



The Master Regulators of the Fla1 and Fla2 Flagella of *Rhodobacter sphaeroides* Control the Expression of Their Cognate CheY Proteins

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ABSTRACT *Rhodobacter sphaeroides* is an alphaproteobacterium that has two complete sets of flagellar genes. The *fla1* set was acquired by horizontal transfer from an ancestral gammaproteobacterium and is the only set of flagellar genes that is expressed during growth under standard laboratory conditions. The products of these genes assemble a single, subpolar flagellum. In the absence of the Fla1 flagellum, a gain-of-function mutation in the histidine kinase CckA turns on the expression of the *fla2* flagellar genes through the response regulator CtrA. The rotation of the Fla1 and Fla2 flagella is controlled by different sets of chemotaxis proteins. Here, we show that the expression of the chemotaxis proteins that control Fla2, along with the expression of the *fla2* genes, is coordinated by CtrA, whereas the expression of the chemotaxis genes that control Fla1 is mediated by the master regulators of the Fla1 system. The coordinated expression of the chemosensory proteins with their cognate flagellar genes highlights the relevance of integrating the expression of the horizontally acquired *fla1* genes with a chemosensory system independently of the regulatory proteins responsible for the expression of *fla2* and its cognate chemosensory system.

IMPORTANCE Gene acquisition via horizontal transfer represents a challenge to the recipient organism to adjust its metabolic and genetic networks to incorporate the new material in a way that represents an adaptive advantage. In the case of *Rhodobacter sphaeroides*, a complete set of flagellar genes was acquired and successfully coordinated with the native flagellar system. Here we show that the expression of the chemosensory proteins that control flagellar rotation is dependent on the master regulators of their corresponding flagellar system, minimizing the use of transcription factors required to express the native and horizontally acquired genes along with their chemotaxis proteins.

KEYWORDS *Rhodobacter sphaeroides*, chemotaxis, bacterial flagellum, horizontal transfer, FleQ, CtrA, RpoN, sigma-54

Chemotaxis is a complex response that enables bacteria to detect and swim toward or away from positive or negative stimuli in the surrounding environment. To achieve chemotaxis, a signal transduction system senses and controls the flagellar motor. Signals are sensed by specialized receptors that are assembled together with the chemotaxis proteins CheW and CheA to form complex arrays. In response to receptor occupancy, CheA phosphorylates the response regulator CheY, which in turn binds to the flagellar switch complex to control flagellar rotation. The response is terminated by lowering the levels of CheY-P, through the action of the phosphatase CheZ. The chemotaxis proteins CheB and CheR play a key role in adaptation by

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chemically modifying the chemotaxis receptors. The methyltransferase CheB is stimulated upon phosphorylation by CheA, and CheR is a constitutive methyltransferase. The activity of these proteins resets the signaling state of the receptor (for reviews, see references 1–5).

This basic chemotaxis system is found in many species other than *Escherichia coli* and *Salmonella enterica*. However, many species have multiple copies of the chemotaxis genes. The products of these additional copies frequently are involved in chemotaxis, but in some species these copies have evolved to control other cellular functions such as development, biofilm formation, and twitching motility mediated by type IV pili (6, 7).

In several bacterial species, the chemotaxis genes are expressed coordinately with the flagellar genes. Therefore, the cytoplasmic components that control flagellar rotation are present only when the flagellum is formed. In the paradigmatic bacteria *E. coli* and *S. enterica*, the chemotaxis genes are expressed when the cytoplasmic and membrane components of the flagella are already formed (8–10). A similar situation has been observed in *Pseudomonas aeruginosa* and *Xanthomonas campestris* (11, 12). In the alphaproteobacteria *Caulobacter crescentus* and *Sinorhizobium meliloti*, chemotaxis genes are expressed under the control of the master activator protein of the flagellar genes (13, 14). Therefore, the chemotaxis genes are expressed early during flagellar biogenesis.

Rhodobacter sphaeroides is an alphaproteobacterium with two different flagellar systems, multiple copies of the chemotaxis genes, and four different *rpoN* genes, encoding functionally different σ^{54} factors. σ^{54-2} has been shown to be required to transcribe one of the flagellar sets (15–17).

This bacterium assembles a single, subpolar flagellum under standard laboratory growth conditions (18), and the products of the *fla1* genes are responsible for the formation and functioning of this structure. The expression of these genes follows a hierarchical pattern. At the top of the hierarchy, the master protein FleQ, together with the RNA polymerase core (E) associated with the σ^{54-2} factor, promotes the expression of the class II flagellar operon *flaT-flaEFGHI*. Subsequently, the heterooligomeric complex FleQ/FleT and E- σ^{54-2} bring about the transcription of genes encoding the proteins that form the hook and the basal body (HBB), *flaA* (which encodes σ^{28}) and *flgM* (encoding the anti-sigma factor of σ^{28}). Once the HBB has been assembled, FlgM is exported out of the cell, and σ^{28} is free to associate with E to transcribe the late flagellar genes, such as those encoding flagellin (FliC) and its scaffold protein, FliD (19).

The other flagellar system present in *R. sphaeroides*, Fla2, enables the formation of several polar flagella (16, 20). The *fla2* genes are not expressed under standard laboratory growth conditions. So far, the Fla2 flagellar system has been studied in mutant strains that are unable to form Fla1 due to a mutation in a regulatory or structural gene and importantly contain a gain-of-function mutation in the histidine kinase CckA. In these strains, it has been determined that the expression of *fla2* is dependent on the two-component system formed by the histidine kinase CckA, the phosphotransferase protein ChpT, and the response regulator CtrA (21).

Phylogenetic studies have shown that the *fla2* genes constitute the native flagellar genes of *R. sphaeroides*, whereas the *fla1* set was acquired by horizontal transfer from an ancestral gammaproteobacterium (16). In *R. sphaeroides*, the multiple copies of the chemotaxis genes are organized in four different operons (15) (Fig. 1).

The control of Fla1 was shown to be mediated by the products of *che* operon 2 (*cheOp2*) and *cheOp3* (15, 22–24), whereas the control of Fla2 is mediated by the chemosensory proteins encoded in *cheOp1* (25, 26). Specifically, the chemotaxis response dependent on Fla1 is mainly controlled by CheY6 (encoded in *cheOp3*), as well as either CheY3 (encoded in *cheOp2*) or CheY4 (24). Fla2 is controlled by CheY2 and CheY5 (both encoded by *cheOp1*) (25).

Regarding the expression of these genes, *cheOp3* expression reportedly depends on the σ^{54-3} factor (27). This is unexpected, given that the expression of flagellar and

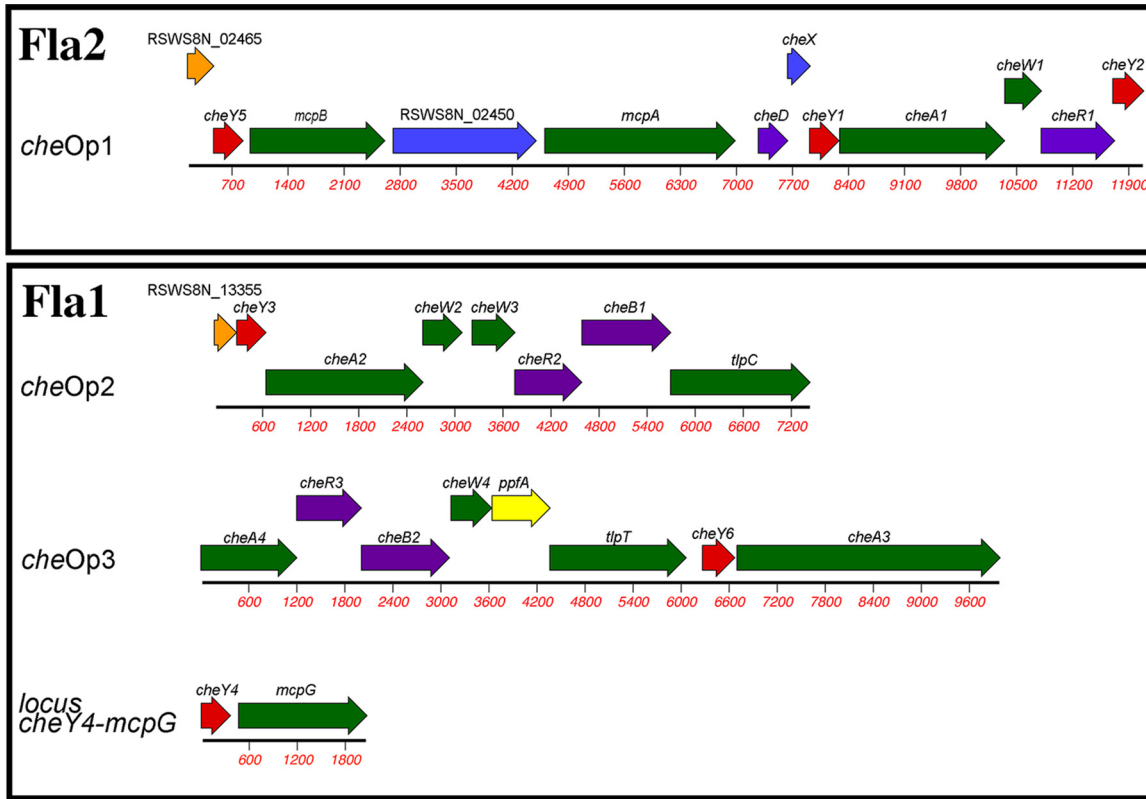


FIG 1 Schematic representation of the organization of the chemotaxis genes in *R. sphaeroides*. The multiple *cheY* genes are in red. Black outlines group the chemotaxis operons that control Fla1 or Fla2.

chemotaxis genes in bacteria is usually coordinated and that σ^{54-3} has not been implicated in the transcription of any *fla1* gene. The expression of *cheOp1* has yet to be studied. The other chemotaxis operon whose expression has already been analyzed is *cheOp2*, which is dependent on σ^{70} and σ^{28} (FliA) (27). Therefore, whether *cheY4* and *cheOp1* are expressed concurrently with *fla1* and *fla2*, respectively, remains to be determined.

