Many bacteria are able to navigate through their local environment using a variety of motility mechanisms. The rotary motion of flagella is utilized by diverse motile bacteria to propel themselves through aqueous media. Flagella are helical extracellular filaments of the protein flagellin that are connected to a flexible, curved segment called the hook, anchored to the membrane via the basal body, and powered by a rotary motor complex (1, 2). For Escherichia coli and other well-studied bacteria, multiple rotating flagella can coalesce into a multifilament bundle, and the concerted motion of this bundle provides propulsion. In E. coli, counterclockwise (CCW) flagellar rotation promotes bundling and drives periods of straight swimming called runs. Clockwise (CW) rotation of one or more flagella leads to polymorphic transitions of the helical flagellar filament, resulting in bundle disruption that causes reorientations known as tumbles. Although many bacterial species initiate tumbles by reversing flagellar rotation like E. coli, others have unidirectional motors, and unbundling occurs when one or more flagella slow their rotation or stop (3–5). For bacteria with multiple flagella, the dynamics and degree of unbundling determine the extent to which the cell is reoriented from its previous course during a tumble (3, 6, 7).

The rotation of the flagellar motor is modulated through its interactions with an intracellular response regulator called CheY that is controlled in turn by the chemotaxis-signaling network. Chemotaxis is a sensory mechanism by which cells bias their movement in response to external chemical gradients.
signal detection is initiated at receptor clusters in the cytoplasmic membrane and transduced through a complex internal signaling pathway comprised of multiple chemotaxis (Che) proteins (8), culminating in a change in the concentration of phosphorylated CheY (CheY-P). CheY-P stimulates tumbles by binding to the FliM protein in the FliM-FliG-FliN flagellar motor switch complex, which in E. coli-type motility systems increases the CW rotational bias. Chemotaxis thereby modulates the frequency of flagellar reversals, regulating the duration of the mean run time and resulting in a biased random walk toward attractants and away from repellents (9–11).

The mechanisms governing the chemotactic response have been intensively studied, and a wide variety of mutants have been isolated. Chemotaxis mutations (e.g., cheA mutations) that decrease tumbling frequency lead to relatively straight, uninterrupted runs, whereas other mutations (e.g., cheB mutants) can increase tumbling, limiting the frequency and duration of runs. For decades, swimming motility has been assayed using semisolid motility agar (a low-concentration, high-porosity, fluid-filled random network of agarose polymers), by inoculating bacteria at a single point and measuring the radial spread of bacterial growth, the so-called swim ring, over time (12–15). As the bacteria deplete nutrients at the site of inoculation, they establish a chemical gradient, and chemotaxis further promotes the outward radial migration of cells toward higher nutrient levels. For straight-swimming, nontumbling mutants, swim ring advancement is greatly diminished, as cells are impeded by the agar network. Hyperswitching mutants are also less efficient in motility agar migration, as they change direction so frequently that their net movement is compromised. In both cases, decoupling the mean run time from Che control abolishes the ability to bias movement in response to gradients (16–20).

Many of the E. coli Che proteins are encoded within the che genetic cluster, and che cluster deletion mutants are straight swimming and spread inefficiently in motility agar. Extended incubation of an E. coli che deletion mutant in these assays was found to generate suppressor mutations that increased the radial spread of the population through the agar, appearing to reverse the chemotaxis deficiency, first shown in a classic work by Wolfe and Berg (21). These secondary mutations were found to give rise to increased tumbling relative to that of the straight swimming parent. Rather than regaining chemotaxis, several of these mutants were found to have elevated flagellar rotation reversals due to point mutations in the fliM and fliG genes, which encode the flagellar switch and rotor, respectively, and together direct flagellar reversal. While the Che system normally stimulates these reversals, these mutants randomly switch rotation independently (16, 22, 23). Hence, the effective migration of nonchemotactic cells in porous agar, termed pseudotaxis, is a purely diffusive spread of motile bacteria that does not respond to chemical gradients.

Analysis of straight-swimming mutants of the alphaproteobacterial plant pathogen Agrobacterium tumefaciens with deletions of the cheA chemotaxis regulator or the entire che gene cluster led to the isolation of suppressor mutants that rescued the decreased che mutant migration through motility agar (24). These were denoted chemotaxis mutation suppressor (cms) mutants and were found to have regained the ability to tumble in suspension. The cms mutations also resulted in a decreased ability of A. tumefaciens to transition from a motile state to a surface-attached biofilm growth mode (24). A. tumefaciens produces a sparse tuft of up to 6 polar flagella (25). In contrast to E. coli and as with other related members of the Rhizobiales family, A. tumefaciens is thought to drive forward propulsion through CW flagellar rotation, resulting in multifilament bundle formation (4, 26–28). Tumbles are thought to occur due to slowing of CW rotation, causing disruption of the flagellar bundle, rather than through rotation reversal (4, 5, 27). These mechanistic differences suggested that cms mutations resulting in A. tumefaciens pseudotaxis might not be in the same switch complex components as those in E. coli. Indeed, the fliM and fliG homologues, as well as several motor and che gene sequences, were wild type (24).

In this work, we analyze a collection of A. tumefaciens cms mutants and utilize whole-genome sequencing to map their mutations. We identify several new mechanisms by which the pseudotaxis phenotype can arise. We find that these mutations affect the structure of the flagellum and the process by which its rotation is powered rather than altering the regulatory switch complex, which can control the speed and direction of flagellar rotation and which is the most common source of pseudotaxis in previous studies on E. coli and Salmonella.

