NOTE

In *Rhodobacter sphaeroides*, Chemotactic Operon 1 Regulates Rotation of the Flagellar System 2[▽][†]

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***Rhodobacter sphaeroides* is able to assemble two different flagella, the subpolar flagellum (Fla1) and the polar flagella (Fla2). In this work, we report the swimming behavior of *R. sphaeroides* Fla2$^+$ cells lacking each of the proteins encoded by chemotactic operon 1. A model proposing how these proteins control Fla2 rotation is presented.**

Chemotaxis is a complex response that allows bacteria to swim toward favorable stimuli and away from repellents. A network of proteins which transmit a signal from the chemoreceptors to the flagellar motor controls this process.

In *Escherichia coli*, membrane chemoreceptors, or MCPs (methyl-accepting chemotactic proteins), are coupled to CheA through CheW. The kinase activity of CheA is controlled by the ligand occupancy state of the MCP (for reviews, see references 1, 7, 17, 28, 46, and 49). When activated, CheA promotes its own phosphorylation; this phosphate is transferred to a specific Asp residue on CheY (18, 36, 53). The phosphorylated form of CheY (CheY-P) suffers a conformational change that increases its affinity for FliM (51, 52). FliM, FliG, and FliN constitute the C-ring or switch apparatus (12, 13, 20–22, 55). Binding of CheY-P to FliM and FliN changes flagellar rotation from counterclockwise (CCW) to clockwise (CW) (5, 37, 53). When the flagellar motor is rotating CCW, cells swim in a linear trajectory, known as a run. However, when the motor switches to the CW direction, swimming is interrupted by a tumbling event (48). Tumbling events are short-lived given the rapid dephosphorylation of CheY-P, caused by the phosphatase activity of CheZ (8, 18, 53).

During adaptation, CheA kinase activity is reset to prestimulus levels. This step involves the reversible methylation of the MCPs that is carried out by the constitutively active methyltransferase CheR (43) and the methylesterase CheB (44). When CheB is phosphorylated by CheA, methylesterase activity increases about 100-fold (3, 23). An increase in methylation counteracts the inhibitory signal generated by attractant binding (6, 14, 26, 47).

Many nonenteric bacterial species show multiple homologues of the chemotactic genes, suggesting that more complex chemosensory pathways are frequently present (16, 54). *Rhodobacter sphaeroides* has become an interesting model with which to study these pathways. The complete genome sequence of *R. sphaeroides* revealed the presence of multiple chemotactic genes arranged mainly in three different loci: *che*Op$_1$, *che*Op$_2$, and *che*Op$_3$ (for a review, see reference 32).

R. sphaeroides swims in liquid medium using a single subpolar flagellum (Fla1) that rotates unidirectionally to produce smooth swimming. Reorientation occurs when flagellar rotation stops briefly. Brownian motion and the slow rotation of the filament coil reorient the cell body before smooth swimming is resumed (4, 29). Genetic and biochemical evidence suggests that the proteins encoded by *che*Op$_2$ and *che*Op$_3$ are responsible for the control of the chemotactic response of Fla1 flagella (15, 24, 25), whereas deletion of *che*Op$_1$ does not have any effect in this system (15, 50). In particular, CheY6 plus CheY3 or CheY4 is required to stop the flagellar motor (33).

We have previously shown that pseudorevertants from a Fla1$^-$ mutant are able to swim in liquid medium by means of multiple polar flagella whose structural components are encoded by a second set of genes (*fla2*). This set is not expressed in the wild-type strain under any laboratory conditions tested so far (30). Cells expressing Fla2 flagella form a ring in swimming plates containing different organic acids. This chemotactic response is mediated only by CheY1, CheY2, and CheY5 (11). Given that these proteins are encoded by *che*Op$_1$, we hypothesized that this locus must control Fla2 flagella.

In this work, we isolated nonpolar mutants for each of the chemotactic genes present in *che*Op$_1$. From the behavioral analysis, we conclude that the majority of the proteins encoded by *che*Op$_1$ are involved in controlling Fla2. A model of the signal-processing circuit controlling Fla2 rotation is presented below.

