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Genetic Determinants of *Silicibacter* sp. TM1040 Motility▽†

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***Silicibacter* sp. TM1040 is a member of the marine *Roseobacter* clade of *Alphaproteobacteria* that forms symbioses with unicellular eukaryotic phytoplankton, such as dinoflagellates. The symbiosis is complex and involves a series of steps that physiologically change highly motile bacteria into cells that readily form biofilms on the surface of the host. The initial phases of symbiosis require bacterial motility and chemotaxis that drive the swimming bacteria toward their planktonic host. Cells lacking wild-type motility fail to establish biofilms on host cells and do not produce effective symbioses, emphasizing the importance of understanding the molecular mechanisms controlling flagellar biosynthesis and the biphasic "swim-or-stick" switch. In the present study, we used a combination of bioinformatic and genetic approaches to identify the genes critical for swimming of *Silicibacter* sp. TM1040. More than 40 open reading frames with homology to known flagellar structural and regulatory genes were identified, most of which are organized into approximately eight operons comprising a 35.4-kb locus, with surprising similarity to the fla2 locus of *Rhodobacter sphaeroides*. The genome has homologs of CckA, CtrA, FlbT, and FlaF, proteins that in *Caulobacter crescentus* regulate flagellum biosynthesis. In addition, we uncovered three novel genes, *flaB*, *flaC*, and *flaD*, which encode flagellar regulatory proteins whose functions are likely to involve regulation of motor function (FlaD) and modulation of the swim-or-stick switch (FlaC). The data support the conclusion that *Silicibacter* sp. TM1040 uses components found in other *Alphaproteobacteria*, as well as novel molecular mechanisms, to regulate the expression of the genes required for motility and biofilm formation. These unique molecular mechanisms may enhance the symbiosis and survival of *Roseobacter* clade bacteria in the marine environment.**

Marine phytoplankton and particularly dinoflagellates are primary producers of a major source of organic sulfur in the ocean called dimethylsulfoniopropionate (DMSP), while *Alphaproteobacteria* phylogenetically related to the *Roseobacter* clade are predominantly responsible for the degradation of DMSP (11, 15, 16, 38). Although roseobacters are ubiquitous in the marine ecosystem, their abundance and activity is significantly correlated with abundance and activity of DMSP-producing dinoflagellates and other phytoplankton (17, 40), establishing a physiological and ecological linkage between dinoflagellates and roseobacters (17, 40).

As part of an investigation to understand the role of the bacteria in dinoflagellate physiology, we analyzed the bacterial community associated with laboratory microcosms of heterotrophic dinoflagellates (1). This bacterial community is a diverse group of more than 30 species numerically dominated by roseobacters. One species, *Silicibacter* sp. TM1040 (hereafter referred to here as TM1040), is notable, since it forms a biofilm on the surface of the dinoflagellate, *Pfiesteria piscicida* (23). The dinoflagellate has an obligate requirement for TM1040 or bacteria physiologically comparable to it, and axenic dinoflagellate cultures lacking bacteria fail to grow and ultimately die (1, 22). Adding back TM1040 to axenic dinoflagellates restores growth to normal levels (1, 22). While the symbiosis is essential for the dinoflagellates, it is much looser for TM1040, which can be grown in both complex and defined minimal media sans phytoplankton. The TM1040-*Pfiesteria* association is the only known "obligate" association between a dinoflagellate and a culturable bacterium and fits the definition of a symbiosis as "the close, permanent relationship between two or more different organisms" (26).

TM1040 is actively motile and swims by means of one to four polar or lophotrichous flagella (Fig. 1) (24). Three main structures form the bacterial flagellum: an engine, a propeller, and a universal joint that connects them. The engine, or basal body, includes a rotor and a stator, both of which are embedded in the cytoplasmic membrane; a rod that acts as a driveshaft and extends from the rotor through the peptidoglycan layer to the outer membrane; and a bushing complex that assembles around the distal rod to form a pore in the outer membrane.

Symbiosis is a stepwise process, and bacterial motility and chemotaxis behavior play a crucial role in the initial phases of symbiosis (22, 24). TM1040 is strongly attracted to chemical compounds synthesized by the dinoflagellate, including DMSP, DMSP metabolites, and amino acids and, to a lesser extent, to tricarboxylic acid cycle intermediates, such as succinate and fumarate (24). These results suggest that TM1040's physiology, motility, and chemotaxis machinery is adapted to ensure that the bacteria are able to find the dinoflagellate in oligotrophic seawater and initiate the symbiosis through swimming to the dinoflagellate surface. The bacteria use chemotaxis behavior to sense these compounds trailing from swimming dinoflagellates to move toward and maintain their interaction with their rapidly swimming host (22, 24, 35). Defects adversely affecting bacterial swimming significantly reduce the ability of TM1040

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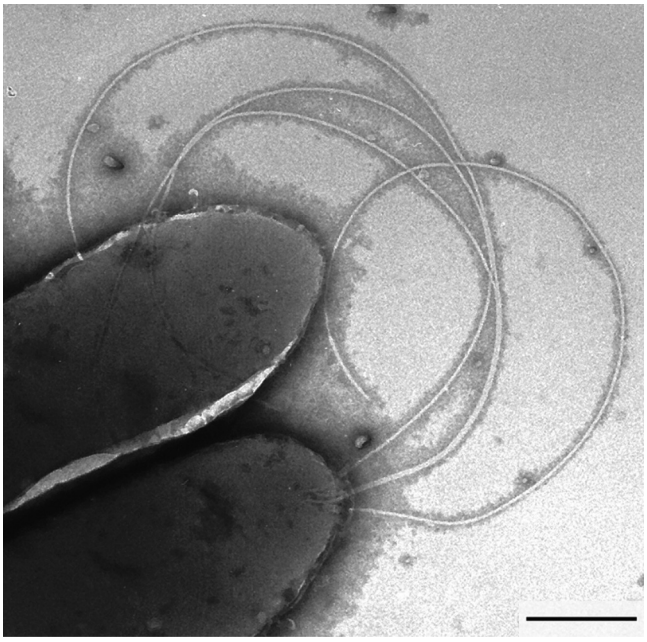

FIG. 1. Transmission electron micrograph of negatively stained TM1040 showing four subpolar or lophotrichous flagella. Bar, 500 nm.

to colonize and form a symbiosis with *P. piscicida* (22). Once near the surface, the symbiosis proceeds with bacteria colonizing the surface of the dinoflagellate (1, 22). Therefore, the bacteria require both motile and sessile phases to produce the symbiosis.