**RESULTS AND DISCUSSION**

Alterations in the A. tumefaciens flagellar hook result in pseudotaxis. A. tumefaciens mutants with chemotaxis mutation suppressors (cms, e.g., $\Delta$cheA mutants or mutants with deletion of the entire Che cluster [$\Delta$che], Atu0514 to Atu0522) after extended incubation on motility agar. Such cms mutants exhibited extended swim rings (Fig. 1; Fig. S1A and C in the supplemental
material) and tumbling swimming in suspension, in contrast to the small swim ring and straight swimming of their parent strain. Although these mutants were phenotypically similar to the pseudotaxis mutants of Wolfe and Berg (21), the \( fliM \) and \( fliG \) genes, encoding the switch and rotor, were wild type in the \( A. \) tumefaciens cms-1 mutant. Whole-genome sequencing the cms-1 mutant, using Illumina high-throughput sequencing, indicated a single base substitution (C1010G) within the 1,278-bp \( flgE \) (Atu0574) gene encoding the flagellar hook (Fig. 2A), resulting in a change from alanine to glycine (A337G) (Fig. 2B; Table 1). Fifteen more cms mutants were independently isolated from \( A. \) tumefaciens cheA and che mutants in two separate screens for suppressors on motility agar. Independent cms mutants were designated with numerical suffixes (Table 1). Twelve of these had mutations in \( flgE \), 11 had single missense mutations, and cms-15 had incurred an 18-bp base duplication. All of these cms mutants were independent, but two pairs had identical mutations (cms-5 and -16 and cms-12 and -18) (Table 1). We designated the \( flgE \) alleles class I cms mutants.

Class I cms mutations map to the FlgE D1 domain. We next mapped the cms mutations onto the hook structure. The three-dimensional structure of the \( A. \) tumefaciens FlgE protein (FlgE\(_{AT} \)) has not been determined; however, the structure of the related \( Salmonella \) enterica serovar Typhimurium FlgE (FlgE\(_{ST} \)) is available, and the amino acid sequences of the two proteins are 31% identical and 46% similar. X-ray crystallographic and cryo-electron microscopy (cryo-EM) studies determined that FlgE\(_{ST} \) consists of four unique domains (Fig. 3A and B) (29, 30), D0, D1, D2, and Dc. The D1 domain (residues 71 to 144 and 285 to 363) is the central domain, flanked on one side by an alpha-helical coiled-coil formed by D0 (residues 1 to 24 and 367 to 402) and Dc (residues 25 to 70) and on the other side by a flexible linker, followed by the D2 domain (residues 145 to 284). The D0 and Dc domains are closely packed in the hook interior, while the D1 and D2 domains are external to the hook central axis (Fig. 3B). We aligned the FlgE\(_{AT} \) and FlgE\(_{ST} \) amino acid sequences (see Fig. S2A in the supplemental material) (31), mapping the cms FlgE\(_{AT} \) mutations onto the homologous positions in the FlgE\(_{ST} \) structure (PDB ID 3A69) (29). Nine of the 11 class I cms mutations we identified clustered together on one face of the central D1 domain (Fig. 3A). For the cms-14 mutation, there was no corresponding FlgE\(_{ST} \) residue, and for the cms-15 mutation, the six additional duplicated amino acids could not be modeled (32, 33).

The arrangement and interaction of the ~120 FlgE monomers that make up the hook are critical to its function. The flexibility of this highly ordered, helical complex has been shown to be important for proper flagellar rotation, bundling, and unbundling (34, 35). We hypothesize that the class I cms mutations in \( flgE \), through

![Flagellar structure with cms mutations and FlgE domain organization.](http://mbio.asm.org/33/3/mbio.asm.org)

**FIG 2** Flagellar structure with cms mutations and FlgE domain organization. (A) Flagellar structure. Black arrowheads denote flagellar substructures, i.e., the filament (A), hook (B), and basal body (C). Roman numerals I, II, and III indicate mutations as follows: class I (FlgE), blue; class II (FliK), orange; class III (MotA), purple. Flagellar proteins whose genes were found to be wild type by direct sequencing are indicated by numbers 1 to 5 as follows: 1 and 2, hook filament junction proteins FlgK and FlgL; 3, hook cap protein FlgD; 4, distal rod protein FlgG; 5, switch protein FliM. FliK is drawn as an extended filament that runs from the basal body through the hook. (B) Domain organization based on the \( Salmonella \) FlgE structure. Approximate boundaries are indicated, and lengths in amino acids are shown under the bar. NC, protein segments (1 to 70 and 370 to 402) that were not crystallizable; I, a region that is invisible in the electron density map (364 to 369). Locations of class I cms mutations in the two regions that comprise domain D1 are indicated, corresponding to the data in Table 1. D2 is the outer domain (30, 68). The \( Salmonella \) FlgE is 403 aa, while \( A. \) tumefaciens FlgE is 425 aa, and this difference is denoted by an asterisk in the figure.
changes in the hook structure and/or flexibility, alter nominal flagellar bundling, causing the cms mutant cells to tumble. One possibility could be that these mutants develop shorter or longer hooks. However, transmission electron microscopy (TEM) analysis of class I cms mutant hooks revealed no obvious differences in length (Fig. 4B). In order to evaluate how the altered FlgE residues in cms mutants might interact between adjacent monomers, FlgE subunits were modeled into the hook structure, based on images of chemically straightened hooks from S. Typhimurium (29, 36). Each turn of the helical hook structure consists of 11 FlgE monomers (Fig. 3B). No predicted intermolecular interactions are revealed between the mutated cms residues and other monomers within one helical turn, but in the assembled straight-hook model, FlgE monomers in one helical turn are in close proximity to monomers in an adjacent helical turn (Fig. 3C). Specifically, residues V93 in cms-9 and Y127 in cms-12 interact with D2, and G372 in cms-8 interacts with D1 of an adjacent FlgE monomer. The distance between the cms mutations and their nearest neighboring residues is 12 Å, precluding specific electrostatic or hydrophobic interactions between individual monomers in the straight-hook model. In a dynamically bending hook, these distances are likely to change, making transient short-range interactions between the helical rings possible as the hook isomerizes in its function as a universal joint. We hypothesize that the cms FlgE proteins modify the dynamic interactions within the hook structure, ultimately altering flagellar motion and promoting frequent unbundling of the flagella.