Another important aspect that remains to be determined is the identity of the activator protein that is responsible for interacting with $E\sigma^{54-3}$ to promote *cheOp3* expression; since $E\sigma^{54}$ is unable to form open complex by itself, the presence of an activator protein of the bacterial enhancer-binding protein (bEBP) family is compulsory (28). In this context, it should be stressed that most bacteria have a single σ^{54} factor (encoded by *rpoN*) that interacts with multiple bEBPs that recognize specific binding sites to achieve the transcription of a specific set of genes (29). In contrast, *R. sphaeroides* has four σ^{54} factors, two of which, σ^{54-1} and σ^{54-2} , are activated by specific bEBPs (i.e., NifA and FleQ/FleT, respectively) (17, 30). Potential bEBP-encoding genes were previously identified *in silico* in the genome of *R. sphaeroides*, revealing that aside from the bEBPs for σ^{54-1} and σ^{54-2} , only two loci remain that encode proteins with bEBP family signatures that could be the activators for σ^{54-3} and σ^{54-4} (19).

In this work, we have determined that the expression of *cheOp3* is dependent on σ^{54-2} and not on σ^{54-3} , as was previously reported (27), and that the bEBP of the *fla1* system (FleQ) is the activator protein for this operon. In addition, we found out that *cheY4* expression is dependent on σ^{28} (FliA) and, consequently, is also a part of the flagellar transcription hierarchy. Our results also show that *cheOp1* expression is controlled by CtrA. Together, these results establish that the expression of the chemotaxis genes in *R. sphaeroides* is coordinated with the expression of their cognate flagellar system.

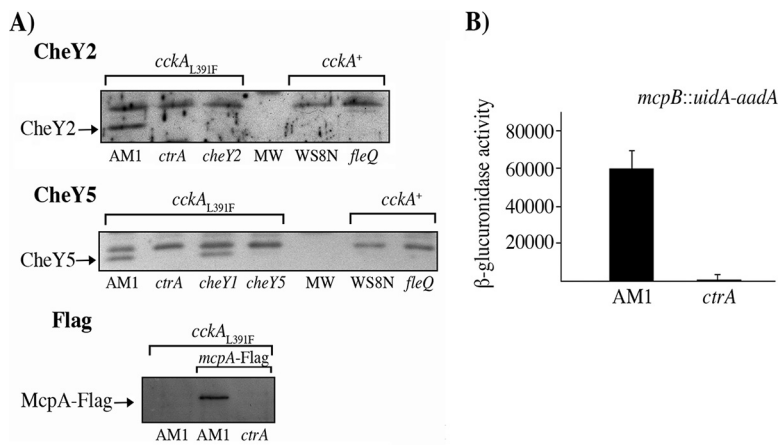


FIG 2 Determination of the presence of proteins encoded in *cheOp1* by immunoblotting and measuring the transcriptional activity of *cheOp1* using a reporter gene. (A) Total cell extracts were obtained from cultures grown in Siström's minimal medium supplemented with 80 μ M succinic acid and harvested at an OD_{600} of 0.3. The notation at the top of the Western blots regarding the status of the *cckA* allele indicates that the WS8N and Δ *fleQ* (SP13) strains do not carry the gain-of-function mutation in CckA that enables the expression of the *fla2* genes whereas the AM1 strain and its derivatives carry the mutations Δ *fleQ* and, importantly, *cckA*_{L391F}, which activates the CckA/ChpT/CtrA two-component system. Each lane is labeled according to the relevant mutation present in the strain. The antibody used for each blot is indicated at the top. MW indicates the lane where the molecular weight marker was loaded. Anti-CheY2 and anti-CheY5 antibodies were used at a 1:3,000 dilution, and anti-Flag antibody (Sigma-Aldrich) was used at a 1:10,000 dilution. (B) β -Glucuronidase activity promoted by the chromosomal fusion *mcpB::uidA-aadA*. AM1 and its Δ *ctrA* derivative (LC7 strain) carrying the transcriptional fusion *mcpB::uidA-aadA* were grown in 80 μ M succinic acid, and total cell extracts were used for the enzymatic reaction. Activity is reported in picomoles of 4-methylumbelliferone produced per minute per milligram of protein. The mean values and standard deviations of the results of three independent determinations are shown.

RESULTS

In this work, we used derivatives of the WS8N and AM1 strains to study the regulation of the flagellar and chemotaxis genes. WS8N is the wild-type strain that constitutively expresses the Fla1 flagellum but is unable to express the *fla2* genes given that the two-component system CckA/ChpT/CtrA is inactive. The AM1 strain is a WS8N derivative that is unable to express the *fla1* genes, given that it carries a mutation in the flagellar master activator *fleQ* and also carries a point mutation in *cckA* that activates the CckA/ChpT/CtrA two-component system (21, 25); therefore, this strain has a Fla1⁻Fla2⁺ phenotype.

***cheOp1* expression is dependent on the master regulator of the *fla2* genes.** The expression of three chemotaxis proteins encoded in *cheOp1* (Fig. 1) was evaluated by Western blotting using total cell extracts obtained from AM1 (25) and its derivative, EA1 (Δ *ctrA::aadA*) (21). The proteins CheY2 and CheY5 were detected in AM1 but not in the absence of *ctrA* (Fig. 2A), indicating that *cheOp1* expression is dependent on the presence of CtrA. To test for another protein encoded in *cheOp1*, we isolated a chromosomal fusion of *mcpA* with the Flag epitope in the AM1 strain. The resulting strain, JV1 (*mcpA-Flag*) and its derivative, JV2 (*mcpA-Flag* Δ *ctrA::hyg*), were tested by immunoblotting using an anti-Flag antibody (Fig. 2A). Consistent with the hypothesis that CtrA is required to transcribe *cheOp1*, McpA-Flag was detected in total cell extracts of JV1 but not of JV2.

To rule out the possibility that an undetected mutation other than Δ *ctrA* was responsible for the lack of CheY2, CheY5, and McpA-Flag, we introduced a plasmid that expresses CtrA (pRK_ctrA) into the EA1 and JV2 strains. In these complemented strains, the expression of CheY2, CheY5, and McpA-Flag was restored (see Fig. S1 in the supplemental material).

Consistent with the proposed model of activation of the CckA/ChpT/CtrA system (21), we did not observe the presence of either CheY2 or CheY5 in the wild-type strain WS8N or in the SP13 (Δ *fleQ::kan*) mutant (Fig. 2A), indicating that *cheOp1* is expressed

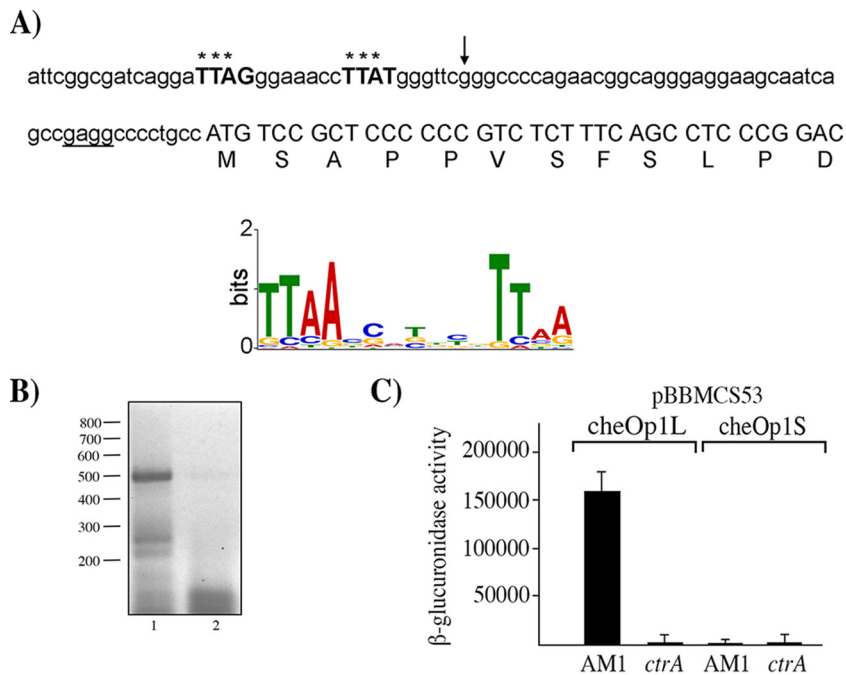


FIG 3 Nucleotide sequence upstream of *cheOp1*, activity of the reporter gene *uidA* fused to different fragments from the regulatory region of *cheOp1* in the pBBMCS53 plasmid, and RT-PCR data for *cheY5* and its upstream gene. (A) Nucleotide sequence upstream of RSW58N_02465. The first residues of the protein encoded by this gene are shown below the corresponding codons. The putative Shine-Dalgarno sequence is underlined, and nucleotides similar to the consensus binding site of CtrA, identified using a previously reported position weight matrix (31), are shown in bold and capital letters. The LOGO sequence of the CtrA-binding site is shown below; it was reported as part of a genomic analysis performed in *C. crescentus* (32). Asterisks above the sequence indicate nucleotides that match with the most frequent base in the LOGO sequence. An arrow indicates the 5' end of the fragment cloned into pBBMCS53_cheOp1S. The 5' end of the fragment cloned into pBBMCS53_cheOp1L includes 25 bp upstream of the sequence shown. (B) Agarose gel showing the RT-PCR products of the primer pair that amplifies the region encompassing *CheY5* and RSW58_02465. A 469-bp product was expected for this reaction. Lane 1, 5 μ l from the RT-PCR; lane 2, 5 μ l from the control reaction carried out in the absence of reverse transcriptase. The relevant molecular sizes from a 100-bp DNA ladder are shown on the left. (C) β -Glucuronidase activity of cell extracts from the AM1 and EA1 (Δ *ctrA::aadA*) strains carrying the indicated plasmids. Cell cultures were grown in Siström's minimal medium supplemented with 80 μ M succinic acid. Activity is reported in picomoles of 4-methylumbelliferone produced per minute per milligram of protein. The mean values and standard deviations (SD) of the results of three independent determinations are shown.