Motile and sessile forms of the bacteria are also seen when TM1040 is incubated in liquid nutrient broth cultures. In these cultures, rapidly swimming motile cells coexist with nonmotile aggregates of cells referred to as rosettes (starlike clusters of cells), a characteristic that defines many species in the *Roseobacter* clade (10). The choice of culturing method greatly influences the composition and physiology of TM1040. For instance, when TM1040 is grown in standing liquid culture conditions and/or is observed in mid to late stationary phase of growth, the population is largely composed of sessile cells. In this condition or phase, formation of biofilms on glass surfaces or cell-cell rosettes is common, as is the synthesis of a yellow-brown pigment and an antibiotic compound, tropodithietic acid (10, 14). Cells in rosettes lack flagella and attach to one another by their poles. In contrast, vigorously shaken liquid cultures of TM1040 are primarily composed of small, flagellated cells that are highly motile (65 to 80 μm/s) and actively chemotactic (10, 14). Cells in the motile phase produce significantly less pigment and tropodithietic acid (10). Cultures started from an inoculum of motile cells give rise to rosettes and vice versa, suggesting that a cycle exists. Thus, TM1040 exhibits a "swim-or-stick" lifestyle, in which the respective percentages of motile and sessile cells are influenced by culturing conditions. The nature of the signal that controls when the cells are motile and when they are sessile—the swim-or-stick switch—is unknown but, presumably, it is also encountered by TM1040 when the bacteria are near the dinoflagellates. We hypothesize that one component of this switch controls flagellar biosynthesis.

A description of the genome of TM1040 was recently published (25) and provides an insight into the genes involved in controlling swimming motility and chemotaxis. The genome is replete with open reading frames (ORFs) encoding possibly more than 20 chemoreceptor proteins, along with multiple copies of cytoplasmic chemotaxis (Che) proteins, emphasizing that TM1040 is proficient in sensing and responding to chemical attractants. ORFs encoding proteins with homology to many known flagellar biosynthetic proteins were also identified in the earlier study (25).

TM1040 also has homologs of the two component regulatory circuit composed of the histidine kinase, CckA, and its response regulator, CtrA, that serves as the master regulator in the dimorphic stalked bacterium *Caulobacter crescentus* (9). *C. crescentus* CckA/CtrA regulates multiple pathways, including cell division, motility, and chemotaxis. Previously, we constructed mutations in both genes, and the resulting phenotypes confirm our suspicions that they are involved in flagellar biosynthesis and motility in TM1040 (22). However, unlike *Caulobacter*, where mutations in *cckA* or *ctrA* are lethal, $CckA^-$ or $CtrA^-$ strains of TM1040 have wild-type growth rates, with no apparent effects to cell division. This leads us to propose that control of flagellar biosynthesis and motility is different from *C. crescentus* or other currently studied models of bacterial motility.

In the present study, through a combination of genomic, bioinformatic, and genetic analyses, we searched for and found genes essential for motility of TM1040, including several regulatory loci. Many of the flagellar biosynthetic proteins are homologous to their counterparts in other motile bacteria, and yet regulation of motility in TM1040 is novel and involves control of the swim-or-stick switch.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type cells (TM1040) and mutant strains derived from it (Table 1) were routinely grown in 2216 marine broth (Difco) with shaking or on 2216 agar medium (2216 marine broth with 15 g of Bacto agar per 1 liter) at 30°C. *Escherichia coli* was cultured using Luria-Bertani (LB) broth (33) or LB agar (LB broth plus 15g of Bacto agar per liter) at 37°C. As required, kanamycin was added at a final concentration of 120 μg/ml for TM1040 and 40 μg/ml for *E. coli.*

Genomic analysis. Annotation of the genome of TM1040 has been published (25) and is available from GenBank (NC_008044) or Roseobase (www.roseobase.org). We confirmed the assignment of each gene using BLASTP searches (4) with the deduced amino acid sequence of each ORF against the GenBank database (www.ncbi.nlm.nih.gov), Roseobase, and the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project (https://research.venterinstitute.org/moore/) databases. Homologs with an E-value cutoff of 10^{-5} or less were considered significant.

Transposon mutagenesis. Random transposon mutagenesis using EZ-Tn*5* (*R6Kγori/KAN-2*) (Epicentre, Madison, WI) was conducted as previously described (22) with minor changes. A 65-μl sample of electrocompetent cells of TM1040 was mixed with 25 ng of the transposome, and the bacteria were electroporated in a 0.2-cm electroporation cuvette at 2.5 kV/cm, 400 Ω, and 25 μF using a Bio-Rad GenePulser (Bio-Rad, Hercules, CA). The cells were suspended in 1 ml of prewarmed HIASW broth (25 g of heart infusion broth [Difco] plus 15 g of Instant Ocean sea salts [Aquarium Systems, Mentor, OH] per liter) and incubated at 30°C with shaking for 2 h. After incubation, 100-μl samples of the culture were spread on HIASW agar (HIASW broth with 1.5% Bacto agar) containing kanamycin and incubated for 48 h at 30°C. Kanamycin-resistant colonies were transferred to a 7-by-7 array on 2216 agar containing kanamycin to facilitate future analysis. The sequence flanking the transposon was obtained by rescue cloning as previously described (22).

Complementation. HG1016 (*flaC*::EZ-Tn*5*) was complemented as follows. PCR with the primers FlaC-F (CAGTCCCATCCTCAGATCCACTC) and