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FIG 3 cms mutations modeled onto the S. Typhimurium FlgE protein and hook. (A) The monomeric FlgE domains determined from the cryo-EM structure are color coded as follows: D0, pink; D1, blue; D2, green; Dc, red. The cms mutation sites are shown as yellow space-filling models. (B) One helical turn of the straight hook composed of 11 FlgE monomers. (C) A side view of four helical turns in the straight-hook model.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Gene product</th>
<th>Base mutation</th>
<th>Amino acid change</th>
<th>Swim ring size (% of WT ± SEM)</th>
<th>Biofilm biomass (% of WT ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δche mutant</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>33.3 ± 1.7</td>
<td>182.4 ± 7.6</td>
</tr>
<tr>
<td>cms-1</td>
<td>FlgE</td>
<td>G1115A</td>
<td>G372D</td>
<td>42.8 ± 0.6</td>
<td>25.5 ± 6.0</td>
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<td>cms-2</td>
<td>FliK</td>
<td>G955C</td>
<td>G319R</td>
<td>68.9 ± 1.1</td>
<td>49.3 ± 2.0</td>
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<tr>
<td>cms-3</td>
<td>MotA</td>
<td>A179G</td>
<td>A375G</td>
<td>50.6 ± 1.5</td>
<td>28.0 ± 3.6</td>
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<tr>
<td>cms-4</td>
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<td>Y127S</td>
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<td>29.5 ± 2.8</td>
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<tr>
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<td>Y127C</td>
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<td>28.7 ± 4.3</td>
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<tr>
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<td>FlgE</td>
<td>Y1115A</td>
<td>Y1115A</td>
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<td>49.3 ± 2.0</td>
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<td>cms-7</td>
<td>FlgE</td>
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<td>49.3 ± 2.0</td>
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<td>cms-8</td>
<td>FlgE</td>
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<td>G277T</td>
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<td>75.6 ± 8.5</td>
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<tr>
<td>cms-9</td>
<td>FlgE</td>
<td>T1102C</td>
<td>T1102C</td>
<td>81.1 ± 3.1</td>
<td>34.8 ± 5.7</td>
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<tr>
<td>cms-10</td>
<td>FlgE</td>
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<td>A380C</td>
<td>86.7 ± 2.5</td>
<td>28.4 ± 2.8</td>
</tr>
<tr>
<td>cms-11</td>
<td>FlgE</td>
<td>T431C</td>
<td>T431C</td>
<td>81.1 ± 1.5</td>
<td>ND</td>
</tr>
<tr>
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<td>T431C</td>
<td>T431C</td>
<td>72.2 ± 0.6</td>
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<td>Duplication of bases 1063–1080</td>
<td>Duplication of aa 355–360</td>
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<td>Duplication of aa 355–360</td>
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<td>75.6 ± 8.5</td>
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<tr>
<td>cms-17</td>
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<td>G428A</td>
<td>86.7 ± 2.5</td>
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<tr>
<td>cms-18</td>
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<td>A380C</td>
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<td>28.4 ± 2.8</td>
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<tr>
<td>cms-19</td>
<td>FlgK</td>
<td>G428A</td>
<td>G428A</td>
<td>86.7 ± 2.5</td>
<td>28.4 ± 2.8</td>
</tr>
</tbody>
</table>

a Swin ring values are from 168 h.
b Biofilm values are from 72 h postinoculation.
c NA, not applicable.
d ND, not determined.

Pseudotaxis due to mutations in the FliK hook length regulator and the MotA flagellar motor protein. Several less frequent cms mutants did not have mutations in flgE or any of several other

TABLE 1 Mutations and phenotypes of cms mutants
flagellar structural genes that were sequenced. Whole-genome se-
quencing revealed that the cms-2 mutant had incurred a single-
base deletion mutation at position 1275 of the \textit{fliK} gene (Atu0571), encoding the putative hook length regulator (37). The deletion resulted in a frameshift within the 3' region of \textit{fliK}, thereby extending the native 449-amino acid (aa) gene product to 620 aa (Table 1). This frameshift mutation fuses \textit{fliK} in frame to the downstream Atu0572 coding sequence (a putative lytic trans-
glycosylase), the 5' end of which overlaps the \textit{fliK} 3' end by 17 bp, thus resulting in a FliK protein that is fused to 8 out-of-frame amino acids and the entire Atu0572 translation product (Fig. 2A and 4I). An additional cms derivative was also due to a \textit{fliK} mutation in which the native stop codon is altered (cms-19, TGA to CGA), extending the coding sequence by 12 codons and produc-
ing a 461-aa mutant FliK protein (Fig. 4J). We designated these non-
flgE cms isolates class II (\textit{fliK}) mutants.