only when the CckA/ChpT/CtrA two-component system is active. To test the hypothesis that *cheOp1* expression could be transcriptionally controlled by CtrA, a transcriptional fusion of *mcpB* to the promoterless *uidA* gene was made. This fusion was used to replace the *mcpB*⁺ gene in AM1 and Δ *ctrA::hyg* (LC7) strains. *uidA* expression was recorded as the amount of β -glucuronidase present in total cell extracts. We noticed that the enzymatic activity was severely reduced in the absence of CtrA (Fig. 2B), implying that transcription of *cheOp1* is directly or indirectly controlled by CtrA.

The control region of *cheOp1* has a potential CtrA binding site relevant for its expression. Given that the expression of several of the proteins encoded in *cheOp1* was dependent on CtrA, we searched for potential CtrA binding sites upstream of the annotated genes in chromosome 1 using a CtrA position weight matrix (PWM) built from the regulatory regions of 54 genes identified to be controlled by CtrA in *Caulobacter crescentus* (31). From this analysis, the sequence TTAGN₇TTAT was found 67 bp upstream of the translational start site of RSW58N_02465, the gene located upstream of *cheY5* (Fig. 1 and 3A). The identified sequence is highly similar to the CtrA consensus binding sequence (TTAAN₇TTAA) previously characterized in *C. crescentus* and other species (32), suggesting that *cheOp1* transcription may be activated by CtrA from this site. This finding implies that RSW58N_02465 might be part of *cheOp1*. To evaluate this

prediction, we performed a reverse transcription (RT)-PCR experiment using specific primers for RSWS8N_02465 and *cheY5*. Using total RNA from AM1, the main product amplified from this reaction had the expected size for a DNA fragment that expands from RSWS8N_02465 to *cheY5* (Fig. 3B). Further supporting the idea that RSWS8N_02465 is part of *cheOp1*, it should be noted that the stop codon of RSWS8N_02465 overlaps the start codon of *cheY5*. RSWS8N_02465 encodes a 109-amino-acid protein with a STAS (sulfate transporter and anti- σ -factor antagonist) domain that has not been implicated in chemotaxis so far.

To evaluate if the upstream region of RSWS8N_02465 exhibits CtrA-dependent promoter activity, we cloned this region upstream of the reporter gene *uidA* in the pBBMCS53 plasmid. The resulting construct (pBBMCS53_*cheOp1L*) promoted high β -glucuronidase expression in the AM1 strain but not in EA1 (Δ *ctrA::aadA*) (Fig. 3C). In addition, we cloned a shorter fragment from the upstream region of RSWS8N_02465, which excludes the putative CtrA binding site, into the pBBMCS53 plasmid. The resulting plasmid (pBBMCS53_*cheOp1S*) did not promote β -glucuronidase expression in either of the two strains (Fig. 3C). The lack of expression of the reporter gene in this construction could be explained by the loss of either the CtrA binding site or the -35 promoter region. In this context, it should be stressed that most of the CtrA-activated promoters in *C. crescentus* show overlapping of these two sequence motifs (32, 33).

***cheOp3* expression is dependent on σ^{54-2} and the master regulator of the *fla1* genes.** CheY6 and either CheY3 or CheY4 are needed to control the chemotaxis response of the Fla1 flagellum (24, 34). CheY6 is encoded in *cheOp3*, which is located within the 56-kb *fla1* gene cluster, and CheY3 is encoded in *cheOp2*, located elsewhere on chromosome I, whereas CheY4 is located on chromosome II (34) (Fig. 1).

cheOp3 expression was previously shown to depend on σ^{54-3} (27); however, *fla1* gene expression is dependent on σ^{54-2} , raising the possibility that the expression of *cheOp3* is unlinked from the expression of Fla1. Given that $E\sigma^{54}$ is unable to form open complex by itself, we decided to identify the bEBP responsible for activating $E\sigma^{54-3}$. The *in silico* identification of bEBP-encoding genes in the *R. sphaeroides* genome revealed only five genes that encode proteins with the characteristic signatures of the σ^{54} transcriptional activators (19): *nifA*, *fleQ*, *fleT*, RSWS8N_RS13335, and RSWS8N_RS04480. We previously reported the isolation of strains with mutations in the genes *nifA*, *fleQ*, and *fleT* (19, 30); therefore, we isolated strains with mutations in RSWS8N_RS13335 and RSWS8N_RS04480.

The expression of CheY6 (encoded in *cheOp3*) was tested in total cell extracts obtained from the wild-type strain, along with cell extracts from the strains with mutations in each of the five bEBP-encoding genes. As shown in Fig. 4A, CheY6 expression was abolished in the strain carrying the Δ *fleQ::kan* allele and was not affected by the absence of RSWS8N_RS13335, RSWS8N_RS04480, or *nifA*, indicating that CheY6 is expressed as a part of the *fla1* transcriptional hierarchy.

An approximately 60% reduction in CheY6 levels was noticed in the absence of FleT and FliA. In both of these strains, the FleQ activator is present and the σ^{28} (FliA) factor is absent, suggesting that wild-type CheY6 levels could arise from the combined activity of a class I FleQ-activated promoter and a class IV σ^{28} -dependent promoter.

In contrast to the proposal that *cheOp3* expression is dependent on σ^{54-3} (27), we observed that the expression of CheY6 (encoded in *cheOp3*) was dependent only on σ^{54-2} and that the absence of σ^{54-3} did not affect CheY6 expression levels (Fig. 4A). Given that *cheY6* and the upstream gene *tlpT* are located 204 bp apart, *cheY6* may be transcribed independently of the upstream chemotaxis genes (Fig. 1). Therefore, to determine if the expression of the genes located upstream of *cheY6* was also dependent on FleQ and σ^{54-2} , we tested the expression of a reporter gene under the control of the region located upstream of *cheA4* which has been reported to carry the *cheOp3* promoter (27). To this end, an 854-bp fragment of the upstream region of *cheA4*, starting from codon 48 of *cheA4*, was cloned upstream of the reporter gene *uidA* in pBBMCS53 (35). The resulting plasmid was introduced into the WS8N, Δ *rpoN2::kan*, and Δ *fleQ::kan* strains. As a control, this plasmid was also introduced into the Δ *rpoN3* strain.

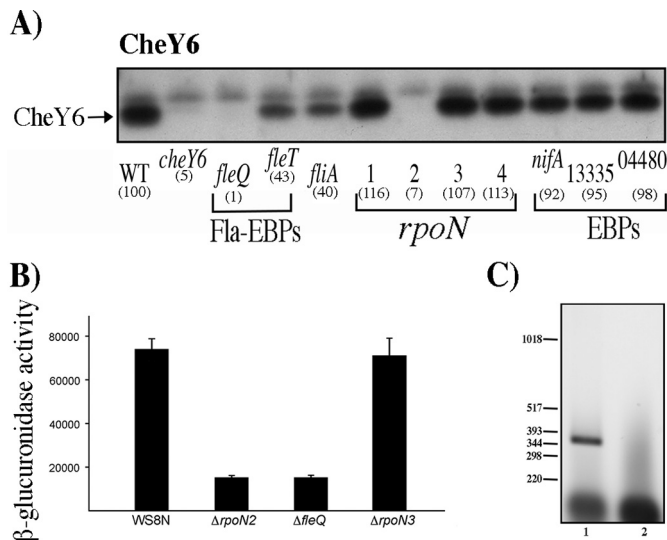


FIG 4 Determination of the presence of CheY6 in different strains, activity of the reporter gene *uidA* fused to the regulatory region of *cheOp3* in the pBBMC53 plasmid, and RT-PCR of *cheY6* and its upstream gene. Total cell extracts obtained from cell cultures grown in Siström's minimal medium and harvested at an OD₆₀₀ of 0.6 were tested as described in Materials and Methods. (A) Total cell extracts were separated by gel electrophoresis, transferred to nitrocellulose, and probed with anti-CheY6 antibodies (1:3,000). WT, WS8N cell extract. Each lane is labeled according to the relevant mutation present in the strain. Square brackets group the samples according to their function. Fla-EBPs indicate the two enhancer binding proteins required for activation of the *fla1* genes, *rpoN* indicates the genes encoding σ^{54-1} , -2, -3, or -4, and EBPs indicate the genes encoding proteins that belong to the enhancer binding protein family in the *R. sphaeroides* genome sequence. The signal identified as CheY was quantified by densitometry using ImageJ; the resulting value was normalized using an unspecific band on the blot. The values shown in parentheses are the average results of three independent experiments and are expressed as a percentage of the WT level. Figure S3 shows the graph of these values with SD. (B) β -Glucuronidase activity of the wild-type and mutant strains carrying the plasmid pBBMC53_cheA4. Activity is reported in picomoles of 4-methylumbelliferone produced per minute per milligram of protein. The mean values and standard deviations of the results of three independent determinations are shown. (C) Agarose gel of the RT-PCR products of the primer pair that amplifies the region encompassing *cheY6* and its upstream gene *tlpT*. A 391-bp product was expected for these primers. Lane 1, 5 μ l from the RT-PCR; lane 2, 5 μ l from the control reaction carried out in the absence of reverse transcriptase. The relevant sizes in base pairs of the DNA molecular size marker X (Roche Life Science) are shown on the left.