TABLE 1. Bacterial strains used in this study

Strains	Genotype[a]	Locus tag[b] of inserted gene	Predicted function	Source or reference
TM1040	Wild type			24
HG1006	*flaB*::EZ-Tn*5*, Kan	TM1040_1206	Regulation	This study
HG1016	*flaC*::EZ-Tn*5*, Kan	TM1040_0051	Regulation	This study
HG1030	*fliM*::EZ-Tn*5*, Kan	TM1040_1506	C-ring	This study
HG1032	*flhA*::EZ-Tn*5*, Kan	TM1040_2953	Export apparatus	This study
HG1033	*motB1*::EZ-Tn*5*, Kan	TM1040_2938	Motor complex	This study
HG1036	*flgL*::EZ-Tn*5*, Kan	TM1040_2941	HAPs	This study
HG1039	*flhB*::EZ-Tn*5*, Kan	TM1040_2955	Export apparatus	This study
HG1046	*flaD*::EZ-Tn*5*, Kan	TM1040_1731	Regulation	This study
HG1063	*flgH*::EZ-Tn*5*, Kan	TM1040_2958	L-ring	This study
HG1091	*fliR*::EZ-Tn*5*, Kan	TM1040_2954	Export apparatus	This study
HG1098	*flgE*::EZ-Tn*5*, Kan	TM1040_2939	Hook	This study
HG1099	*fliG*::EZ-Tn*5*, Kan	TM1040_1009	C-ring	This study
HG1100	*flaI*::EZ-Tn*5*, Kan	TM1040_2975	Unknown function	This study
HG1101	*fliC3*::EZ-Tn*5*, Kan	TM1040_2974	Flagellin	This study
HG1118	*flaF*::EZ-Tn*5*, Kan	TM1040_2973	Regulation	This study
HG1135	*pflI*::EZ-Tn*5*, Kan	TM1040_2949	Motor associated	This study
HG1139	*flgF2*::EZ-Tn*5*, Kan	TM1040_2971	Proximal Rod	This study
HG1148	*fliF*::EZ-Tn*5*, Kan	TM1040_2947	MS-ring	This study
HG1162	*flgG*::EZ-Tn*5*, Kan	TM1040_2960	Distal rod	This study
HG1192	*fliL*::EZ-Tn*5*, Kan	TM1040_2948	Motor associated	This study
HG1205	*flaA*::EZ-Tn*5*, Kan	TM1040_2952	Motor associated	This study
HG1212	*motA1*::EZ-Tn*5*, Kan	TM1040_2951	Motor complex	This study
HG1216	*flgK*::EZ-Tn*5*, Kan	TM1040_2940	HAPs	This study
HG1234	*flgI*::EZ-Tn*5*, Kan	TM1040_2942	P-ring	This study
HG1241	*cckA*::EZ-Tn*5*, Kan	TM1040_1228	Regulation	This study
HG1245	*fliK*::EZ-Tn*5*, Kan	TM1040_2977	Hook-length control	This study
HG1246	*fliI*::EZ-Tn*5*, Kan	TM1040_2966	Export ATPase	This study

[a] Kan, kanamycin resistance.
[b] NCBI GenBank descriptor.

FlaC-R (GACAGGGAGGATGCATATCGTGAC) was used to amplify a 1,022-bp segment of genomic DNA containing 235 bp upstream and 100 bp downstream of *flaC*. The resulting DNA was ligated into pCR2.1 (Invitrogen, Carlsbad, CA), electroporated into *E. coli* DH5α, with subsequent selection for kanamycin-resistant colonies, producing pRSI505. The *flaC* fragment was digested from pRSI505 with EcoRI, ligated into pRK415, and electroporated into *E. coli* S17-1 λ*pir*. The resulting tetracycline-resistant transformants were screened on LB agar with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml) producing pRSI506 (*flaC*$^+$). Biparental mating (22) was used to move pRSI506 into HG1016 and a rifampin-resistant spontaneous mutant of TM1040 called RSI01. Phenotypic analysis of the resulting strains was conducted as described in the following sections.

Motility analysis. Mutants with defects in wild-type swimming (Mot$^-$) were screened as described by Miller and Belas (22) using semisolid 2216 motility agar (2216 marine broth supplemented with 0.3 g of Bacto agar per liter) or marine motility agar (0.1% peptone, 200 mM NaCl, 50 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2.2H_2O$, 240 μM K_2HPO_4, 70 mM Tris-HCl [pH 7.5], 0.3% agar per 100 ml), followed by incubation at 30°C. Although TM1040 swam well through both semisolid media, marine motility agar was preferred in cases where inorganic salt precipitation in 2216 motility agar interfered with measurements or photography. Swimming measurements in semisolid agar media were confirmed by examining cultures incubated overnight in 2216 marine broth using phase-contrast microscopy (Olympus BX60, Center Valley, PA).

Measurement of the percentage of motile cells versus sessile cells in the population. The percentages of motile and sessile cells, respectively, in broth cultures were measured by phase-contrast microscopy of wet mounts of cells grown in broth culture using computer-assisted image and motion analysis. Five random fields were selected from each sample, and the motion of the population within the field was recorded for 3 s by using a Qicam Fast 1394 camera at a capture rate of ca. 22 frames per s. Analysis of the motion of the cells within the field was conducted using Volocity software (v4.1.0; Improvision, Coventry, England) to estimate the percentages of motile and nonmotile cells. The software was "trained" to distinguish motile single cells from nonmotile single cells and from cells in rosettes. To accomplish this, we divided the cells within the field into two populations based on size of the object: single cells (objects ≤5 μm^2) and cells in rosettes (objects >5 μm^2). Next, the population of single cells was analyzed for swimming motility, using Volocity motion analysis software, separating it into single motile and single sessile cells. Third, the number of cells in rosettes was measured from the subpopulation of objects >5 μm^2. Statistical analyses were performed by using a Student unpaired *t* test (Prism 4.0; GraphPad).

Light microscopy detection of flagella. Flagellar filaments on cells obtained from broth culture were stained using the RYU flagellar stain (Remel, Lenexa, KS) according to the recommendations of the manufacturer and examined by phase-contrast microscopy.

Transmission electron microscopy (TEM). Cells were harvested after 5 h of growth by low centrifugation (5,000 rpm for 5 min) and suspended in distilled water. Resuspended cells were immediately stained with 2% (wt/vol) phosphotungstic acid (pH 7.0) and observed with a JEM-1200EXII electron microscope (JEOL, Tokyo, Japan). There was no fixation process, so that we could observe cells as fresh as possible. Micrographs were taken at an accelerating voltage of 80 kV.

Detection of flagellin proteins. Flagella were purified by using a modification of the procedure described by Kanbe et al. (18). After overnight incubation in broth, cells were pelleted by centrifugation (10,000 × *g* for 5 min at 4°C). Detached flagella in the supernatant were precipitated by adding polyethylene glycol (8% PEG 8000 [Sigma] in 0.4 mM NaCl) to a final concentration of 2% (wt/vol). After 60 min on ice, flagella were pelleted by centrifugation at 17,400 × *g* for 15 min at 4°C. Flagella were resuspended in 50 mM Tris (pH 7.5), and the protein in each sample determined (BCA kit; Pierce, Rockford, IL).

Flagellin proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% polyacrylamide gel. Protein bands were stained with Coomassie Fluor Orange protein gel stain (Molecular Probes/Invitrogen, Carlsbad, CA) and visualized with a 555-nm emission filter and a 488-nm excitation filter by using a Typhoon 9410 (Amersham Biosciences, Piscataway, NJ).

Measurement of antibiotic activity. Antibiotic activity was measured by the well diffusion method with *Vibrio anguillarum* strain 90-11-287 as described by Bruhn et al. (10).