Bacterial strains and plasmids are listed in Table 1. *R. sphaeroides* (40) was grown in liquid or solid Sistrom's minimal medium (39) as described previously (30).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
TOP10	Cloning strain	Invitrogen
S17-1	*recA endA thi hsdR* RP4-2 Tc::Mu-Kn::Tn7 Tp^r Sm^r	38
R. sphaeroides		
WS8-N	Wild type, spontaneous Nal^r	40
RS1Y5	WS8-N derivative, *cheY5::aadA*	This study
RS1B	WS8-N derivative, *mcpB::aadA*	This study
RS1S	WS8-N derivative, *tlpS::aadA*	This study
RS1A	WS8-N derivative, *mcpA::aadA*	This study
RS1D	WS8-N derivative, *cheD::aadA*	This study
RS1Y1	WS8-N derivative, *cheY1::aadA*	This study
RS1A1	WS8-N derivative, *cheA1::aadA*	This study
RS1W1	WS8-N derivative, *cheW1::aadA*	This study
RS1R1	WS8-N derivative, *cheR1::aadA*	This study
RS1Y2	WS8-N derivative, *cheY2::aadA*	This study
RS12432	WS8-N derivative, RSP2432::*aadA*	This study
AM1	SP13 derivative, *fla2*$^+$	11
RS2Y5	AM1 derivative, *cheY5::aadA*	11
RS2B	AM1 derivative, *mcpB::aadA*	This study
RS2S	AM1 derivative, *tlpS::aadA*	This study
RS2A	AM1 derivative, *mcpA::aadA*	This study
RS2D	AM1 derivative, *cheD::aadA*	This study
RS2Y1	AM1 derivative, *cheY1::aadA*	11
RS2A1	AM1 derivative, *cheA1::aadA*	This study
RS2W1	AM1 derivative, *cheW1::aadA*	This study
RS2R1	AM1 derivative, *cheR1::aadA*	This study
RS2Y2	AM1 derivative, *cheY2::aadA*	11
RS22432	AM1 derivative, RSP2432::*aadA*	This study
Plasmids		
pTZ19R	Cloning vector; pUC derivative; Ap^r	Pharmacia
pJQ200mp18	Suicide vector used for gene replacement; Gm^r	34
pRK415	pRK404 derivative, used for expression in *R. sphaeroides*; Tc^r	19
pRKtlpS	pRK415 derivative expressing *tlpS*	This study
pRKmcpA	pRK415 derivative expressing *mcpA*	This study
pRKcheD	pRK415 derivative expressing *cheD*	This study
pRKcheA1	pRK415 derivative expressing *cheA1*	This study
pRKcheW1	pRK415 derivative expressing *cheW1*	This study
pRKcheR1	pRK415 derivative expressing *cheR1*	This study
pRK2432	pRK415 derivative expressing RSP2432	This study

Rotation and energy source of Fla2 flagella. To analyze the rotational bias of Fla2 flagella, AM1 cells (Fla1$^-$ Fla2$^+$) were tethered to a microscope slide using antiflagellin antibodies. Cells were recorded using a digital camera attached to a high-intensity dark-field microscope (Olympus BH-2). Video recordings of tethered cells were analyzed using CellTrak 1.5 (Motion Analysis Corp., Santa Rosa, CA). A total of 40 cells were analyzed for at least 10 s, and results from a single representative cell are shown in Fig. 1. The orientation (measured in degrees) accumulates as a function of time, suggesting that Fla2 flagella rotate only in one direction and in a stop-go fashion, as has been shown for the Fla1 system (4). Additionally, we investigated the energy source of the Fla2 motor. AM1 cells were grown on Sistrom's minimal medium devoid of succinate under photoheterotrophic conditions. When the culture

reached an optical density at 600 nm of approximately 0.3, a 500-μl aliquot of cells was removed and treated with the protonophore carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) or with the sodium channel blocker amiloride. We added 10 μM CCCP or 0.5 mM amiloride to the cell culture, as described previously (27, 45). Motility was completely inhibited by CCCP (see Movie S1 in the supplemental material). In contrast, cells continued swimming in the presence of amiloride. These results demonstrate that Fla2 flagella rotate unidirectionally and that the proton motive force is responsible for motor rotation.

Isolation and swimming phenotype of *che*Op$_1$ mutants. The *che*Op$_1$ locus (Fig. 2A) contains 11 genes, including one unassigned open reading frame and genes encoding the following putative proteins: three response regulators, one sensor kinase, one methyltransferase, one adaptor protein, two membrane chemoreceptors, one cytoplasmic chemoreceptor, and one receptor deamidase. We mutated each gene, with the exception of the *cheY* genes, which were mutated previously (11). Mutants were generated by the amplification of the desired gene followed by the insertion of a nonpolar Spc^r cassette (11). These fragments were subcloned into the suicide plasmid pJQ200mp18 (34) and mobilized into WS8-N (Fla1$^+$ Fla2$^-$) and AM1 (Fla1$^-$ Fla2$^+$) by conjugation with the S17-1 strain (10, 38). The Spc^r Gm^s transconjugants were selected, and the allelic exchange was confirmed by PCR.

