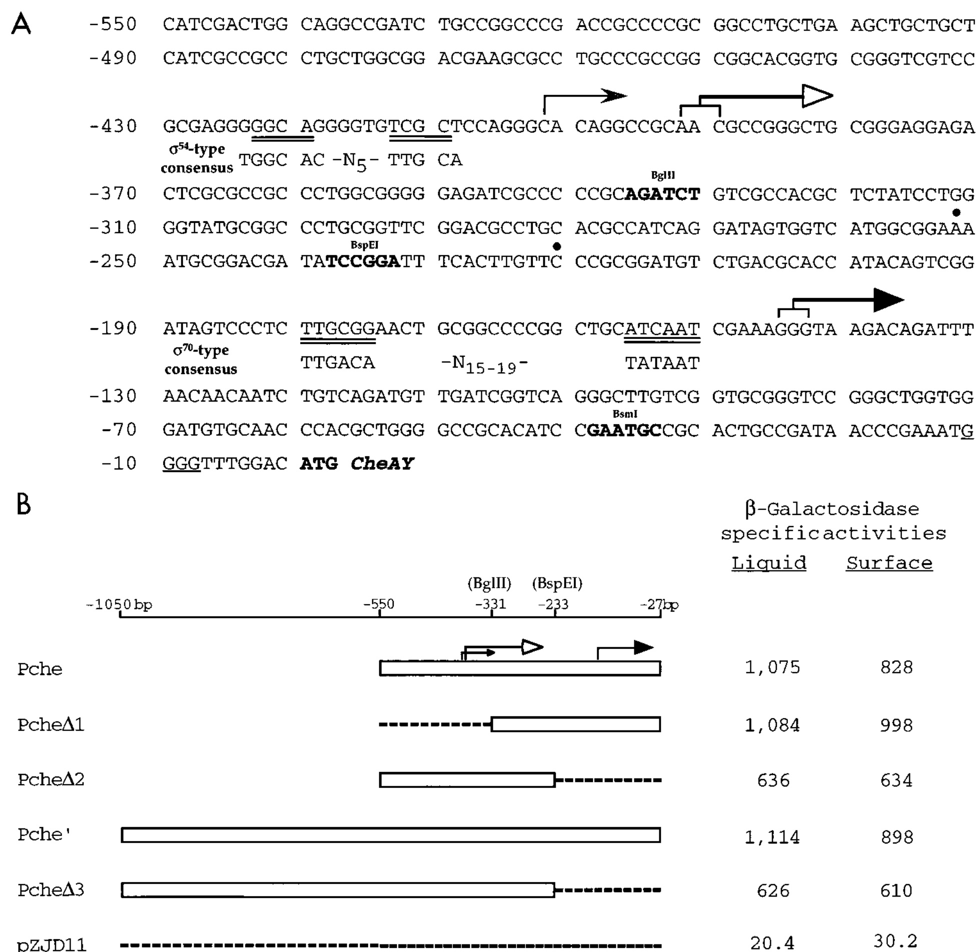

FIG. 6. Nucleotide sequence of the 540-bp PCR fragment upstream of the *cheAY* gene that was cloned in reporter plasmid pZJD11. Numbering is relative to the putative translational start site of *cheAY*. The putative σ^{54} and σ^{70} motifs are double underlined; their consensus sequences are given below. Strong signals and direction of transcription from primer extension are marked by arrows above the start sites; weak signals are indicated by dots above them. The putative ribosome binding site of *cheAY* is underlined; the translational start codon of *cheAY* and relevant restriction sites used for constructing deletions are marked in boldface. (B) Schematic view of *lacZ* reporter constructs and β-galactosidase specific activities (micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed/minute/milligram of total protein in cell extract). Liquid, samples collected from cells grown in liquid medium; plate, samples collected from cells grown on 0.8% agar plates. Each sample was assayed in quadruplicate, and average values are presented.

The *che* operon has two transcription start sites. In *E. coli* and *Caulobacter crescentus*, *che* gene expression relies on a σ^{28} subunit of RNA polymerase for promoter recognition (for reviews, see references 5 and 22). σ^{28} activity is dependent on synthesis and assembly of the flagellar basal body; thus, *che* gene expression is coordinated in a hierarchic fashion with flagellin biosynthesis (22). To characterize the promoter region of the *R. centenum che* operon, we performed primer extension analysis as well as deletion analysis of the promoter region. The results of primer extension analysis (Fig. 5 and 6A) demonstrate the presence of two strong 5′-end signals located at bp −391 and −144 upstream of the *cheAY* start codon as well as weaker signals at bp −402, −231, and −192. The signals at bp −402 and −391 can be clearly resolved with a long sequencing gel run (data not shown). Inspection of the DNA sequence upstream of these regions indicated no obvious σ^{28} promoter consensus sequences. Instead, the sequence upstream of the strong signal at bp −144 exhibits putative σ^{70} promoter motif, whereas the region upstream of the signal at bp −402 exhibits a σ^{54} motif (Fig. 6A).