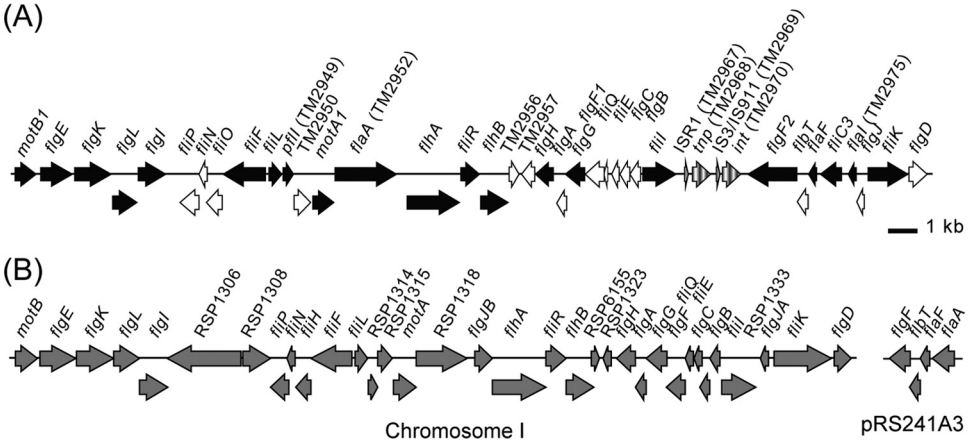

FIG. 2. The 35.4-kb flagellar locus of TM1040 is similar to the fla2 locus of *R. sphaeroides*. (A) Most of the flagellar biosynthetic genes required for swimming motility of TM1040 are located in ca. eight operons within one large genetic locus. Arrows indicate the direction of transcription of ORFs. An arrow located beneath the main line indicates that the gene overlaps the adjacent gene. White arrows indicate those genes identified solely by bioinformatic analyses, black arrows are used to indicate mutated genes producing a Mot$^-$ phenotype, and hatched arrows signify nonflagellar genes encoding insertion sequences (ISR1 and IS*3*/IS*911*), transposase (*tnp*), and integrase (*int*). GenBank locus tags have been abbreviated. (B) The fla2 locus of *R. sphaeroides* (30). The names of the genes have been shortened by removal of "2" to simplify comparisons.

CR binding. Congo Red (CR) binding was used to assess changes in the cell surface of Mot$^-$ mutants. A 2-μl inoculum of an overnight broth culture was applied to the surface of 2216 agar containing 100 μg of CR per ml. After overnight incubation, the color of the mutant colony was compared to that of TM1040, which served as a reference.

Biofilm formation. Biofilm formation was assessed by crystal violet (CV) staining (28). Bacteria were incubated for 16 h at 30°C with shaking in 30-by-100-mm borosilicate (Pyrex) glass culture tubes containing 0.5 ml of 2216 broth. After incubation, the liquid culture was discarded and each tube rinsed with artificial seawater (40 g of Instant Ocean in 1 liter of distilled water) to remove loosely attached cells. The biofilm attached to the test tube wall was stained with 1 ml of CV solution (Becton Dickinson, Sparks, MD) for 30 min at room temperature. Unbound dye was removed with a subsequent wash using artificial seawater. A 1:1 solution of dimethyl sulfoxide (DMSO; Fisher Scientific, Hanover Park, IL) and ethanol (95% [vol/vol]) was used to elute the dye, which was measured by spectroscopy at 560 nm using a Bio-Rad Model 680 microplate reader.

RESULTS

Organization of the flagellar loci. In a recent study (25), we identified 48 ORFs in the genome of TM1040 with homology to genes encoding flagellar biosynthetic proteins. To continue this effort, we analyzed these flagellar ORFs by genomic and bioinformatic approaches to provide insight into the genetic organization and potential regulation of flagellar biosynthesis.

Most (75%) of the 48 genes encoding homologs of proteins involved in synthesis of the TM1040 flagellum are located in a single 35.4-kb (kb) region of the chromosome (Fig. 2). An additional 10 genes are scattered around the 3.2-Mb chromosome in seven small loci, while two more genes (*fliY* and *fliC1*) are found on pSTM1, an 823-kb megaplasmid, in two unlinked loci. No cytoplasmic chemotaxis (*che*) genes or genes encoding methyl-accepting chemoreceptor proteins (MCPs) are found associated with flagellar genes, although multiple copies of both *che* and *mcp* genes have been identified in an earlier bioinformatic analysis of the genome (25).

The majority of the 36 genes found in the 35.4-kb locus encode proteins that are likely involved in structural components of the flagellum, principally proteins of the hook-basal body and motor (Fig. 2A). There are approximately eight operons within this region with genes encoding flagellar type III secretion system components (*flhA*, *flhB*, *fliI*, *fliO*, *fliP*, *fliQ*, and *fliR*); C-, MS-, P-, and L-rings (*fliN*, *fliF*, *flgI*, and *flgH*, respectively); motor stator proteins (*motA1* and *motB1*); rod components (*flgB*, *flgC*, *flgF1*, *flgF2*, *flgG*, *flgJ*, and *fliE*); a rod-associated protein (*fliL*); hook (*flgE*) and hook-associated proteins (*flgD*, *flgK*, *flgL*, and *fliK*); and flagellin (*fliC3*). The novel flagellar gene *flaA*, previously identified by us (22) and required for flagellar function, is located in a putative class II *fliL* operon (Fig. 2A) within the large locus. The *fliL* operon (Fig. 2A) also contains an ORF (TM2949) with homology to PflI, a protein involved in the cellular positioning of flagella in *C. crescentus* (27). Because of its homology and similar position within the operon, we have chosen to call this gene *pflI*. In addition, the 35.4-kb flagellar region contains four hypothetical proteins (TM2950, TM2956, TM2957, and TM2975) lacking known functional domains that may function in flagellar export or biogenesis, based on their association within operons containing homologs of genes with known flagellar function in other bacteria.

As previously observed (22, 25), the genome contains genes that encode homologs of *C. crescentus* CckA and CtrA, a two-component master regulator of flagellar and other genes, as well as homologs FlaF and FlbT (Fig. 2A). In *C. crescentus*, these latter two proteins function at the posttranscriptional level to control flagellin mRNA stability (20). We were unable to identify homologs of *fliA* or *flgM*, encoding the σ^{28} flagellar-gene-specific sigma factor and anti-sigma factor, respectively, of enteric bacteria, nor does the genome have *flhDC* or obvious homologs to the known flagellar regulatory proteins of the *Alphaproteobacteria Rhodobacter sphaeroides* or *Sinorhizobium meliloti* (31, 37).