Total cell extracts of these strains were assayed for β -glucuronidase activity. A strong reduction in the amount of β -glucuronidase was detected for the $\Delta rpoN2::kan$ and $\Delta fleQ::kan$ mutants compared with that in the wild-type WS8N or $\Delta rpoN3::kan$ strains (Fig. 4B), suggesting that the expression of *cheA4* is also dependent on σ^{54-2} and FleQ. In addition, RT-PCR revealed that *cheY6* is cotranscribed with *tlpT*, suggesting that these genes could form a single operon (Fig. 4C).

σ^{28} is involved in the expression of *cheY4*. The expression of CheY3 and CheY4 was analyzed in each of the mutant strains lacking either one of the bEBP or σ^{54} factors. We observed that the expression of CheY4 was severely reduced in $\Delta fleQ::kan$ (SP13), $\Delta fleT::aadA$ (SP12), $\Delta rpoN2::kan$ (SP7), and $\Delta fliA::kan$ (SP15) strains (Fig. 5A). Since it has been shown that the expression of *fliA* is dependent on FleQ, FleT, and σ^{54-2} (19), these results suggest that *cheY4* expression is dependent on σ^{28} (*fliA*) and that the absence of CheY4 in the $\Delta fleQ$, $\Delta fleT$, and $\Delta rpoN2$ strains is the expected consequence of this regulation.

To verify that the expression of *cheY4* was dependent on the presence of σ^{28} , we carried out an RT-PCR assay using specific oligonucleotides for *cheY4*. The expected product of 361 bp was detected when the total RNA from WS8N was included in the reaction, whereas a faint product was detected when RNA from the *fliA::kan* strain was used (Fig. 5B, lanes 1 and 2, respectively). It should be noted that for this RNA, a similar signal was detected when no reverse transcriptase was included in the reaction mixture (Fig. 5B, lane 4), indicating that this slight signal is the product of a small amount of

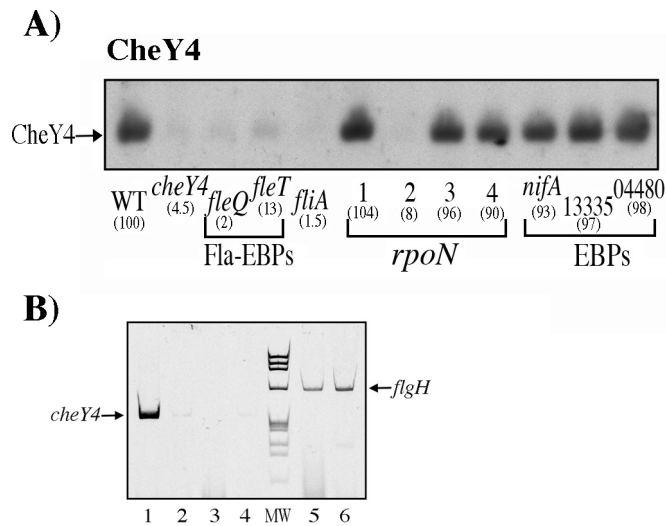


FIG 5 Determination of the presence of CheY4 in different mutant strains by immunoblotting. Total cell extracts obtained from cell cultures grown in Siström's minimal medium and harvested at an OD₆₀₀ of 0.6 were tested as described in Materials and Methods. Anti-CheY4 antibodies were used at 1:3,000. WT denotes the WS8N cell extract. Each lane is labeled according to the relevant mutation present in the strain. Other labels are the same as described in the legend for Fig. 4A. (B) Acrylamide gel electrophoresis of the RT-PCR products corresponding to the primer pair that amplifies *cheY4* (lanes 1 to 4) and the primer pair that amplifies *flgH* (lanes 5 and 6). Lanes: 1, 3, and 5, total RNA from WS8N; 2, 4, and 6, total RNA from the *fliA*:kan mutant strain. Control reactions were carried out in the absence of reverse transcriptase (lanes 3 and 4). Lane MW corresponds to PhiX174 DNA digested with HaeIII and was used as a molecular size marker (fragment sizes, 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, and 118 bp). The expected sizes for the *cheY4* and *flgH* products are 361 and 639 bp, respectively).

DNA in the sample. To be certain that both samples of RNA had the appropriate quality to yield an RT-PCR product, we performed a reaction using a pair of oligonucleotides that amplified a region of the *flgH* gene (Fig. 5B, lanes 5 and 6).

Given that our results suggest that CheY4 is expressed from a σ^{28} -dependent promoter, we searched upstream of *cheY4* for a sequence similar to the σ^{28} consensus promoter. We analyzed a 159-bp region from the stop codon of RSWS8N_17744 (RSP_3301) to the start codon of *cheY4* by using a PWM built from 24 σ^{28} -dependent promoters (36). This analysis revealed a possible σ^{28} -promoter located 104 bp upstream of the start codon of *cheY4* (Fig. S2). The sequence of this putative promoter is similar to that of previously reported σ^{28} -dependent promoters in *R. sphaeroides* (19).

A more complex situation was observed for CheY3, where we detected a reduction in the amount of CheY3 in the $\Delta fliA$ mutant strain but no reduction in the absence of FleQ or FleT (Fig. 6A); this result was unexpected given that the expression of *fliA* depends on FleQ and FleT (19). It has been shown that *cheOp2* contains a regulatory region with two promoters, one dependent on σ^{70} and the other dependent on σ^{28} (27), which explains the reduction of CheY3 in the absence of σ^{28} (FliA). However, the CheY3 expression level in the absence of FleQ and FleT (in these strains, *fliA* is not expressed [19]) is similar to that observed in the wild-type strain, suggesting that FleQ and FleT may have a negative effect on CheY3 expression.

To evaluate this possibility, we carried out a primer extension experiment using the oligonucleotide *cheOp2B*, which targets the first gene of *cheOp2* (i.e., RSWS8N_13355). The reaction mixtures were subjected to gel electrophoresis, and the products were analyzed using ImageJ. The plot profile of the sample from the wild-type strain revealed the previously reported σ^{28} and σ^{70} promoters (27) (Fig. 6B). From two independent experiments, a 26% average increase was observed in the amount of the σ^{70} -dependent transcript in the $\Delta fleQ$ mutant strain compared with that in the wild-type strain. This increase is consistent with a negative role for FleQ on *cheOp2* expression. Unexpectedly, we detected a low-abundance transcript in both strains that initiates

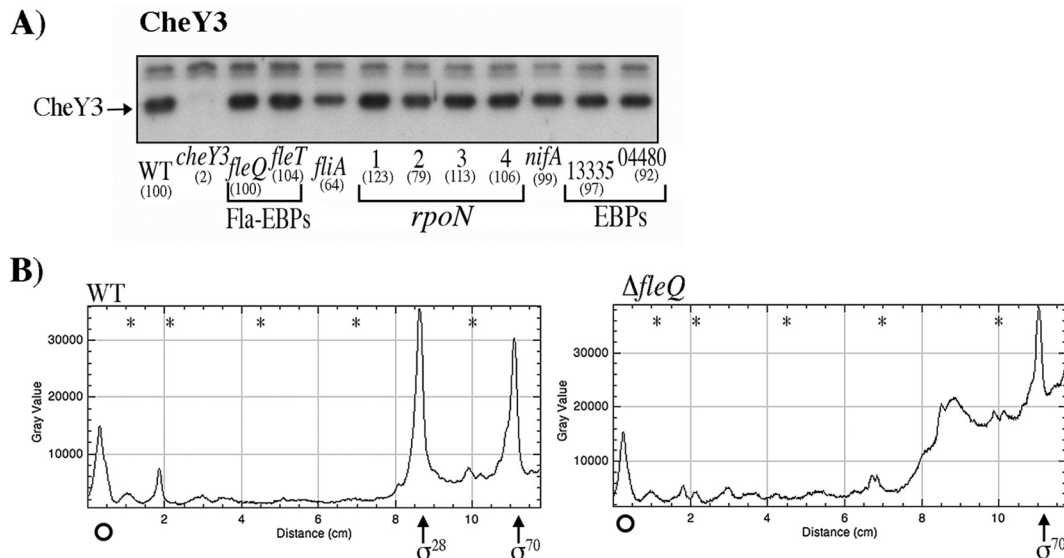


FIG 6 Determination of the presence of CheY3 in different strains by immunoblotting and plot profile of the primer extension experiment for *cheOp2*. (A) Total cell extracts obtained from cell cultures grown in Sistrom's minimal medium and harvested at an OD_{600} of 0.6 were tested as described in Materials and Methods. Anti-CheY3 antibodies were used at 1:3,000. WT, WS8N cell extract. Each lane is labeled according to the relevant mutation present in the strain. Other labels are the same as described in the legend for Fig. 4A. (B) Total RNA was isolated from cell cultures of WS8N and SP13 ($\Delta fleQ::kan$) strains grown in Sistrom's minimal medium. After hybridization of the RNA samples with the 5'-end-labeled oligonucleotide, cDNA synthesis was performed using AMV reverse transcriptase. The reactions were subjected to gel electrophoresis, and the images were visualized using a Typhoon scanner (GE Healthcare Life Science) and quantified using ImageJ. The plot profiles initiate immediately below the largest transcript detected (position -155 from the translational start site of RSWS8N_13355), denoted by an open circle. The transcripts that originated from the σ^{70} and σ^{28} promoters are indicated by vertical arrows. The asterisks correspond to the migration of the 151-, 140-, 118-, 100-, and 82-bp (from left to right) DNA fragments of PhiX174 that were obtained after digestion with *Hin*I (Promega) and 5' end labeled.

approximately 155 nt upstream of the translational start of RSWS8N_13355. The amounts of transcript are similar for the two samples.