The two class II cms mutations extend the \textit{fliK} coding sequence and are predicted to produce a longer FliK protein (Fig. 4I and J). FliK functions in the determination of the flagellar hook length, and mutants with null mutations of \textit{fliK} in \textit{Sinorhizobium melloti}, a close relative of \textit{A. tumefaciens}, produce abnormally large and aberrant flagellar hook structures called polyhooks (37). We therefore compared the flagellar structures of class II mutants to the structures of the wild type and an \textit{A. tumefaciens} \textit{fliK} deletion mutant using TEM. Wild-type hook structures are only occasion-
ally visible as small bulbs of material at the end of sheared or dislodged flagellum filaments (Fig. 4A). The deletion of \textit{fliK} in \textit{A. tumefaciens} results in nonmotile derivatives that shed large coiled structures of \(\approx 200\) nm, readily visible using TEM, that we define as polyhooks (Fig. 4C). The two class II mutants cms-2 and cms-19, with mutations that encode C-terminally extended FliK proteins, produce large, readily visible filaments with much more heterogeneous structures, some coiled, some with extended polyhook filaments, and some normal flagella (Fig. 4D to G). However, in contrast to the \textit{fliK} deletion mut-
tants, these mutants are motile and, therefore, must generate functional flagella. The FliK C terminus is thought to interact with the FliB protein in the basal body in order to switch from hook assembly to filament assembly (38). The class II muta-
tions may reduce the efficiency of this switching process, fos-
tering the formation of polyhooks and polyhook filaments. As with class I mutants, alteration of the hook structure is likely to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Flagellar hook morphologies of cms mutants, organization of \textit{fliK} locus, and FliK mutant variants. (A to G) TEM images with uranyl acetate staining of wild-type filaments showing the hook (black arrowhead) (A), class I cms (\textit{flgE}) mutant showing a normal hook (black arrowhead) (B), coiled polyhooks in a \textit{fliK} mutant (C), and polyhook and polyhook filaments in class II mutants cms-2 (D and E) and cms-19 (F and G). The scale bar in panel A applies to all TEM images. (H) Gene organization of \textit{fliK} and the overlapping (17 bp) downstream gene Atu0572, shown as solid black arrows, and FliK polypeptide, shown as a light green bar. (I) White arrow indicates the position of the single-base frameshift (FS) in \textit{fliK} cms-2 that alters the polypeptide sequence, resulting in the 620-aa FliK-FS polypeptide. The striped region indicates the 8 out-of-frame amino acid residues that are followed by the Atu0572 polypeptide (dark green). (J) White arrow indicates the stop codon mutation (SCM) in \textit{fliK} cms-19 that extends the polypeptide by 12 aa residues, indicated by the striped region, to produce a 461-aa FliK-SCM polypeptide.}
\end{figure}
impede flagellar bundling, leading to tumbles in an otherwise straight-swimming background.

In contrast to the flk mutations, the cms-3 mutant had a single base substitution (C536G) in the motA gene (873 bp, Atu0560), encoding a mutated (A179G) flagellar motor protein (Fig. 2A; Table 1; Fig. S2B in the supplemental material). This non-flgE cms isolate was designated a class III (motA) cms mutant. All of the cms mutants exhibited more efficient swim ring expansion than their Che− parents (Fig. 1; Table 1). We introduced the identified muta-
tions into a naive background by allelic exchange for represen-
tative class I, II, and III mutants in the A. tumefaciens Δche mutant, and the swimming deficiency was suppressed in these derivatives, as with the isolated cms mutants (Fig. 5A and C; Fig. S1A and C).

cms mutants exhibit random and erratic swimming behavior. Comparisons of the swimming behavior of cms mutants with that of wild-type A. tumefaciens and other derivatives by single-cell microscopic tracking of cells in suspension were performed. Wild-type A. tumefaciens exhibits periodic switching between runs and sharp directional changes or turns (Fig. 6A), whereas the Δche mutant shows markedly smooth swimming behavior (Fig. 6B). Representatives of the three different cms mutant classes were examined in parallel: mutants in class I (cms-9 [flgE V93F]), class II (cms-2 [FliK frameshift]), and class III (cms-3 [MotA A179G]) all exhibit notably discontinuous motion, with clear runs and abrupt reorientations or directional changes (Fig. 6C to E). This swimming behavior is consistent with the tumble phenotype observed for the cms-1 (FlgE A337G) mutant under light microsco-
py (24).

Single-cell trajectories were tracked in the particle tracker plug-in of the Fiji image analysis package (39, 40). Trajectory plots were obtained by utilizing a MatLab program developed by our group. We examined at least 20 separate trajectories for class I, II, and III representatives (see Materials and Methods for details on tracking analysis). The number of reorientations made by the cell over time was manually recorded from the single-cell traces by eye, and the swimming speeds were calculated from the trajectory reports generated by the particle tracker. The Δche parent strain exhibited virtually no reorientations in an unobstructed aqueous environment (other than a gradual change in direction due to rotational Brownian motion) and a high swimming speed relative to that of the wild type (median value of ~46 μm/s compared to ~29 μm/s) (Fig. 6F and G). The cms mutants exhibited signifi-
cantly higher reorientation frequencies relative to that of their Δche parent, comparable to that of the wild type, with the class III motA mutant being the least tumble (in comparison to the results for the wild type, the P value was <0.01) (Fig. 6F).