Analysis of the chemotactic behavior of the *che*Op$_1$ mutants in soft agar swimming plates revealed that strains RS2Y5, RS2S, RS2A, RS2D, RS2Y1, RS2A1, RS2W1, RS2R1, RS2Y2, and RS22432 showed a smaller swimming ring than AM1 (Fig. 2B). This result is consistent with the idea that the proteins encoded by *che*Op$_1$ control the chemotactic response of Fla2. The same mutations in the genetic background where only the Fla1 flagellum is expressed have no significant effect on chemotaxis, as reported previously (data not shown) (11, 15, 24, 25, 33, 50).
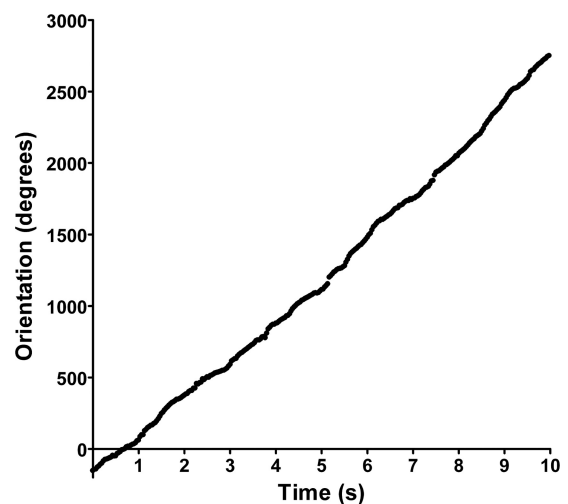

FIG. 1. Tethered-cell analysis of Fla2 flagella from *Rhodobacter sphaeroides*. Cells were analyzed using CellTrak 1.5 to determine the rotational bias of flagella. The direction of orientation of individual cells is measured in degrees and given as a function of time in seconds. As cells rotate in one direction, the orientation value increases linearly. Results for an individual representative cell are shown in this graph.
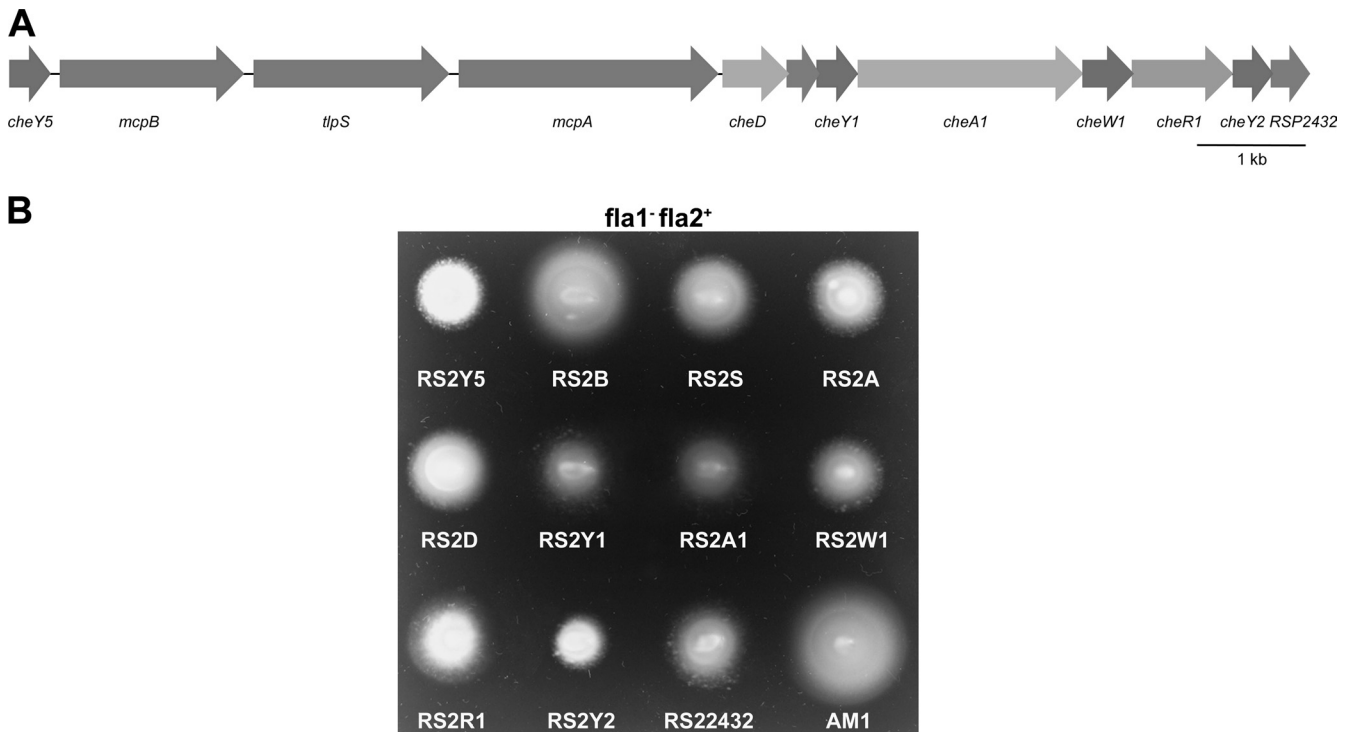
A