Flagellar organization resembles the fla2 locus of *R. sphaeroides*. The organization of the genes within the 35.4-kb flagellar locus, as shown in Fig. 2, is nearly identical to the fla2 locus of *R. sphaeroides* (Fig. 2B) (30). Recently, Poggio et al. (30) noted the similarity of the *R. sphaeroides* fla2 locus to the

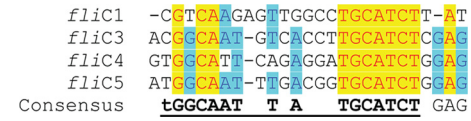

FIG. 3. The nucleotide sequence 5′ to the start codon of *fliC1*, *fliC3*, *fliC4*, and *fliC5*. CLUSTAL W was used to align the upstream regulatory regions of *fliC1* and *fliC3* to *fliC5*, resulting in a consensus sequence that has partial homology to a σ^{54} binding site, TGGC-N7-TTGC. The red font with yellow highlighting indicates perfect matches, while partial matches are noted with a black font and blue highlighting. The resulting consensus sequence is shown in boldface beneath the alignment.

flagellar locus of a near relative of TM1040, *Silicibacter pomeroyi* DSS-3, suggesting that the organization of flagellar biosynthetic genes is common and conserved among the *Roseobacter* clade.

While the overall structure and organization is similar, there are localized differences between the major flagellar loci of *R. sphaeroides* and TM1040. The most striking example is the insertion of four genes (*fliC3*, *flaF*, *flbT*, and *flgF2*) in the 35.4-kb flagellar locus nestled between *fliI* and TM2975 (renamed *flaI* as shown in Fig. 2A). In *R. sphaeroides*, the homologs of these genes (*flaA*, *flaF*, *flbT*, and *flgF*) are located on a plasmid, pRS241A3 (Fig. 2B) (30). Located adjacent to *fliC3-flgF2* are ORFs with homology to two insertion sequences (TM2967 and TM2969), a transposase (TM2968), and an integrase (TM2970), supporting the idea that an integration and insertion of a plasmid-borne *fliC3*, *flaF*, *flbT*, and *flgF2* took place in a common ancestor of TM1040 and *R. sphaeroides*.

Six flagellin-encoding genes are scattered around the genome. The remaining seven flagellar biosynthetic loci are scattered around the chromosome and notably contain multiple copies of some genes (25). We have maintained the nomenclature originally used by Moran et al. (25) for multiple alleles potentially encoding the same proteins, such as *motA1* and *motA2* or *flgF1* and *flgF2*. In particular, our bioinformatic analysis found six *fliC* alleles scattered across the genome. Only *fliC3*, located in the 35.4-kb locus (Fig. 2A), is associated with other known flagellar genes, while the other copies are scattered either around the chromosome (*fliC2* to *fliC6*) or in the case of *fliC1* on pSTM2.

The deduced amino acid sequence of each FliC protein shows remarkable conservation of properties. The FliC proteins are similar in size (283 ± 4 amino acids), mass (29,210 ± 616 Da), and isoelectric point (4.06 ± 0.14 pI). Moreover, the nucleotide sequence 5′ to the start codon of *fliC1* and *fliC3* to *fliC5*, but not *fliC2* or *fliC6*, is similar and contains a nucleotide sequence (Fig. 3 and see Fig. S1 in the supplemental material) with homology to the −24 region (TGGC) of a σ^{54} binding site, TGGC-N7-TTGC (6). When the entire upstream regulatory sequences of *fliC1*, *fliC3*, *fliC4*, and *fliC5* are compared (see Fig. S1 in the supplemental material), additional conservation of sequence is evident. The sequence similarity between these regulatory regions hints that the expression of *fliC1*, *fliC3*, *fliC4*, and *fliC5* may be controlled by the same mechanism.

Distribution of the flagellar genes among the *Roseobacter* clade. We analyzed the distribution of flagellar genes found in TM1040 among other roseobacters. Overall, the flagellar biosynthetic and regulatory genes found in TM1040 are well conserved within the *Roseobacter* clade (see Table S1 in the supplemental material). Nearly 80% (11 of the 14) of the *Roseobacter* genomes analyzed possess homologs of the C-ring, MS-ring, export apparatus, L-ring, P-ring, rod, hook, hook-associated proteins, filament, and regulatory proteins. Only six species (*Jannaschia* sp. strain CSS1, *Silicibacter pomeroyi* DSS-3, *Sagittula stellata* E-37, *Oceanicola granulosus* HTCC2516, *Roseovarius* sp. strain HTCC260, and *Roseovarius* sp. strain TM1035) plus TM1040 have homologs to *fliL*, *pflI*, and *flaA*.

Transposon mutagenesis of genes involved in motility. To provide functional significance to the flagellar ORFs found by genomics and to identify novel genes involved in motility, we screened a mutant library containing 11,284 transposon mutants for defects in swimming motility (Mot$^-$). A total of 183 Mot$^-$ mutants—ca. 1.6% of the total mutant library—were identified, and the sequence of 77 of these genes was determined.

Multiple independent transposon insertions were found in 21 of the 36 flagellar structural genes in the 35.4-kb locus (black arrows in Fig. 2A) adding experimental support to our bioinformatic analysis. In addition to mutating ORFs with strong homology to known flagellar genes, the transposon also inserted in several ORFs within the large flagellar locus (for example, TM2949, TM2952, and TM2975) that have limited or no homology to know proteins (Fig. 2A). These insertions gave rise to a Mot$^-$ phenotype implicating these genes as critical in the biosynthesis or rotation of the flagellum. One of these ORFs, TM2952, has been previously characterized by us and given the name *flaA* (22), while the other two are newly described. The first, *pflI* (TM2949), is the second gene in the predicted *fliL* operon and encodes a protein with weak homology to *C. crescentus* PflI, while the second, TM2975, has been named "*flaI*" to indicate that empirical evidence links its activity to the motile phenotype.

No mutations were found in any of the other *fliC* genes despite observation of multiple independent mutations in several other biosynthetic genes, including *fliC3*. This suggests that *fliC3* is essential to flagellar biosynthesis under laboratory conditions but does not necessarily mean that one or more of the other *fliC* genes is not involved in swimming motility. Rather, denaturing SDS-PAGE separation of flagellar filaments routinely yielded at least two protein bands (Fig. 4), underscoring the possibility that the flagellum is composed of more than FliC3 protein.