The swimming speeds for class I and class II mutants did not differ significantly, but the class III motA mutant was slower than the wild type (median value of ~17 μm/s; P < 0.0001) (Fig. 6G). The same motA mutation in the wild-type background also resulted in a similar slower swimming speed (median value of ~18 μm/s; P < 0.0001). In S. meliloti, coordination between Arg90 and Glu98 of MotA is important for chemokinesis (control of speed modulation) and Glu150 is important for torque generation (5). The alanine residue that is mutated in cms-3 (MotA A179G) is in this region and is conserved in E. coli MotA (see Fig. S2B in the supplemental material) but is of unknown function. This seg-
ment of MotA is predicted to be helical, and glycine residues are known to disfavor helix formation (41). The Flig rotor protein interacts with this region of the MotA motor protein, and the slower swimming speed of cms-3 (Fig. 6G) may be due to a decreased rate of flagellar rotation. This alteration in the flagellar rotation rate was shown in numerical studies to com-
promise flagellar bundling, likely leading to the tumbles ob-
served in these mutants (42).

cms alleles affect motility even in strains proficient for che-
motaxis. The cms mutants were isolated from straight-swimming, chemotactically deficient parents. Wild-type A. tumefaciens Che+ derivatives engineered to harbor cms mutations were evaluated for motility and biofilm formation. Interestingly, the class I cms (flgE) allele moderately but significantly enhanced migration effi-
ciency compared to that of the wild type (Fig. 5D and F). We hypothesize that this is due to the additive effect of chemotaxis and the activity of the altered cms hook that promotes more efficient
expansion through the agar matrix. The class II cms (fliK) mutation compromises the motility agar phenotype in a wild-type background (Fig. 5D and F). The class III cms (motA) allele exhibited only a modest decrease relative to that of the wild type (Fig. 5D and F). These mutant phenotypes clearly indicate that the characteristics of the original cms isolates were the net effect of the chemotaxis deficiency and the changes to flagellar function caused by the cms mutation combining to improve migration through
motility agar. The ability to perform chemotaxis improves motility even with these additional flagellar mutations.

**Complementation of cms mutants: tests for dominant-negative effects.** The original cms mutations were haploid. It seemed possible that the cms mutations in merodiploids would be dominant over the wild-type copies of the mutated genes (flgE, fliK, and motA). Plasmid-borne expression of the flgE, fliK, and motA cms alleles from the lacZ promoter (P lac) in otherwise wild-type A. tumefaciens cells that have chromosomal copies of the corresponding genes did not result in any notable decrease in motility, even at the strongest level of induction (see Fig. S3A and B in the supplemental material). Conversely, plasmid-borne copies of the flgE and motA wild-type alleles expressed from P lac decreased the motility of the corresponding cms mutants to levels similar to that of the Che− parent (Fig. S3C and D). This suggests that the flgE and motA cms alleles are recessive to the wild-type genes, perhaps indicating modest deficiencies that favor the incorporation and/or functions of the nonmutated proteins over the mutant proteins.

Both of the class II cms mutations were different from the class I and class III mutations in that the frameshift and the stop codon mutations in the fliK gene are likely to affect the expression of the downstream gene Atu0572. These mutations would disrupt the translation initiation of the overlapping Atu0572 start codon, 14 bp upstream from the fliK stop codon. The plasmid-borne fliK gene weakly complemented the cms-2 mutant, and plasmid-borne expression of Atu0572 alone had no effect (see Fig. S3E and F in the supplemental material). However, a plasmid with both genes (P lac fliK-Atu0572 fusion) in their native configuration achieved nearly full complementation, suggesting that disruption of Atu0572 expression in the class II cms-2 mutant contributes to the strength of its suppressor phenotype (Fig. S3C and D). The Atu0572 gene product has homology to lytic transglycosylases and, thus, might be involved in the remodeling of peptidoglycan and, thus, might be involved in the remodeling of peptidoglycan.

**Conclusions.** In the alphaproteobacterium A. tumefaciens, we have identified spontaneous mutations that restore effective tumbling in straight-swimming chemotaxis-deficient mutants to approximately the level in the wild type via mechanisms that are distinct from those previously identified in enteric bacteria (21–23). In contrast to mutations in the flagellar switching mechanism as previously described, these mutants alter the tumbling propensity through structural changes to the flagellar hook and through a change to the MotA motor protein that drives the rotation of each flagellum. These mutants are also deficient in attachment to surfaces, possibly due to impairment of flagellum-mediated surface sensing mechanisms.

For bacteria with multiple flagella, the formation and stability of the flagellar bundle are major determinants of swimming behavior, and bundle disruption causes reorientation of the cell. Reigh et al. (7) computationally investigated the stability of a flagellar bundle by modeling Agrobacterium sp. strain H13-3 (highly related to Agrobacterium tumefaciens) with 5 to 10 peritrichous flagella and a mesoscale hydrodynamics simulation method for the fluid. This and other studies suggest that unbundling events and the resulting cellular tumbles are due to differences in the torque generated from individual flagellar filaments and their organization relative to each other on the cell surface (3, 7, 44).