B

fla1⁻ fla2⁺

FIG. 2. (A) Organization of the *che*Op₁ locus of *R. sphaeroides*. The arrows represent genes and their direction of transcription. (B) Phenotypic analysis of the *che*Op₁ mutants in the *fla1⁻ fla2⁺* genetic background. Soft agar motility assays were carried out in 0.25% agar supplemented with 100 μM succinate. Cells were grown under aerobic conditions at 30°C for 48 h.

In order to demonstrate that the observed phenotypes in the Fla1⁻ Fla2⁺ background were due only to the absence of each of the *che*Op₁ genes, we carried out a complementation test introducing the wild-type gene cloned into pRK415 (19). Figure 3 shows that all the strains were complemented by the corresponding wild-type gene, except for the mutant strain RS2A, which was partially complemented, indicating that the

impairment in motility produced by each mutation was recovered.

Characterization of swimming motility. To better characterize the *che*Op₁ mutants, we analyzed free-swimming behavior. Swimming paths were obtained with CellTrak software by analyzing video recordings of motile cultures of *R. sphaeroides* strains as described above. The stopping frequency and aver-
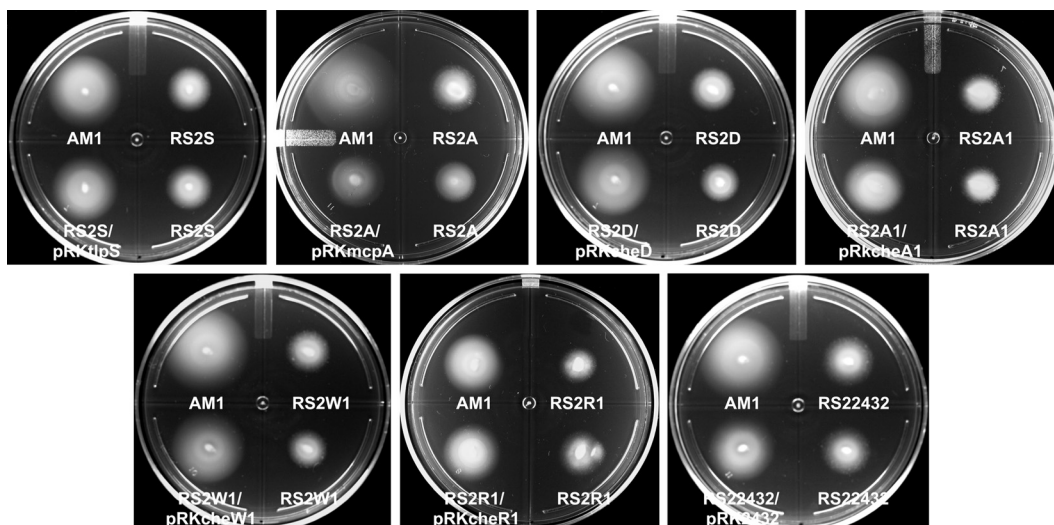