Transposon insertions producing a Mot$^-$ phenotype were also found in ORFs located at disparate sites around the genome remote from the 35.4-kb flagellar locus. These ORFs largely encode proteins of unknown function (Table 2), except for three proteins—FlaB (TM1206), FlaC (TM0051), and FlaD (TM1731)—that harbor amino acid domains suggestive of regulatory functions. The other nonregulatory ORFs lying outside of the 35.4-kb flagellar regulon are not discussed further.

Identification of three novel flagellar regulatory genes. Bioinformatic characterization of the first regulatory gene, *flaB*, indicates that it encodes an acidic (pI = 4.84) 21.1-kDa protein with an N-terminal dimerization and phosphoacceptor domain frequently associated with sensory histidine kinases;

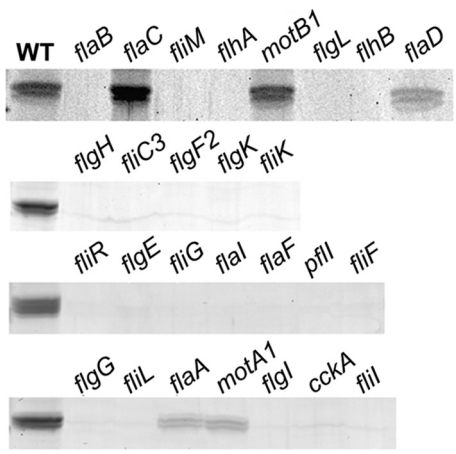

FIG. 4. Flagellar filament production by Mot$^-$ mutants. Flagellum filament bundles were harvested from overnight cultures of TM1040, and each Mot$^-$ mutant by precipitation and analyzed by SDS-PAGE for the presence of flagellin bands (29 and 30 kDa).

however, FlaB lacks a C-terminal kinase domain typically found on these proteins (8). The putative cellular localization of FlaB is unclear from PSORT analysis (13). Based on its characteristics, we hypothesize that FlaB is a histidine phosphotransferase similar to *C. crescentus* ChpT, which has weak homology (E = 5e−7) to FlaB. *flaB* is monocistronic, and the genes and operons around *flaB* do not provide much help in determining FlaB function.

flaC (TM0051) is a monocistronic gene. FlaC is predicted to be a 25.8-kDa protein with domain architecture of a two-component signal transduction transcriptional response regulatory protein. FlaC has an N-terminal CheY-like receiver domain (29) and a C-terminal DNA-binding domain (21). FlaC is predicted to be phosphorylated by an unknown histidine kinase.

BLASTP analysis shows that FlaC has significant homology (65% identity with an E-value of 3e−81) to *C. crescentus* CenR (36), a response regulator that, along with its cognate histidine kinase (CenK), plays a critical role in coordinating cell cycle progression and development by controlling cell envelope biogenesis and structure (36).

The third gene, called *flaD*, encodes a slightly basic (pI = 7.86), 19-kDa flagellar regulatory protein containing a MarR-type DNA-binding protein with a helix-turn-helix domain (Table 1) (3). *flaD* is the last gene in a three-gene locus (TM1733, TM1732, and *flaD*) and may be part of an operon. The genes upstream of *flaD* are predicted to encode a transcriptional regulator of the XRE (for xenobiotic-responsive element) family (39) and a protein with a domain (DUF339) of unknown function, respectively.

Phenotype of Mot$^-$ mutants. Our analysis of motility consisted of a stepwise approach that used a semisolid motility agar swimming assay, microscopy of swimming cells in liquid culture and, when required, detailed motion analysis of the population. All mutants produced nonmotile colonies when incubated in semisolid marine agar for 24 h and were therefore designated Mot$^-$. However, one of the mutants, HG1016 (*flaC*), was able to swim out from the point of inoculation when incubated for longer periods (>2 days at 30°C). Swimming of the *flaC* mutant in semisolid agar was uniform, albeit significantly less than the parent strain and lacked signs of motile flares, suggesting that the aberrant, weak swimming pattern was due to the effect of the *flaC* mutation and not reversion back to wild-type motility. Similarly, when the *flaC* mutant cells were transferred from the periphery of a motile swimming zone to fresh semisolid nutrient agar, the *flaC* mutant retains its defect in swimming.

Recapitulating the results obtained using semisolid media, all but one of the Mot$^-$ mutants failed to swim in liquid cultures; the exception was the FlaC$^-$ strain, HG1016. When incubated overnight in marine broth or other nutrient broth media, we observed many motile cells, whose velocity and behavior were identical to TM1040. The seeming contradiction between a nearly Mot$^-$ phenotype when in semisolid media compared to a highly Mot$^+$ phenotype when grown in broth is reproducible.

The ability of the Mot$^-$ mutants to synthesize intact flagella was assessed by staining followed by light microscopy, by SDS-PAGE analysis of flagellar bundles precipitated from spent media after overnight incubation, and by examining negatively stained cells using TEM, as required (Materials and Methods). As shown in Table 2, several of the Mot$^-$ strains synthesize flagellar filaments, including those with defects in *motB1*, *fliL*, *pflI*, *motA1*, *flaA*, *flaC*, and *flaD*. Cells with mutations in *fliL* or *pflI* possessed flagella at a low frequency—ca. 1 in 1,000 cells had a flagellum—when observed by light microscopy (Table 2), but we were unable to detect flagellin in either of these strains

TABLE 2. Phenotype of Mot$^-$ mutants

Predicted function	Mutation	Flagellum stain[a]	External flagellin[b]	Motility in broth/ semisolid agar
C-ring	*fliM*	–	–	–/–
	fliG	–	–	–/–
Export apparatus	*flhA*	–	–	–/–
	flhB	–	–	–/–
	fliR	–	–	–/–
Export ATPase	*fliI*	–	–	–/–
MS-ring	*fliF*	–	–	–/–
P-ring	*flgI*	–	–	–/–
L-ring	*flgH*	–	–	–/–
Rod	*flgF2*	–	–	–/–
	flgG	–	–	–/–
Hook	*flgE*	–	–	–/–
Hook-length control	*fliK*	–	–	–/–
Hook associated	*flgK*	–	–	–/–
	flgL	–	–	–/–
Flagellin	*fliC3*	–	–	–/–
Motor complex	*motA1*	+	+	–/–
	motB1	+	+	–/–
Regulation	*flaB*	–	–	–/–
	flaC	+	+	+/(+/–)
	flaD	+	+	–/–
	flaF	–	–	–/–
	cckA	–	–	–/–
Unknown function	*flaA*	+	+	–/–
	flaI	–	–	–/–
	fliL	+	–	–/–
	pflI	+	–	–/–

[a] +, Flagellum filaments present; –, no detectable filaments (n = 1,000 cells).
[b] +, Flagellin protein bands present; –, no detectable protein bands.