DISCUSSION

Our work shows that the expression of chemotaxis proteins in *R. sphaeroides* is coordinated with the expression of the cognate flagellar system. In many bacteria, chemotaxis genes are expressed under the control of the flagellar regulator proteins, and in *E. coli* and other related bacteria, their expression is dependent on σ^{28} (FlhA), indicating that these genes belong to the lower class of the flagellar hierarchy. In contrast, chemotaxis genes in other bacteria are expressed under the control of the master regulator of the flagellar hierarchy; therefore, the chemotaxis proteins are synthesized during the early steps of flagellar formation. These opposing patterns suggest that the timing of chemotaxis protein expression may be irrelevant for the function of the system or that this regulation may reflect different regulatory needs. Nonetheless, the coordinated expression of flagellar proteins and chemotaxis proteins seems to be a ubiquitous scenario, and this scheme prevails in the case of *R. sphaeroides*, even though one flagellar system was horizontally acquired, indicating a strong pressure to coordinate the presence of the flagellar and chemotaxis proteins. In agreement with this view, it has been proposed that the gene encoding σ^{54-2} (*rpoN2*) is a duplication from the vertically inherited *rpoN1* gene (37).

In *R. sphaeroides*, the expression of the *fla2* genes is dependent on the transcriptional activator CtrA (21). We demonstrated that *cheOp1* expression is also dependent on CtrA. Our data identified a 652-bp fragment that contains the *cheOp1* promoter and revealed the possible site through which CtrA mediates transcriptional activation. Our results also showed that RSWS8N_02465 is part of *cheOp1*; the product of this gene has yet to be implicated in chemotaxis, but its presence in *cheOp1* suggests its potential involvement in this response. Interestingly, a gene containing a STAS domain is also

located upstream of *cheY3* in *cheOp3*, strengthening the idea that the products of these genes might be involved in chemotaxis. In addition, our results show that the *cheOp1*, *CheY5*, *CheY2*, and *McpA* gene products are not expressed when the CckA/ChpT/CtrA two-component system is inactive (in the WS8N strain or its derivative, SP13 [$\Delta fleQ::kan$]), further supporting the notion that *cheOp1* is expressed only when the *fla2* genes are expressed.

In contrast to the Fla2 flagella, where the expression of all the CheY proteins involved in its control are dependent on the master flagellar regulator CtrA, the expression of the chemotaxis proteins that control Fla1 appears to be more complex. The expression of *cheOp3*, in which *CheY6* is encoded, was previously suggested to depend on the sigma factor σ^{54-3} (27); however, our results show that *cheOp3* expression is dependent on σ^{54-2} and the master regulator FleQ. We speculate that a mistake in the nomenclature of the σ^{54} factors might be behind this disparity. The relevant point that should be stressed is that the expression of *cheOp3*, which encodes *CheY6*, the only CheY able to bind the Fla1 flagellar motor, is controlled by the master flagellar regulator FleQ and the σ^{54-2} factor.

We also observed a reduction in the amount of *CheY6* in cell extracts from the $\Delta fleT::aadA$ (SP12) and $\Delta fliA::kan$ (SP15) strains (Fig. 4A), suggesting that *cheOp3* should have an additional σ^{28} -dependent promoter and that *cheOp3* might be expressed from both a FleQ-dependent promoter and from a σ^{28} (FliA)-dependent promoter. If this hypothesis is correct, the observed reduction of *CheY6* in the absence of FleT and FliA can be explained by the absence of σ^{28} in these two strains, leaving only the FleQ promoter active. The fact that *CheY6* expression depends on the master flagellar regulator FleQ and probably also on σ^{28} (FliA) highlights the importance of keeping a high and sustained level of this protein as long as the Fla1 flagellum is present, consistent with the idea that *CheY6* is the main chemotaxis regulator of Fla1 (34).

The response regulators *CheY3* and *CheY4*, which act along with *CheY6* to control Fla1, are also expressed through the action of the transcriptional factors that regulate the Fla1 gene hierarchy. We observed that *CheY4* expression is dependent on σ^{28} (FliA), and consequently we did not detect this protein in the absence of either σ^{28} or the FleQ, FleT, and σ^{54-2} proteins, which are required to transcribe *fliA* (19). Accordingly, no RT-PCR product of *cheY4* was obtained from RNA of the *cheY4::aadA* strain, and a sequence similar to the σ^{28} consensus promoter was identified upstream of *cheY4*.

The expression of *CheY3*, which is encoded in *cheOp2*, depends on σ^{70} and σ^{28} , and a part of its expression is dependent on the σ^{28} promoter (27). Our results also showed a reduction in the level of *CheY3* in the $\Delta fliA$ mutant strain. However, the lack of a reduction in the $\Delta fleQ$ and $\Delta fleT$ strains suggests that these proteins have a negative effect on *CheY3* expression. A primer extension experiment designed to detect expression by the two *cheOp2* promoters showed a 26% increase in the amount of the σ^{70} -dependent transcript in the absence of FleQ, which could explain the Western blot results. The mechanism of this negative regulation remains to be determined, but the sustained expression of *cheOp2* mediated by σ^{70} , as well as its possible increase in the absence of FleQ, makes this operon unique among the four chemotaxis operons, suggesting that the products of *cheOp2* might be required to accomplish another physiological role.

Figure 7 presents a model summarizing the conclusions of this work. This model illustrates the transcriptional relationship between the expression of flagellar genes and their cognate chemosensory proteins. Notably, only one transcriptional regulator directly controls the expression of the proteins involved in the chemotaxis response of Fla2, whereas the chemosensory proteins that control Fla1 are directly or indirectly regulated by two different transcription factors, FleQ and σ^{28} . Whether there is a physiological reason for this multiple regulation remains to be elucidated, but obviously, a pathway controlled by several regulators offers multiple checkpoints for finely tuned responses. Nevertheless, it should be kept in mind that this global regulation may simply reflect the evolutionary history of these genes.

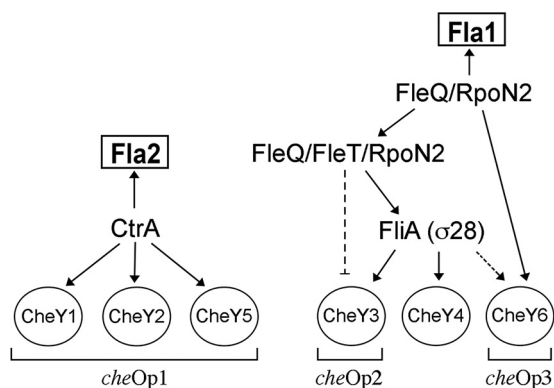


FIG 7 Model for the transcriptional control of genes encoding the CheY proteins of *R. sphaeroides* through the master regulators of *fla1* and *fla2*. Solid arrows represent the confirmed regulatory pathways established here and in reference 27. Broken lines represent the regulation inferred from our results. The brackets at the bottom indicate the chemotaxis operon to which each *cheY* gene belongs.

MATERIALS AND METHODS

Genome sequences, plasmids, bacterial strains, and growth conditions. All plasmids and bacterial strains used in this work are listed in Table 1. The genome sequence analyzed in this work corresponds to *R. sphaeroides* WS8N (NCBI reference sequence NZ_CM001161.1, GI:332561612). *R. sphaeroides* WS8N (38) and its derivatives were grown chemoheterotrophically at 30°C in Siström's minimal medium (39). AM1 and its derivatives were grown photoheterotrophically in Siström's minimal medium with 80 μM succinic acid as the carbon source. Photoheterotrophic liquid cultures were grown under continuous illumination and in completely filled screw-cap tubes. Heterotrophic liquid cultures were incubated in the dark with orbital shaking at 200 rpm. *Escherichia coli* was grown in Luria-Bertani (LB) medium (40) at 37°C. When required, antibiotics were added at the indicated concentrations: for *R. sphaeroides*, kanamycin (25 μg/ml), tetracycline (1 μg/ml), spectinomycin (50 μg/ml), and hygromycin (20 μg/ml for liquid cultures and 150 μg/ml for plates); for *E. coli*, kanamycin (50 μg/ml), spectinomycin (50 μg/ml), ampicillin (100 μg/ml), and hygromycin (20 μg/ml for liquid cultures and 200 μg/ml for plates).

Oligonucleotides. The oligonucleotides used in this work are listed in Table S1 in the supplemental material.