FIG. 3. Complementation of each *che*Op₁ mutant by the corresponding wild-type gene cloned in pRK415. Soft agar motility assays were carried out in 0.25% agar supplemented with 100 μM succinate. Cells were grown under aerobic conditions at 30°C for 48 h.
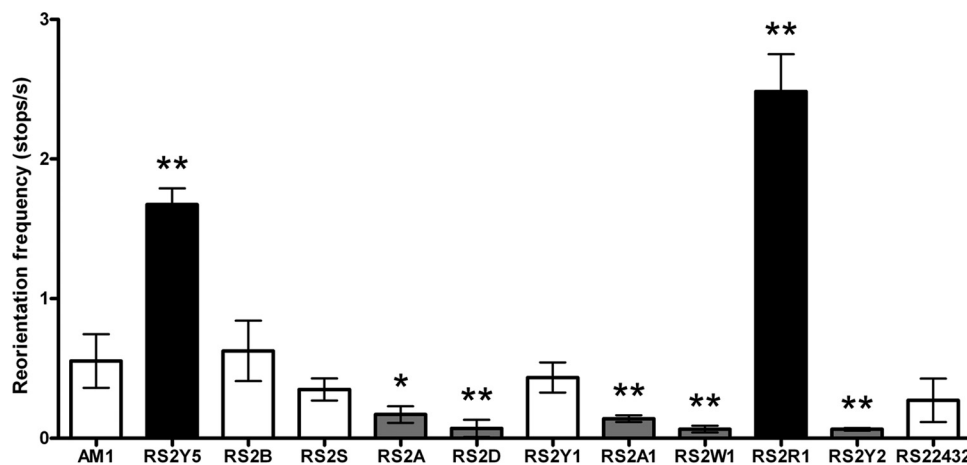
FIG. 4. Reorientation frequency of free-swimming cells. Values are means ± standard deviations from 100 cells. Significance was assessed by one-way analysis of variance; ∗∗, $P < 0.01$; ∗, $P < 0.05$.

age run velocity were determined by analyzing for 100 cells from three independent cultures for 2 s. As shown in Fig. 4, AM1 cells reorient under these conditions approximately once every 2 s (0.55 stop/s). Mutant strains RS2A (*mcpA*), RS2D (*cheD*), RS2A1 (*cheA1*), RS2W1 (*cheW1*), and RS2Y2 (*cheY2*) showed a lower reorientation frequency, which is equivalent to a smooth swimming bias. In contrast, RS2Y5 (*cheY5*) and RS2R1 (*cheR1*) stop at a higher frequency, similar to a tumbling behavior. Furthermore, strains RS2Y1 (*cheY1*), RS2B (*mcpB*), RS2S (*tlpS*), and RS22432 (*RSP2432*) had essentially the same reorientation frequency as the parent strain. This suggests that under this particular condition, none of these products participate in the Fla2 chemotaxis signaling pathway.

From these results we propose that CheY2 is mainly responsible for stopping the Fla2 flagellar motor. Given that RS2A1 and RS2W1 show the same phenotype as RS2Y2, it could be assumed that CheA1, CheW1, and CheY2 comprise the signal transduction pathway that controls Fla2 (Fig. 5). In agreement with this idea, it has been shown *in vitro* that CheA1 is able to phosphorylate the response regulators encoded by *che*Op₁ (CheY1, CheY2, and CheY5) (31). Therefore, it is likely that the phosphorylated form of CheY2 (CheY2-P) is the signal that stops the Fla2 flagellar motor. Moreover, we observed that the absence of *mcpA* also reduces the stopping frequency significantly; hence, it is possible that this MCP together with CheW1 modulates CheA1 activity.

Conversely, in the absence of CheY5, a marked increase of the stopping frequency was detected, suggesting that the intracellular concentration of CheY2-P was increased. Given that the phenotype shown by RS2Y5 cells evokes that observed for *cheZ* mutants in *E. coli*, it is tempting to suggest that CheY5 could act as a phosphate sink. A similar situation occurs in *Sinorhizobium meliloti*, which has two different CheY proteins and no CheZ. In this case, CheA phosphorylates both CheYs, but only CheY2 binds to the flagellar motor to control rotation. The response is terminated when CheY2-P transfers its phosphate group back to CheA, and then it is transferred to CheY1 (41, 42). In this regard, CheY5 in *R. sphaeroides* could play a role similar to that of CheY1 in *S. meliloti* (Fig. 5).

Another strain that showed a high reorientation frequency is

the *cheR1* mutant (strain RS2R1), indicating that CheR1 is a relevant component of the chemotactic pathway that controls the Fla2 motor.