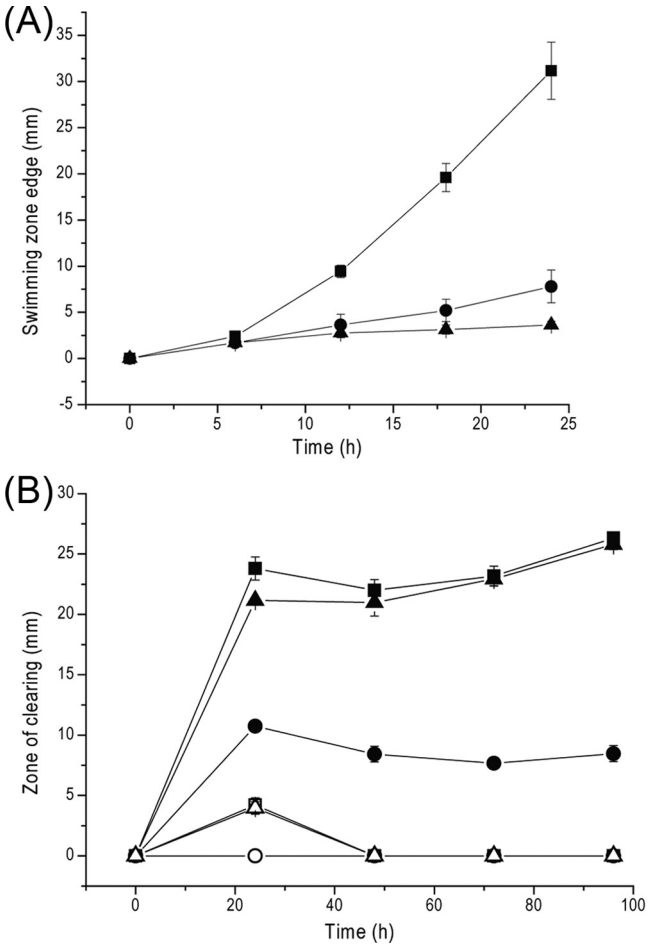

FIG. 5. Defects in *flaC* result in cells that are motility-phase biased. (A) FlaC$^-$ cells are inhibited in their swimming through semisolid nutrient agar. The migration through semisolid motility agar of the wild type (■), a *flaC* mutant (●), and a *fliC3* mutant (▲) was measured after 24 h of incubation at 30°C. Wild-type cells swam approximately six times farther than did cells with defects in *flaC* (30 mm versus 5 mm). Cells lacking flagella (*fliC3*) are nonmotile and do not move past the point of inoculation. (B) FlaC$^-$ cells produce less antibiotic activity. Incubation in standing liquid culture (closed symbols) results in the production of antibiotic activity from wild-type (■) and *fliC3* (▲) cells, while the antibiotic activity is about 2.5 times less in the FlaC$^-$ mutant (●). The antibiotic activity is downregulated when any of these strains is incubated in shaking liquid culture (open symbols).

using our precipitation method and denaturing SDS-PAGE (Fig. 4).

During the SDS-PAGE analysis, it became evident that strains that synthesized a flagellum yielded two protein bands of ca. 29 and 30 kDa (Fig. 4). This is unexpected if FliC3, as indicated by mutagenesis, is the only flagellin composing the filament. The deduced amino acid sequence of FliC3 produces a protein of 29 kDa. Both protein bands were extracted from the gel and subjected to proteomic analysis. Fragmentation of the upper (30-kDa) band produced a peptide, SXILTNNGA MVALQT, while the lower (29-kDa) band generated SLILT NNGAMXA. Both of these amino acid sequences align perfectly within the first 16 amino acids of FliC1, FliC3, FliC4, and FliC5. These data strengthen our bioinformatic analyses of the upstream regulatory regions of these genes and suggest that, in addition to FliC3, at least one other flagellin encoded by *fliC1*, *fliC4*, or *fliC5* is incorporated into the filament.

Mutation in *flaC* bias the cell toward the motile phase. We next focused our attention on the FlaC$^-$ mutant (strain HG1016, *flaC*), since its odd swimming behavior—i.e., poor swimming in semisolid media and proficient swimming in liquid media—was curious and unlike that of any other mutant. Also, in analyzing the relative abundance of flagellin obtained from the various mutants, the *flaC* strain consistently produced an ~2-fold increase in flagellin, as measured by band intensity, compared to the wild type (Fig. 4). The increase in flagellin was not due to an increase in flagella per cell, since *flaC* cells have the same number of flagella as the wild type (ca. two to four flagella per cell), as determined by TEM examination.

Although initially scored as nonmotile, we subsequently learned that *flaC* cells swim through semisolid agar media, albeit poorly relative to their parent TM1040 (Fig. 5A). While such a phenotype could result from a mutation affecting chemotaxis, HG1016 (*flaC*) responds appropriately to all known chemoattractant molecules (see Fig. S2 in the supplemental material); therefore, *flaC* must affect other functions. When grown in nutrient broth, individual FlaC$^-$ cells swam as well as the parent strain (TM1040), and the population contained more motile cells than with the wild type. As shown in Table 3, the *flaC* population had ca. three times more motile cells than did the wild type (20.85% versus 7.24% motile cells) and ca. two times fewer cells in rosettes (14.47% versus 30.03%). These results suggested that the *flaC* mutation affects whether the population is in the motile or sessile phase. Since antibiotic activity is a hallmark of the sessile phase and nearly absent in conditions that favor the motile phase, we reasoned that defects in *flaC* should adversely affect production of the antibiotic, and this is the case. When grown in conditions that favor sessile-phase cells and antibiotic activity, the *flaC* mutant produced 60% less antibiotic activity than did wild-type or *fliC3* cells (Fig. 5B). Cells incubated under shaking culture conditions produced little to no antibiotic in both wild-type and *flaC* strains. The FlaC$^-$ strain also causes defects in other phenotypes associated with the sessile phase, including the ability of the cells to synthesize pigment (Fig. 6A) and form a biofilm (Fig. 6B). These changes in physiology are correlated with changes in the outer surface of the cells, as indicated by a slight increase in the binding of the dye Congo red (Fig. 6C). Taken together, these results suggest that FlaC is involved in the biphasic switch between motility and sessility in TM1040.