Isolation of mutant strains. In general terms, to inactivate RSW58N_RS13335 and RSW58N_RS04480, which encode proteins with characteristic bEBP signatures, a suicide vector (41) carrying the target gene disrupted with an antibiotic resistance gene was used to perform allelic exchange. Specifically, to inactivate RSW58N_RS13335, which encodes a 446-residue protein, the oligonucleotides 159-1 and 159-2 (both carrying an XbaI recognition site at the 5' end), were used to PCR amplify a 2,541-bp product containing the complete coding region of RSW58N_RS13335, as well as regions upstream and downstream of this gene. This product was cloned into pTZ19RBam⁻, and the resulting plasmid was digested with BamHI and partially digested with BglII to remove a 573-bp portion of RSW58N_RS13335 (residues 229 to 420). The resulting fragment was ligated to a BamHI-digested, 1.4-kb hygromycin resistance cassette (hyg), obtained by PCR from the pB45 plasmid (42). The plasmid carrying the ΔRSW58N_RS13335::hyg allele was digested with XbaI, and the fragment containing the mutant allele was subcloned into pJQ200mp18 (41). This plasmid was then introduced into *E. coli* S17-1 (43) and subsequently transferred to *R. sphaeroides* by conjugation (44). Since pJQ200mp18 cannot replicate in *R. sphaeroides*, the double-recombination event was selected directly on LB agar plates in the presence of hygromycin and 5% sucrose. The mutant was verified by PCR.

A similar strategy was used to inactivate RSW58N_RS04480, which encodes a 645-residue protein. The oligonucleotides 187-1 and 187-2 (both carrying an XbaI recognition site at the 5' end) were used to PCR amplify a 2,968-bp product containing the complete coding region of RSW58N_RS04480, as well as regions upstream and downstream of the target gene. This product was cloned into pTZ18REcoRI⁻SacI⁻, and the resulting plasmid was digested with EcoRI and SacI to remove a 1,098-bp segment of RSW58N_RS04480 (residues 4 to 370). The resulting fragment was end repaired with T4 DNA polymerase and ligated with a kanamycin resistance cassette (kan) obtained from the pUC4K plasmid. The plasmid carrying the ΔRSW58N_RS04480::kan allele was digested with XbaI, and the fragment containing the mutant allele was subcloned into pJQ200mp18. This plasmid was then introduced into *E. coli* S17-1 and subsequently transferred to *R. sphaeroides* by conjugation. The mutant strains were selected, and the gene replacement was verified by PCR.

To obtain strain JV1 (*mcpA*-Flag), a 764-bp PCR product corresponding to the 3' end of *mcpA* without its stop codon but including an extra guanine was obtained using the oligonucleotides *mcpAtagFw* and *mcpAtagRv*; the *McpA*-Flag fusion was obtained by cloning the PCR product into the pFlgC1 plasmid (45). The resulting plasmid was introduced into *R. sphaeroides* by conjugation, and integration into the chromosome by single recombination was selected. The *Spc*^c transconjugants were tested by PCR and Western blotting by using monoclonal antibodies against the Flag epitope. Subsequently, the *ctrA*⁺ allele

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>Rhodobacter sphaeroides</i> strains		
AE1	AM1 derivative; $\Delta fleQ::kan$ $cckA_{L391F}$ $\Delta ctrA::aadA$	21
AM1	WS8N derivative; $\Delta fleQ::kan$ $cckA_{L391F}$	25
JV1	AM1 derivative; $\Delta fleQ::kan$ $cckA_{L391F}$ <i>mcpA</i> -Flag	This work
JV2	JV1 derivative but $\Delta ctrA::hyg$	This work
JV3	AM1 derivative; <i>mcpB::uidA-aadA</i>	This work
JV4	AM1 derivative; <i>mcpB::uidA-aadA</i> $\Delta ctrA::hyg$	This work
LC7	AM1 derivative; $\Delta ctrA::hyg$	This work
RS1Y3	WS8N derivative; <i>cheY3::aadA</i>	25
RS1Y4	WS8N derivative; $\Delta cheY4::aadA$	25
RS1Y6	WS8N derivative; $\Delta cheY6::aadA$	25
RS2Y1	AM1 derivative; <i>cheY1::aadA</i>	25
RS2Y2	AM1 derivative; <i>cheY2::aadA</i>	25
RS2Y5	AM1 derivative; <i>cheY5::aadA</i>	25
SP7	WS8N derivative; $\Delta rpoN2::kan$ <i>rpoN2</i> (RSWS8N_05555)	17
SP8	WS8N derivative; $\Delta rpoN1::aadA$ <i>rpoN1</i> (RSWS8N_07950)	17
SP12	WS8N derivative; $\Delta fleT::aadA$	19
SP13	WS8N derivative; $\Delta fleQ::kan$	19
SP15	WS8N derivative; $\Delta flia::kan$	19
SP16	WS8N derivative; $\Delta nifA::kan$	30
SP21	WS8N derivative; $\Delta rpoN3::kan$ <i>rpoN3</i> (RSWS8N_19584)	17
SP22	WS8N derivative; $\Delta rpoN4::kan$ <i>rpoN4</i> (RSWS8N_04590)	17
SP23	WS8N derivative; Δ RSWS8N_RS13335::hyg	This work
SP24	WS8N derivative; Δ RSWS8N_RS04480::kan	This work
WS8N	Wild-type strain; spontaneous NaI^r	38
<i>Escherichia coli</i> strains		
S17-1	<i>recA endA thi hsdR</i> RP4-2-Tc::Mu::Tn7	53
TOP10	Cloning strain	Invitrogen
Plasmids		
pB45	Plasmid source of the hygromycin cassette	42
pBBMCS53	Transcriptional <i>uidA</i> fusion vector; Gm^r	35
pBBMCS53_cheA4	pBBMCS53 carrying the <i>cheOp3</i> promoter	This work
pBBMCS53_cheOp1L	pBBMCS53 carrying 652 bp upstream of <i>cheOp1</i>	This work
pBBMCS53_cheOp1S	pBBMCS53 carrying 591 bp upstream of <i>cheOp1</i>	This work
pCR2.1-TOPO	Cloning vector	Invitrogen
pFlgC1	Integration vector used to create a C-terminal fusion with the FLAG tag epitope; Spc^r	45
pJQ200_ $\Delta ctrA::hyg$	pJQ200mp18 carrying $\Delta ctrA::hyg$	This work
pJQ200_ <i>mcpB::uidA-aadA</i>	pJQ200mp18 carrying <i>mcpB::uidA-aadA</i>	This work
pJQ_ Δ RSWS8N_RS04480::kan	pJQ200mp18 carrying Δ RSWS8N_RS04480::kan	This work
pJQ_ Δ RSWS8N_13335::hyg	pJQ200 mp18 carrying Δ RSWS8N_13335::hyg	This work
pJQ200mp18	Mobilizable suicide vector for <i>R. sphaeroides</i> ; Gm^r	41
pRK_ctrA	pRK415 derivative expressing <i>ctrA</i>	21
pTZ_ctrAupdown	pTZ19R Bam^- carrying <i>ctrAupdown</i>	21
pTZ EcoRI $^-$ SacI $^-$	pTZ19R without EcoRI and SacI sites	Laboratory collection
pTZ_Eco $^-$ SstI $^-$ Δ RSWS8N_RS04480::kan	pTZ19R EcoRI $^-$ SacI $^-$ carrying Δ RSWS8N_RS04480::kan	This work
pTZ_Eco $^-$ SacI $^-$ RSWS8N_RS04480	pTZ19R EcoRI $^-$ SacI $^-$ carrying RSWS8N_RS04480	This work
pTZ19R	Cloning vector; Ap^r	Thermo Scientific
pTZ19R Bam^-	pTZ19R without BamHI site	Laboratory collection
pTZ19R BamHI^- Δ RSWS8N_RS13335::hyg	pTZ19R Bam^- carrying Δ RSWS8N_RS13335::hyg	This work
pTZ19R BamHI^- RSWS8N_RS13335	pTZ19R Bam^- carrying RSWS8N_RS13335	This work
pTZ18R_ <i>mcpB</i>	pTZ18R carrying <i>mcpB</i>	26
pTZ18R_ <i>mcpB::uidA-aadA</i>	pTZ18R carrying <i>mcpB::uidA-aadA</i>	This work
pUC4K	Plasmid source of the kanamycin cassette	Pharmacia
pWM5	Vector source of the <i>uidA-aadA</i> cassette	46

in the JV1 strain was replaced with the $\Delta ctrA::hyg$ allele by using the plasmid pJQ200_ $\Delta ctrA::hyg$. This plasmid was obtained by inserting a BglII fragment carrying the hyg cassette into the BamHI site of the pTZ_ctrAupdown plasmid (21).

To obtain the mutant strains JV3 (*mcpB::uidA-aadA*) and JV4 (*mcpB::uidA-aadA* $\Delta ctrA::hyg$), we used the plasmid pTZ18R_*mcpB* (26), which carries a 1,830-bp fragment of the coding region of *mcpB* plus 107 bp upstream of the start codon and 40 bp downstream of the stop codon. This plasmid was digested with NcoI, end repaired with T4 DNA polymerase, and ligated to a 4-kb SmaI fragment obtained from pWM5, which carries the *uidA-aadA* cassette (46). The fragment containing the *mcpB::uidA-aadA* allele

was subcloned into pJQ200mp18. This plasmid was introduced into AM1 and LC7 strains to replace the chromosomal *mcpB* gene by following the procedure described above.

Plasmids used in this work. A transcriptional fusion between the regulatory region of *cheOp1* and the reporter gene *uidA* was obtained by cloning a 652-bp fragment of the upstream region of *cheOp1* into the pBBMCS53 plasmid (35). This fragment was obtained by PCR using the oligonucleotides *cheOpromFw* and *cheOpromRv*. The resulting plasmid was named pBBMCS53_*cheOp1L*. The pBBMCS53 plasmid was designed to generate transcriptional fusions with the *uidA* gene, which encodes the enzyme β -glucuronidase (35). The plasmid pBBMCS53_*cheOp1S* was obtained by cloning a 591-bp PCR product carrying the regulatory region of *cheOp1* (without the putative CtrA binding site identified in this work) into the pBBMCS53 plasmid. The PCR product in this plasmid was generated using the oligonucleotides *cheOprommut* and *cheOpromRv*. Both plasmids were introduced into AM1 and EA1 strains by conjugation.