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Simulation predicted that hydrodynamic interactions between flagella, short-range volume exclusion, and flagellar flexibility are key physical factors governing their synchronization and bundling. For small differences in adjacent motor torques, bundle formation was robust, but as the torque difference was increased, a flagellar phase lag occurred, followed by intermittent slippage and, finally, unbundling. The cms mutations isolated in this study generate tumbles in otherwise straight-swimming mutants through alterations that affect the universal joint and the hook and through mutation of the motor. Thus, while the chemotaxis-deficient mutants are trapped in the agar matrix and cannot make the directional changes to escape, the cms mutants apparently overcome this barrier via reorientation and, hence, exhibit expansion and improved migration. Our work suggests that the class I (hook) and class II (hook length regulator) mutations generate tumbles by altering the dynamics of flagellar bundling. Mutations in the MotA protein of the related Agrobacterium sp. H13-3 have been reported to slow flagellar rotation and to cause motility to be so jiggly that migration through motility agar is compromised (45). The class III motA mutant we identify here may cause a less dramatic but related modification of the motility apparatus, whereby a decrease in the rate of flagellar rotation alters bundling, leading to sufficient tumbles to facilitate migration through motility agar.

Why did selection for increased migration through motility agar in A. tumefaciens result in mutations that alter the hook or motor proteins, presumably altering the mechanical properties of the flagellum, whereas the identical selection in E. coli yielded regulatory mutations in the switch complex? No switch mutants were identified in the A. tumefaciens screen, and no hook or motor protein mutants were obtained in the E. coli studies (21–23). We hypothesize that this reflects the profound mechanistic differences between motility systems that generate tumbles through reversal of flagellar rotation, as in E. coli (1), and systems that slow or stop unidirectional rotation to induce tumbles (3). The polar arrangement of flagella in A. tumefaciens, in contrast to a peritrichous organization, may also contribute to differences in the details of the bundling and unbundling processes that govern transitions between runs and tumbles and associated tumble angle distribution. The related bacterium S. meliloti has a peritrichous flagellar organization, and despite extensive studies examining its chemotaxis (46, 47), including analysis of straight-swimming mutants, the suppression observed for the A. tumefaciens cms mutants described here has not been reported.

Our findings point to the importance of the tumbling process in navigating complex environments. Even in the absence of a chemotactic response, the intrinsic tumbling rates and tumble angle distributions are likely to be evolutionarily adapted for enhanced performance under specific conditions. Chemotaxis acts to modulate this basal reorientation rate to provide directionality to motility. It seems certain that natural selection has acted upon these properties for each specific bacterial system and that the basal flagellar dynamics are to some extent optimized for the habitats in which these bacteria evolve.

The intense selective pressure exerted in motility agar, in which those bacteria that can better migrate outwards experience a significant growth advantage, made it possible to isolate apparent suppressor mutants of diminished migration resulting from disabled chemotaxis. Indeed, recent work has highlighted the difference in chemotactic behavior in liquid medium versus agar from the standpoint of optimal concentrations of chemotaxis proteins (CheR and CheB) determining the adaptation rate and tumbling frequency (48). These strong selective pressures are likely recapitulated in natural environments, with heterogeneous distributions of chemical stimuli and complex microarchitecture. While most bacteria in which motility has been studied appear to be chemotactic (49), it has been suggested that organisms may separate motility from the chemotaxis system (50). In the evolutionary path from motility to directed cell movement under the control of a sensory network, an intriguing point that our work highlights is that environments of different porosity provide strong selection for specific reorientation dynamics, and this evolutionary tuning of basal motility almost certainly provides the ground state on which chemotaxis developed.

We observe that the cms mutations, in contrast to their positive effect on migration through motility agar, have a uniformly detrimental impact on surface colonization and biofilm formation. Many bacteria, including A. tumefaciens, undergo a complex physiological acclimation to contact with surfaces, such as the production of alternative flagellar systems and adhesin production (51, 52). Indeed, as bacteria get close to surfaces, the dynamics of flagellar rotation can dictate the pattern of interactions (53). While it is known that flagella drive surface colonization through the promotion of collisions with surfaces and, in some cases, by acting directly as adhesins (24, 54), the mechanisms underlying surface detection in bacteria—likely mediated by flagella in some cases—are as yet poorly understood. The cms mutations, with the resulting compromised surface attachment, appear to disrupt the normal surface acclimation process. High-resolution microscopic comparison of these mutants at surfaces would provide insights into the mechanistic basis for their adhesion deficiency and, more generally, the biophysical mechanisms underlying surface detection.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S1 and S2, respectively, in the supplemental material. Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). DNA was manipulated as described previously (35), and DNA sequencing was performed on an ABI 3730 sequencer at Indiana Molecular Biology Institute, Bloomington, IN. DNA purification was done using E.Z.N.A. plasmid miniprep kits (Omega Bio-Tek, Norcross, GA). All restriction enzymes and molecular biology reagents were obtained from NEB (Ipswich, MA). Electroporation to introduce plasmids into A. tumefaciens was performed as described previously (56, 57). E. coli was grown in LB medium, and A. tumefaciens was grown in AT minimal medium (58) supplemented with 0.5% (wt/vol) glucose and 15 mM ammonium sulfate (ATGN). For sacB counterselection, 0.5% sucrose was used as the sole carbon source (ATSN) instead of glucose. The antibiotic concentrations used for E. coli were 100 µg·ml⁻¹ ampicillin (Ap), 25 µg·ml⁻¹ streptomycin (Sm), and 25 µg·ml⁻¹ kanamycin (Km); for A. tumefaciens, they were 300 µg·ml⁻¹ Km and 3 mg·ml⁻¹ Sm. Media were supplemented with isopropyl-thiobetagalactoside (IPTG) for Prac induction. Reagents, antibiotics, and microbiological media were obtained from Fisher Scientific (Pittsburg, PA) and Sigma-Aldrich (St. Louis, MO).