To functionally analyze promoter location and to confirm results of the primer extension assays, we also constructed several transcriptional fusions of the *che* promoter region to the *E. coli* β-galactosidase gene (*lacZ*), using a vector that is capable of replicating in *R. centenum*. For this analysis, five plasmids were constructed. Plasmids pZJDH-Pche and pZJD11-Pche′ contain 536 and 1,033 bp, respectively, of DNA upstream of the start of *cheAY*, which spans all of the 5′ ends mapped by primer extension analysis. As shown in Fig. 6B, these constructs clearly contain *che* promoter function, as indicated by similar high levels of β-galactosidase activity. The existence of two promoters is indicated by plasmid pZJD11-PcheΔ1, which contains a DNA segment from bp −27 to −331 with only the putative σ^{70} promoter, and by plasmids pZJD11-PcheΔ2 and pZJD11-PcheΔ3, which contain only the putative σ^{54} promoter (DNA segments from bp −233 to −540) (Fig. 6). As indicated in Fig. 6B, strains containing either plasmid exhibit high levels of β-galactosidase activity. Taken together with the primer extension data, these data lead us to conclude that the *che* operon is indeed expressed from two promoters. One promoter appears to be of the σ^{70} type that is responsible for the strong primer extension signal located at bp −144. The

second appears to be initiated from a σ^{54}-like promoter that is responsible for initiating the uppermost primer extension signals at bp −402. We also observed no significant difference in expression of either of these promoters in swimmer (liquid-grown) versus swarm (agar surface-grown) cells (Fig. 6B).

DISCUSSION

***R. centenum* contains a unique chemotaxis signal transduction cascade.** Sequence analysis of the *R. centenum* chemotaxis gene cluster indicated the existence of five ORFs that exhibit homology to chemotaxis genes from other species. Many of the conserved components of the signal transduction cascade (CheW, CheY, CheB, and CheR) are represented. An absence of a CheZ homolog is not unexpected since it has not been found in nonenteric species. One unexpected finding was the existence of an ORF coding for a CheA-CheY hybrid polypeptide. A related CheA-CheY hybrid, termed FrzE, has been observed in the gliding eubacterium *M. xanthus* (24). Mutational analysis has demonstrated that FrzE is involved in controlling the gliding reversal frequency (24). Similar analysis of *R. centenum* indicates that CheAY is involved in controlling the tumbling frequency, thereby indicating that CheAY and FrzE may have comparable functions (17).

The observation that a flagellated organism contains a CheAY hybrid raises interesting questions such as how such a hybrid is capable of efficiently communicating with chemo- and photoreceptors as well as with the flagellar motor switch complex. In vitro protein-protein interaction experiments indicate that CheW and CheA from *E. coli* are capable of forming a thermodynamically stable complex with chemoreceptors under physiological conditions (11, 35). If *R. centenum* CheW-CheAY forms a stable complex with receptors, communication with the polar flagellar motor would require that receptors be located near flagella. This possibility is supported by protein localization studies which have indicated a polar localization for chemoreceptors in *E. coli* and *C. crescentus* (1, 23).

The existence of a second CheY homolog also raises questions about the role of the separate CheY domain versus that of the CheY domain of CheAY. Since the presence of more than one CheY homolog has been found in *R. meliloti* and *R. sphaeroides*, the existence of two proteins with CheY sequence similarities is not unprecedented. Mutational analysis of the CheY in *R. centenum* indicates that it has a much more pronounced effect on swarmer cell motility than on swimmer cell motility (17). One model emerging from our mutational studies is that CheAY may control rotation of the polar motor and CheY may control the lateral motor. While we have no direct evidence for separate motors in *R. centenum*, we have observed that the polar and lateral flagella are composed of different flagellin and basal ring subunits (16, 32). Evidence for different specificities of the *R. centenum* CheY homologs to the motor complex can also be obtained by inspection of the motor docking domains that have been identified by mutational and crystallographic analyses of CheY homologs from *E. coli* and *S. typhimurium* (31, 37, 38, 43). As shown in the CheY alignment in Fig. 2, 7 of 10 residues which are thought to be involved in docking to the motor polypeptides FliM and FliG (37) are not conserved between CheAY and CheY. Indeed, half of the amino acid substitutions involve alterations in hydrophobicity or charge. Taken together with the results of our mutational analyses (17), these results indicate that the CheY domain of CheAY and CheY must serve distinct roles in chemotactic signal transduction in *R. centenum*.

The *R. centenum che* operon promoters deviate from the norm. In enteric bacteria as well as in *Caulobacter*, expression of genes that contribute to motility are highly ordered and responsive to the state of flagellar assembly. Expression of chemotaxis and flagellin genes, which are grouped into the lowest class, is dependent on σ^{28} for promoter sequence recognition. Therefore, it is surprising to observe that the *R. centenum che* operon has two promoters, one with a σ^{70}-like sequence motif and the other with a σ^{54}-like sequence motif. We observe little difference in activity of either promoter when the cells are shifted from a liquid to a solid surface, and it thus remains unclear what significance, if any, these promoter motifs may have in regulating *che* gene expression. To determine if *R. centenum che* gene expression is controlled in a hierarchic fashion with flagellar assembly, it will be necessary to analyze *che* expression in mutants that fail to synthesize polar and lateral flagella. Such analyses await characterization of these types of mutants in *R. centenum*.

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