Molecular biology techniques. Standard methods were used to obtain chromosomal or plasmid DNA (40). Restriction and other DNA-modifying enzymes were acquired from New England Biolabs (NEB), Roche, or Invitrogen. For sequencing, plasmids were purified using the Illustra plasmidPrep mini spin kit (GE Healthcare Life Sciences). Chromosomal or plasmid DNA was amplified with the appropriate oligonucleotides using PrimeSTAR HS DNA polymerase (TaKaRa Bio Inc.) according to the manufacturer's recommendations.

RNA isolation and RT-PCR assays. Total RNA was isolated from cells grown to an optical density at 600 nm (OD_{600}) of 0.5 in Sistrostom's minimal medium using hot phenol (47). After purification, the samples were incubated with RNase-free DNase I using the DNA-free DNA removal kit (Thermo Fisher Scientific). Reverse transcription and PCR amplification were performed with the Access RT-PCR system (Promega) according to the manufacturer's instructions. A control reaction mixture lacking reverse transcriptase was run in parallel. The PCR products were analyzed by agarose or acrylamide gel electrophoresis. The oligonucleotides *cheY5XbaFw* and *cheY5PstRev* were used to test the region between RSW58N_02465 and *cheY5*. *CheY6UP* and *tlpTdown* were used to test the region between *cheY6* and *tlpT*. The oligonucleotides *cheY4Fw* and *cheY4Rev* were used to amplify an internal region of *cheY4*. *FlgHFw* and *flgHRv* were used as a control to amplify an internal region of *flgH*.

Primer extension. Total RNA was purified as previously described (47). The oligonucleotide *cheOp2B* was 5' end labeled with [γ - 32 P]ATP. Then, 50 μ g of total RNA was hybridized with the labeled oligonucleotide at 42°C in the presence of 50% formamide. The hybrid RNA-DNA was ethanol precipitated and resuspended in a 30- μ l reaction volume. The cDNA was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase. The products were subjected to electrophoresis on a 6% polyacrylamide–8 M urea gel. As a molecular marker, we used DNA from PhiX174 digested with *HinfI* (Promega) and 5' end labeled with [γ - 32 P]ATP. The images were visualized using a Typhoon scanner (GE Healthcare Life Science) and quantified using ImageJ software (48).

Western blot analysis. Samples were obtained from exponentially growing cultures at an OD_{600} of 0.6 from heterotrophically grown cultures and at an OD_{600} of 0.3 for photoheterotrophic cultures grown in 80 μ M succinic acid. Whole bacterial cells were lysed by boiling in a solution containing 2% SDS, 1% β -mercaptoethanol, and 50 mM Tris (pH 7.5). These samples were separated on 12% polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes (49). The membranes were then probed with antibodies raised in rabbits against *CheY2*, *CheY3*, *CheY4*, *CheY5*, and *CheY6* (1:3,000) or probed with a commercial mouse antibody against the Flag epitope tag (1:10,000) (Sigma-Aldrich). Anti-rabbit or anti-mouse IgG antibodies (1:30,000) (Sigma-Aldrich) coupled to alkaline phosphatase were used for chemiluminescent detection with CDP-star as the substrate (Applied Biosystems). His-tagged *CheY* proteins were purified by following previously reported protocols (50). Rabbit immunizations were performed as reported elsewhere (49). Representative results of three independent experiments are shown in Fig. 2A, S1, 4A, 5A, and 6A.

β -Glucuronidase activity assay. β -Glucuronidase assays employed 4-methylumbelliferyl- β -D-glucuronide (MUG) as the substrate along with sonicated cell extracts, as previously described (51). Samples of 100 μ l were taken at three time points between 5 and 20 min and then mixed with 0.9 ml of stop buffer (0.2 M Na_2CO_3). Fluorimetric determinations were made on a Perkin-Elmer LS-5 apparatus (excitation, 360 nm; emission, 446 nm). The fluorimeter was calibrated using 4-methylumbelliferone (Sigma-Aldrich) standards. Specific enzyme activity in cell extracts was expressed as picomoles of methylumbelliferone per minute per milligram of protein. Protein content was determined with a Bio-Rad protein assay kit, using bovine serum albumin as the standard.

Identification of the CtrA and σ^{28} binding sites. The sequence upstream of *cheY4* was analyzed using a position weight matrix (PWM) built using the σ^{28} promoters from *E. coli* (36) with the tool matrix-scan (52) included in the Regulatory Sequence Analysis Tools (RSAT) (<http://embnet.ccg.unam.mx/rsa-tools/>). The sequence upstream of the first gene in the *cheOp1* operon, RSW58N_02465, was analyzed using the same tool, with a PWM built from the regulatory region of 54 genes identified to be controlled by CtrA in *C. crescentus* (31).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00670-16>.

TEXT S1, PDF file, 0.4 MB.