In *E. coli*, an attractant detected by an MCP inhibits CheA activity, and the reduction in the concentration of CheY-P allows bacteria to swim toward the attractant; in the adaptation phase, CheR methylates the MCP, and in this state, CheA autophosphorylation is reactivated (6, 26). In *Bacillus subtilis*, a
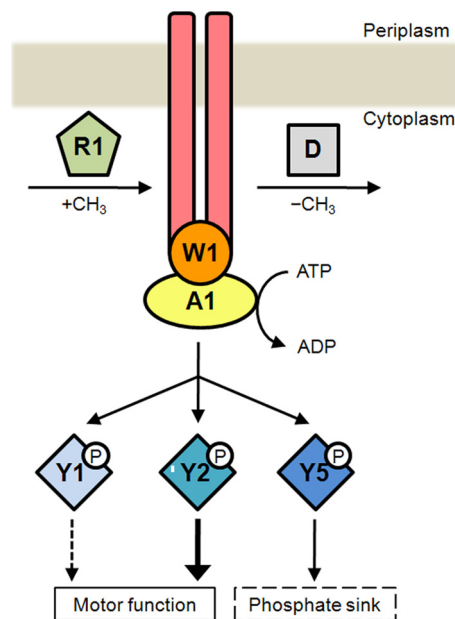

FIG. 5. Chemotactic control of Fla2 flagella is mediated by the proteins encoded in *che*Op₁. In this model, a chemoreceptor, which could be McpA, controls the kinase activity of CheA1. When the MCP is methylated by the product of CheR1, autophosphorylation of CheA1 is inhibited and flagella rotate continuously for a longer time. CheD could demethylate the chemoreceptor and promote CheA1 activation, bringing about the phosphorylation of CheY1, CheY2, and CheY5. The accumulation of CheY2-P, in turn, increases the probability of reorientation, whereas CheY5 could modulate the intracellular levels of CheY2-P by acting as a phosphate sink.

TABLE 2. Free-swimming behavior of *R. sphaeroides*
*che*Op$_1$ mutants

Strain	Run speed (μm/s)[a]
AM1	61.35 ± 7.98
RS2Y5	51.67 ± 0.91
RS2B	62.62 ± 5.94
RS2S	53.92 ± 2.97
RS2A	55.83 ± 6.13
RS2D	61.24 ± 8.34
RS2Y1	61.40 ± 2.19
RS2A1	64.42 ± 2.68
RS2W1	62.57 ± 5.53
RS2R1	39.51 ± 7.73[b]
RS2Y2	57.53 ± 3.39
RS22432	55.37 ± 3.19

[a] Values are averages ± standard deviations for 100 cells from three independent experiments.
[b] $P < 0.01$; significance was assessed by one-way analysis of variance.

more complex situation has been observed, given that methylation of some residues of certain MCPs inhibits CheA, whereas methylation of other residues activates it (35). In our case, the phenotype of RS2R1 (Fig. 4) leads us to propose that methylation of a yet-unknown receptor (that may be McpA) inhibits CheA1 activity (Fig. 5).

Under our experimental conditions, the absence of CheR and McpA produced a bias in flagellar rotation, whereas the absence of McpB did not. This finding could indicate that McpB senses different signals that were absent under these conditions.

Adaptation also requires a methylesterase that counteracts the methylating activity of CheR. In *E. coli*, CheB accomplishes this function, although in other bacteria, such as *Thermotoga maritima*, the protein CheD could act as a methylesterase (9). In *R. sphaeroides*, the absence of CheD induces a reduction in the stopping frequency, which is in agreement with the possibility that a demethylated receptor would activate CheA1, whereas a methylated receptor would inhibit its activity (Fig. 5).

The average run speed, measured as the average velocity between stops, for each one of these mutants was determined. Table 2 shows that RS2Y5 and RS2R1 were the only strains with a lower swimming speed value than AM1. This reduction in swimming speed correlates with the fact that these strains show a very high stopping frequency, so it is possible that maximal speed cannot be reached between stops. A similar effect was observed in *E. coli*, where overexpression of CheY-P not only caused an increase in the tumbling frequency but also affected the swimming speed negatively (2).

Intriguingly, the stop frequencies and swimming speeds of RS2Y1 (*cheY1*), RS2S (*tlpS*), and RS22432 (RSP2432) are similar to those of AM1 (Fig. 4 and Table 2); nonetheless, in a swimming plate, these mutants showed a different phenotype (Fig. 2B). It is possible that chemotactic signals in soft agar plates could be stronger than in liquid medium (due to different diffusion rates), so it is still possible that CheY1, TlpS, and RSP2432 may control Fla2 flagella when the chemotactic stimulus is strong. Further research will determine if this hypothesis is adequate to explain the observed phenotypes.

Based on the results obtained in this work, we propose the model shown in Fig. 5.

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