**Construction of complementation constructs.** Complementation constructs were generated by cloning wild-type coding sequences into the IPTG-inducible expression vector pSRK-Km (59). For optimal gene expression, 5’ primers were designed to fuse the Ndel site of pSRK-Km in frame with the lacZa start codon. Coding sequences for the desired genes, i.e., wild-type fliK, Atu0572, fliK-Atu0572, and motA and their corre-
sponding cms mutant alleles, were PCR amplified with primers comp P1 and comp P2 from AtC58 genomic DNA or cms mutants using Phusion high-fidelity DNA polymerase. PCR fragments were gel purified and ligated to pGEM-T Easy, confirmed by sequencing, and then cleaved with the appropriate restriction enzymes to be religated to pSRK-Km, which was previously cleaved with the corresponding restriction enzymes. An fglE pPM110 expression plasmid (pBBR1-MCS-2 Pspac::fglE) from our previous study (24) was used as the source of the fglE gene, and this was ligated into pSRK-Km and electroporated into A. tumefaciens as described previously (56).

Construction of nonpolar markerless deletions. Nonpolar markerless deletions were constructed as described previously (24, 60). Briefly, about 500 to 1,000 bp of upstream and downstream DNA sequences flanking the desired gene were PCR amplified with P1 and P2 primers for the upstream sequence and with P3 and P4 primers for the downstream sequence. Primers were designed to delete a desired gene without affecting the adjacent genes, including any potential translational coupling in operons. Primers P2 and P3 for each gene were designed to have about 18 bp of homology (the overlap indicated in Table S2 in the supplemental material), enabling splicing by overlapping extension (SOEing) as described previously (24, 60, 61). The flanking sequences were amplified with Phusion high-fidelity DNA polymerase (NEB, Ipswich, MA), agarose gel purified, and used as the starting template and primer for a short, 5-cycle PCR. A final PCR round was performed using 2 μl of the product of the above-described PCR as the template and primers P1 and P4 in order to fuse these flanking sequences. This resultant full-length product of about 1 kb was cloned into pGEM-T Easy (Promega, Madison, WI), agarose gel purified, and used as the starting template and primer for a short, 5-cycle PCR. A final PCR round was performed using 2 μl of the product of the above-described PCR as the template and primers P1 and P4 in order to fuse these flanking sequences. This resultant full-length product of about 1 kb was cloned into pGEM-T Easy (Promega, Madison, WI), confirmed by DNA sequencing, digested with the appropriate restriction enzymes, and ligated into the suicide vector pNPTS138, which was previously digested with the same set of enzymes. The pNPTS138 plasmid (62; M. R. K. Alley, unpublished data) confers Km resistance (KmR) and sucrose sensitivity (SucR). pNPTS138 uses a ColE1 replication origin that does not function in A. tumefaciens, and thus, the plasmid must recombine into a stable endogenous replicon to impart the KmR phenotype. Derivatives of pNPTS138 were introduced into A. tumefaciens by conjugation, and recombinants were selected for growth on ATGN Km plates. KmR isolates were restreaked on ATGN Km plates and ATSN Km plates to confirm plasmid integration and sucrose sensitivity. Colonies with normal growth on ATGN Km plates and very poor growth on ATSN Km plates were selected. A single KmR SucR colony was grown overnight in ATGN broth without antibiotic selection and then plated on ATSN to select for mutants that had excised the integrated plasmid via a second recombination event. The resultant SucR colonies were patched on ATSN and ATGN Km to verify plasmid excision. Primers P1 and P4 (the external primers designed to flank the targeted gene) were used to perform diagnostic PCR to verify plasmid excision. Primers P1 and P4 (the external primers designed to flank the targeted gene) were used to perform diagnostic PCR to verify plasmid excision. Preceding the appropriate restriction enzymes to be religated to pSRK-Km, which was previously cleaved with the corresponding restriction enzymes. An fglE pPM110 expression plasmid (pBBR1-MCS-2 Pspac::fglE) from a previous study (24) was used as the source of the fglE gene, and this was ligated into pSRK-Km and electroporated into A. tumefaciens as described previously (56).

Site-directed mutagenesis for combining cms mutations. Site-directed mutagenesis was performed as described previously (62). The QuikChange protocol obtained from Stratagene Corp. was used to perform site-directed mutagenesis on the cms-5 (fglE Y127C) coding sequence. Long, self-complementary mutagenic primers cms-9 Flmut and cms-9 R1mut (melting temperature [Tm] = −68°C) harboring the desired cms-9 mutation (fglE V93F) in the middle (underlined) were designed (GCAAGGGTTTTCCTTGGTTACCGAATGGTCCGGGC to GCAAGGGTTTTCCTTGGTTACCGAATGGTCCGGGC for Val to Phe) (see Table S2 in the supplemental material). pGEM-T Easy plasmid pRC103 containing the cms-5 mutation (Table S1) was used as a template to perform PCR with the mutagenic primers using Phusion DNA polymerase. The parental wild-type and hemimethylated plasmid DNA were removed by digesting the reaction products with DpnI, leaving only the newly synthesized, uniformly nonmethylated mutated DNA. DNA sequencing was performed to confirm the mutations, and then the mutated DNA fragment (the same fglE fragment, now harboring cms-5 Y127C and cms-9 V93F mutations) was used to mutagenize the cms-9 mutant by allelic replacement as described above.