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REFERENCES

- Baker MD, Wolanin PM, Stock JB. 2006. Signal transduction in bacterial chemotaxis. *Bioessays* 28:9–22. <https://doi.org/10.1002/bies.20343>.
- Wadhams GH, Armitage JP. 2004. Making sense of it all: bacterial chemotaxis. *Nat Rev Mol Cell Biol* 5:1024–1037. <https://doi.org/10.1038/nrm1524>.
- Szurmant H, Ordal GW. 2004. Diversity in chemotaxis mechanisms among the bacteria and archaea. *Microbiol Mol Biol Rev* 68:301–319. <https://doi.org/10.1128/MMBR.68.2.301-319.2004>.
- Sourjik V, Wingreen NS. 2012. Responding to chemical gradients: bacterial chemotaxis. *Curr Opin Cell Biol* 24:262–268. <https://doi.org/10.1016/j.ceb.2011.11.008>.
- Hazelbauer GL, Falke JJ, Parkinson JS. 2008. Bacterial chemoreceptors: high-performance signaling in networked arrays. *Trends Biochem Sci* 33:9–19. <https://doi.org/10.1016/j.tibs.2007.09.014>.
- Wuichet K, Zhulin IB. 2010. Origins and diversification of a complex signal transduction system in prokaryotes. *Sci Signal* 3:ra50. <https://doi.org/10.1126/scisignal.2000724>.
- He K, Bauer CE. 2014. Chemosensory signaling systems that control bacterial survival. *Trends Microbiol* 22:389–398. <https://doi.org/10.1016/j.tim.2014.04.004>.
- Fitzgerald DM, Bonocora RP, Wade JT. 2014. Comprehensive mapping of the *Escherichia coli* flagellar regulatory network. *PLoS Genet* 10:e1004649. <https://doi.org/10.1371/journal.pgen.1004649>.
- Arnosti DN, Chamberlin MJ. 1989. Secondary sigma factor controls transcription of flagellar and chemotaxis genes in *Escherichia coli*. *Proc Natl Acad Sci U S A* 86:830–834. <https://doi.org/10.1073/pnas.86.3.830>.
- Kalir S, McClure J, Pabbaraju K, Southward C, Ronen M, Leibler S, Surette MG, Alon U. 2001. Ordering genes in a flagella pathway by analysis of expression kinetics from living bacteria. *Science* 292:2080–2083. <https://doi.org/10.1126/science.1058758>.
- Dasgupta N, Wolfgang MC, Goodman AL, Arora SK, Jyot J, Lory S, Ramphal R. 2003. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* 50:809–824. <https://doi.org/10.1046/j.1365-2958.2003.03740.x>.
- Yang TC, Leu YW, Chang-Chien HC, Hu RM. 2009. Flagellar biogenesis of *Xanthomonas campestris* requires the alternative sigma factors RpoN2 and FljA and is temporally regulated by FlhA, FlhB, and FlgM. *J Bacteriol* 191:2266–2275. <https://doi.org/10.1128/JB.01152-08>.
- Laub MT, Chen SL, Shapiro L, McAdams HH. 2002. Genes directly controlled by CtrA, a master regulator of the *Caulobacter* cell cycle. *Proc Natl Acad Sci U S A* 99:4632–4637. <https://doi.org/10.1073/pnas.062065699>.
- De Nisco NJ, Abo RP, Wu CM, Penterman J, Walker GC. 2014. Global analysis of cell cycle gene expression of the legume symbiont *Sinorhizobium meliloti*. *Proc Natl Acad Sci U S A* 111:3217–3224. <https://doi.org/10.1073/pnas.1400421111>.
- Porter SL, Wadhams GH, Armitage JP. 2011. Signal processing in complex chemotaxis pathways. *Nat Rev Microbiol* 9:153–165. <https://doi.org/10.1038/nrmicro2505>.
- Poggio S, Abreu-Goodger C, Fabela S, Osorio A, Dreyfus G, Vinuesa P, Camarena L. 2007. A complete set of flagellar genes acquired by horizontal transfer coexists with the endogenous flagellar system in *Rhodobacter sphaeroides*. *J Bacteriol* 189:3208–3216. <https://doi.org/10.1128/JB.01681-06>.
- Poggio S, Osorio A, Dreyfus G, Camarena L. 2002. The four different σ^{54} factors of *Rhodobacter sphaeroides* are not functionally interchangeable. *Mol Microbiol* 46:75–85. <https://doi.org/10.1046/j.1365-2958.2002.03158.x>.
- Armitage JP, Macnab RM. 1987. Unidirectional, intermittent rotation of the flagellum of *Rhodobacter sphaeroides*. *J Bacteriol* 169:514–518. <https://doi.org/10.1128/jb.169.2.514-518.1987>.
- Poggio S, Osorio A, Dreyfus G, Camarena L. 2005. The flagellar hierarchy of *Rhodobacter sphaeroides* is controlled by the concerted action of two enhancer-binding proteins. *Mol Microbiol* 58:969–983. <https://doi.org/10.1111/j.1365-2958.2005.04900.x>.
- de la Mora J, Uchida K, del Campo AM, Camarena L, Aizawa S, Dreyfus G. 2015. Structural characterization of the Fla2 flagellum of *Rhodobacter sphaeroides*. *J Bacteriol* 197:2859–2866. <https://doi.org/10.1128/JB.00170-15>.
- Vega-Baray B, Domenzain C, Rivera A, Alfaro-Lopez R, Gomez-Cesar E, Poggio S, Dreyfus G, Camarena L. 2015. The flagellar set Fla2 in *Rhodobacter sphaeroides* is controlled by the CckA pathway and is repressed by organic acids and the expression of Fla1. *J Bacteriol* 197:833–847. <https://doi.org/10.1128/JB.02429-14>.
- Shah DS, Porter SL, Martin AC, Hamblin PA, Armitage JP. 2000. Fine tuning bacterial chemotaxis: analysis of *Rhodobacter sphaeroides* behaviour under aerobic and anaerobic conditions by mutation of the major chemotaxis operons and *cheY* genes. *EMBO J* 19:4601–4613. <https://doi.org/10.1093/emboj/19.17.4601>.
- Porter SL, Armitage JP. 2002. Phosphotransfer in *Rhodobacter sphaeroides* chemotaxis. *J Mol Biol* 324:35–45. [https://doi.org/10.1016/S0022-2836\(02\)01031-8](https://doi.org/10.1016/S0022-2836(02)01031-8).
- Porter SL, Wadhams GH, Martin AC, Byles ED, Lancaster DE, Armitage JP. 2006. The CheYs of *Rhodobacter sphaeroides*. *J Biol Chem* 281:32694–32704. <https://doi.org/10.1074/jbc.M606016200>.
- del Campo AM, Ballado T, de la Mora J, Poggio S, Camarena L, Dreyfus G. 2007. Chemotactic control of the two flagellar systems of *Rhodobacter sphaeroides* is mediated by different sets of CheY and Flim proteins. *J Bacteriol* 189:8397–8401. <https://doi.org/10.1128/JB.00730-07>.
- Martínez-del Campo A, Ballado T, Camarena L, Dreyfus G. 2011. In *Rhodobacter sphaeroides*, chemotactic operon 1 regulates rotation of the flagellar system 2. *J Bacteriol* 193:6781–6786. <https://doi.org/10.1128/JB.05933-11>.
- Martin AC, Gould M, Byles E, Roberts MA, Armitage JP. 2006. Two chemosensory operons of *Rhodobacter sphaeroides* are regulated independently by sigma 28 and sigma 54. *J Bacteriol* 188:7932–7940. <https://doi.org/10.1128/JB.00964-06>.
- Yang Y, Darbari VC, Zhang N, Lu D, Glyde R, Wang YP, Winkelman JT, Gourse RL, Murakami KS, Buck M, Zhang X. 2015. Structures of the RNA polymerase- σ^{54} reveal new and conserved regulatory strategies. *Science* 349:882–885. <https://doi.org/10.1126/science.aab1478>.
- Francke C, Groot Kormelink T, Hagemeyer Y, Overmars L, Sluijter V, Moezelaar R, Siezen RJ. 2011. Comparative analyses imply that the enigmatic sigma factor 54 is a central controller of the bacterial exterior. *BMC Genomics* 12:385. <https://doi.org/10.1186/1471-2164-12-385>.
- Poggio S, Osorio A, Dreyfus G, Camarena L. 2006. Transcriptional specificity of RpoN1 and RpoN2 involves differential recognition of the promoter sequences and specific interaction with the cognate activator proteins. *J Biol Chem* 281:27205–27215. <https://doi.org/10.1074/jbc.M601735200>.
- Brilli M, Fondi M, Fani R, Mengoni A, Ferri L, Bazzicalupo M, Biondi EG. 2010. The diversity and evolution of cell cycle regulation in alpha-proteobacteria: a comparative genomic analysis. *BMC Syst Biol* 4:52. <https://doi.org/10.1186/1752-0509-4-52>.
- Zhou B, Schrader JM, Kalogeraki VS, Abeliuk E, Dinh CB, Pham JQ, Cui ZZ, Dill DL, McAdams HH, Shapiro L. 2015. The global regulatory architecture of transcription during the *Caulobacter* cell cycle. *PLoS Genet* 11:e1004831. <https://doi.org/10.1371/journal.pgen.1004831>.
- Ouimet MC, Marczynski GT. 2000. Analysis of a cell-cycle promoter bound by a response regulator. *J Mol Biol* 302:761–775. <https://doi.org/10.1006/jmbi.2000.4500>.
- Porter SL, Wadhams GH, Armitage JP. 2008. *Rhodobacter sphaeroides*:

- complexity in chemotactic signalling. *Trends Microbiol* 16:251–260. <https://doi.org/10.1016/j.tim.2008.02.006>.
35. Girard L, Brom S, Davalos A, Lopez O, Soberon M, Romero D. 2000. Differential regulation of *fixN*-reiterated genes in *Rhizobium etli* by a novel *fixL*-*fixK* cascade. *Mol Plant Microbe Interact* 13:1283–1292. <https://doi.org/10.1094/MPMI.2000.13.12.1283>.
 36. Grote A, Klein J, Retter I, Haddad I, Behling S, Bunk B, Biegler I, Yarmolinetz S, Jahn D, Munch R. 2009. PRODORIC (release 2009): a database and tool platform for the analysis of gene regulation in prokaryotes. *Nucleic Acids Res* 37:D61–D65. <https://doi.org/10.1093/nar/gkn837>.
 37. Domenzain C, Camarena L, Osorio A, Dreyfus G, Poggio S. 2012. Evolutionary origin of the *Rhodobacter sphaeroides* specialized RpoN sigma factors. *FEMS Microbiol Lett* 327:93–102. <https://doi.org/10.1111/j.1574-6968.2011.02459.x>.
 38. Sockett RE, Foster JCA, Armitage JP. 1990. Molecular biology of the *Rhodobacter sphaeroides* flagellum. *FEMS Symp* 53:473–479.
 39. Siström WR. 1962. The kinetics of the synthesis of photopigments in *Rhodospseudomonas sphaeroides*. *J Gen Microbiol* 28:607–616. <https://doi.org/10.1099/00221287-28-4-607>.
 40. Ausubel FM, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1987. *Current protocols in molecular biology*. John Wiley and Sons, New York, NY.
 41. Quandt J, Hynes MF. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram negative bacteria. *Gene* 127: 15–21. [https://doi.org/10.1016/0378-1119\(93\)90611-6](https://doi.org/10.1016/0378-1119(93)90611-6).
 42. Servín-González L, Sampieri AI, Cabello J, Galván L, Juárez V, Castro C. 1995. Sequence and functional analysis of the *Streptomyces phaeochromogenes* plasmid pJV1 reveals a modular organization of *Streptomyces* plasmids that replicate by rolling circle. *Microbiology* 141(Part 10): 2499–2510. <https://doi.org/10.1099/13500872-141-10-2499>.
 43. Priefer UB, Simon R, Pühler A. 1985. Extension of the host range of *Escherichia coli* vectors by incorporation of RSF1010 replication and mobilization functions. *J Bacteriol* 163:324–330.
 44. Davis J, Donohue TJ, Kaplan S. 1988. Construction, characterization, and complementation of a Puf⁻ mutant of *Rhodobacter sphaeroides*. *J Bacteriol* 170:320–329. <https://doi.org/10.1128/jb.170.1.320-329.1988>.
 45. Thanbichler M, Iniesta AA, Shapiro L. 2007. A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in *Caulobacter crescentus*. *Nucleic Acids Res* 35:e137. <https://doi.org/10.1093/nar/gkm818>.
 46. Metcalf WW, Wanner BL. 1993. Construction of new β -glucuronidase cassettes for making transcriptional fusions and their use with new methods for allele replacement. *Gene* 129:17–25. [https://doi.org/10.1016/0378-1119\(93\)90691-U](https://doi.org/10.1016/0378-1119(93)90691-U).
 47. Aiba H, Adhya S, de Crombrugge B. 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J Biol Chem* 256: 11905–11910.
 48. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675. <https://doi.org/10.1038/nmeth.2089>.
 49. Harlow E, Lane D. 1988. *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 50. Ferre A, De La Mora J, Ballado T, Camarena L, Dreyfus G. 2004. Biochemical study of multiple CheY response regulators of the chemotactic pathway of *Rhodobacter sphaeroides*. *J Bacteriol* 186:5172–5177. <https://doi.org/10.1128/JB.186.15.5172-5177.2004>.
 51. Jefferson RA, Burgess SM, Hirsh D. 1986. β -Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc Natl Acad Sci U S A* 83: 8447–8451. <https://doi.org/10.1073/pnas.83.22.8447>.
 52. Turatsinze JV, Thomas-Chollier M, Defrance M, van Helden J. 2008. Using RSAT to scan genome sequences for transcription factor binding sites and *cis*-regulatory modules. *Nat Protoc* 3:1578–1588. <https://doi.org/10.1038/nprot.2008.97>.
 53. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. *Nat Biotechnol* 1:784–791. <https://doi.org/10.1038/nbt1183-784>.