Whole-genome sequencing of cms mutants. The cms suppressors obtained from motility agar flares were double-streak purified on ATGN. Total genomic DNA from the cms suppressor mutants was used to generate a paired-end library, following the modified protocol of Lazinski and Camilli (http://tucf-genomics.tufts.edu/home/libraryprep) as described in an earlier study (60). Approximately 20 μg of sheared genomic DNA was blunt ended using the NEB Quick Blunting kit (New England Biolabs). The Klenow fragment of DNA polymerase I was used to add one deoxadenosine to the 3’ ends of the DNA preparation, and the DNA preparation was ligated with an adapter mix consisting of primers OLJ131 and OLJ137, using the NEB Quick Ligation kit (New England Biolabs). Finally, library amplification was performed by PCR with primers OLJ139 and OLJ140. Sequencing was performed on an Illumina HiSeq 2000 at the Tufts University Core Facility.

Motility assays. Flagellar swimming phenotypes were tested with ATGN motility (0.25%) agar in 100-mm petri dishes with 20 ml of Bacto agar (BD, Sparks, MD), with the addition of Km and IPTG as necessary (63). Fresh colonies were used to inoculate swim plates, using a toothpick that was stabbed into the agar at the center of the plate. The plates were incubated at 28°C for up to 7 days, and their swim ring diameters were measured daily.

Cultivation and analysis of static-culture biofilms. For a static-culture biofilm assay, biofilms were grown as described previously (60, 64). Briefly, biofilms were grown on sterile polystyrene (CovR); coverslips were placed vertically in the walls of UV-sterilized 12-well polystyrene cell culture plates (Corning, Inc.). Overnight bacterial cultures grown in ATGN were subcultured to an optical density at 600 nm (OD600) of 0.2, grown at 28°C until exponential phase, and then diluted to an OD600 of 0.05 in about 3 ml of ATGN, followed by 48 h of room temperature incubation. Coverslips were rinsed, rinsed with water, and stained with 0.1% (wt/vol) crystal violet (CV) for 5 to 7 min. The coverslips were further washed with water to remove excess stain. Coverslip biofilms were soaked in 1 ml of 33% acetic acid to solubilize the CV stain, and the absorbance at 600 nm (A600) was measured in a Bio-Tek Synergy HT microplate reader. The A600 value of solubilized CV was normalized to the OD600 of the planktonic cells to obtain the A600/OD600 ratio, and this ratio was normalized to the wild type or parent mutant value as appropriate.

Transmission electron microscopy. Transmission electron microscopy was performed as described previously with a few modifications (65). A. tumefaciens cells were grown in appropriate medium (ATGN) to an OD600 of ~1.0 and were diluted 1:10 before being used to coat 300 mesh, 3-mm copper grids with carbon-formvar films for 5 min (Electron Microscopy Sciences, Hatfield, PA). Each grid was dried with filter paper
and then negatively stained with 2% uranyl acetate for another 5 min. The grids were dried to remove excess stain and then examined with a JEOL JEM-1010 transmission electron microscope set to 80 kV in the Indiana University Electron Microscopy Center.

Sequence comparisons and threading of class I cms mutations through the Salmonella hook crystal structure. The amino acid sequences of A. tumefaciens and S. Typhimurium FlgE were compared via BLASTp analysis (66). The FASTA A. tumefaciens and S. Typhimurium FlgE and A. tumefaciens and E. coli MotA amino acid sequences were imported from the PubMed Protein database to Clustal Omega and aligned using the default settings (31). The class I cms mutations were mapped to the hook structure as described in detail above (see Results, “Class I cms mutations map to the FlgE D1 domain”).

Tracking swimming behavior of the bacteria. Overnight bacterial cultures of A. tumefaciens cells grown in ATGN were subcultured to mid-exponential phase. Swimming movies were captured using a 10× dark-field objective on a Nikon 90i microscope. Movies were captured with an exposure of 30 ms and frame duration of 0.03283 s. The movies were each ~10.6 s long. The raw files were imported into the Fiji image analysis package and processed with the Mosaic two-dimensional single-particle tracking tool plug-in to track the individual cells; only trajectories longer than 40 frames were analyzed further, and a trajectory report was generated (39, 40). Twenty separate single-cell trajectories (of at least 50 μm in length) obtained for each strain were analyzed to calculate their reorientation frequencies over time and their swimming speed. The trajectory report obtained from the Mosaic particle tracker plug-in was used to calculate the length, time scale, and swimming speed from the trajectories. Trajectory reports were then imported into MatLab version 8.1.0.640 (67) to obtain trajectory plots. The number of reorientations made by a single cell over time was counted by eye from the traces. Trajectories are plotted as a square plot centered around 0. Box plots are used (Fig. 6F and G) to indicate the speed and reorientation frequencies of swimming cells. Reorientations are expressed per second and are only counted if they changed the overall direction of the cell, i.e., jittery swimming that maintained the basic trajectory is not considered a reorientation. The swimming speed is in micrometers per second and includes times when cells were actively tumbling. Evaluation of the swimming speeds that did not include the reorientation periods revealed the same basic trend that the class III mutant was slower than the wild type and the other cms mutants.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi=10.1128/mBio.00005-15/-/DCSupplemental.

Figure S1, PDF file, 0.6 MB.
Figure S2, PDF file, 0.5 MB.
Figure S3, PDF file, 0.7 MB.
Figure S4, PDF file, 1 MB.
Figure S5, PDF file, 0.5 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.

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