Complementation of *flaC* mutants restores swimming in semisolid media. A plasmid bearing ~1 kb of genomic DNA containing 235 bp upstream and 100 bp downstream of the wild-type *flaC* gene (*flaC*$^+$) was constructed and moved into the *flaC* mutant (HG1016). As shown in Fig. 7, when *flaC*$^+$ in

TABLE 3. Percentages of motile and rosette-forming cells

Strain	Mean % cells ± SD		
	Motile cells	Rosette-forming cells	Nonmotile single cells
TM1040	7.24 ± 4.66	30.03 ± 12.75	62.73 ± 11.73
flaC mutant	20.85 ± 9.01	14.47 ± 13.61	64.68 ± 9.27

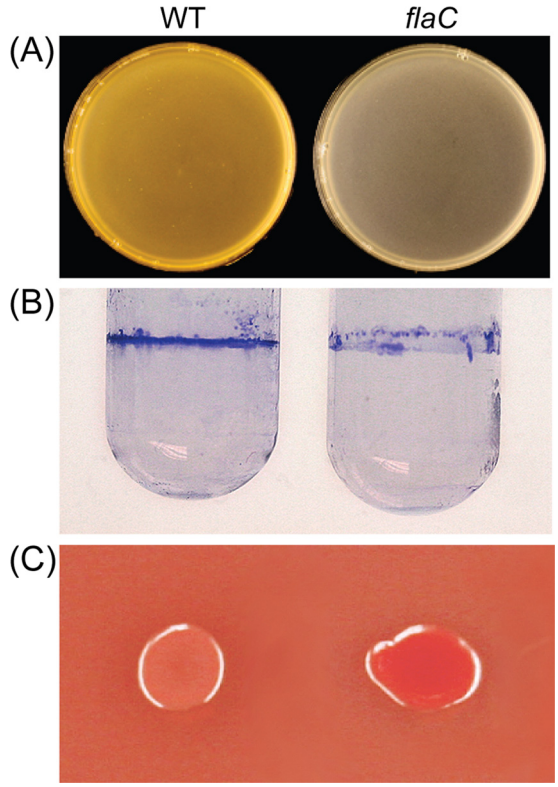

FIG. 6. Defects in *flaC* are pleiotropic. Loss of FlaC function results in a dramatic decrease in the production of pigment (A), a decrease in the formation of biofilms (B), and an increase in the binding of Congo red (C).

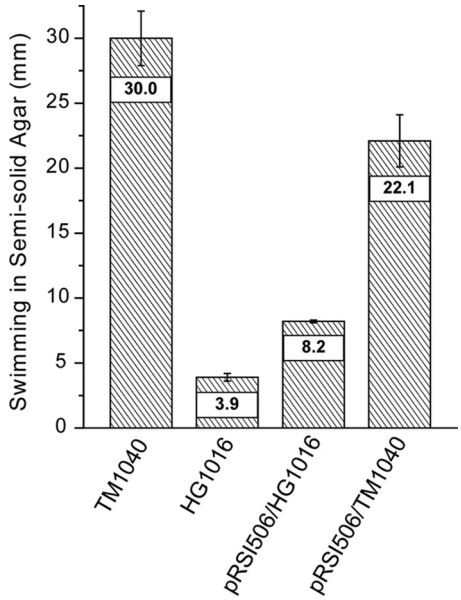

FIG. 7. Complementation of *flaC* restores swimming through semisolid nutrient agar. A plasmid, pRSI506, bearing an intact copy of *flaC*$^+$ was transformed into TM1040 (wild type) and HG1016 (*flaC*), and swimming migration through semisolid motility agar was measured after 24 h of incubation. The mean is displayed for in each bar, with the standard deviation indicated by the error bars. The *flaC*$^+$ complemented strain (pRSI506/HG1016) migrated twice as far as the mutant lacking the plasmid. Overexpression of *flaC*$^+$ in TM1040(pRSI506/TM1040) decreased movement by 74% (22.1 mm versus 30.0 mm).

trans was introduced into the FlaC$^-$ strain, partial complementation of motility resulted, and the efficiency of swimming through semisolid medium increased by twofold (3.9 mm for the *flaC* mutant compared to 8.2 mm in the complemented strain). Similarly, when *flaC*$^+$ was overexpressed in a wild-type background (TM1040), there was a reproducible 25% decrease (22.1 mm versus 30.0 mm for the wild type) in the ability of the cells to move through semisolid nutrient agar. No changes were observed in the number of flagella per cell, which remained the same as observed on the parent (TM1040) at ca. two to four filaments per cell, in either the *flaC* strain harboring *flaC*$^+$ in *trans* or the wild-type overexpressing *flaC*$^+$.

We also determined the percentage of motile cells in the populations of *flaC*$^+$-complemented and *flaC*$^+$-overexpressed cells, respectively. Partial complementation was again observed, and an overall reduction in the percentage of motile cells in HG1016 complemented with *flaC*$^+$ in *trans* was found, but the results were variable and lacked statistical significance (data not shown). Consistent with the partial complementation of motility, in *trans* complementation of *flaC* resulted in only small changes to antibiotic production, pigment, or Congo red binding in the *flaC* mutant. Overexpression of *flaC*$^+$ in the wild type consistently resulted in larger rosettes, but this observation was also variable.

Based on these results, we conclude that the migration defect is due to disruption of *flaC* and suggest the possibility that the *flaC*$^+$ plasmid expresses too much FlaC that adversely affects the swim-or-stick switch, which is sensitive to the amount of FlaC or when this regulator is expressed. We hypothesize that constitutive expression of *flaC* interferes with the timing of flagellum synthesis and motility (and other motile-phase phenotypes), resulting in the partial complementation phenotype we have observed.

DISCUSSION

The ability of roseobacters, such as TM1040, to follow a trail of chemoattractant molecules, swim into close proximity to a suitable phytoplankton cell, and then transition from a motile to a sessile existence is an important mechanism in the symbiosis and survival of these bacteria in the marine environment (22). The present study highlights the genes required for swimming motility, emphasizing the genes involved in the biosynthesis of the flagellum, as well as several regulatory genes.

The bulk of the flagellar biosynthetic genes reside within a 35.4-kb flagellar locus of TM1040 that we predict contains about eight operons. This estimate is based on the orientation of the respective genes, the presence of ribosome-binding sites (Shine-Dalgarno sequences), and the size of the intergenic gap between the genes within the putative operons. The clustering of genes within the flagellar operons of TM1040 resembles the arrangement of operons within the fla2 locus of *R. sphaeroides* (30) and shows obvious synteny with the flagellar loci of that bacterium and the closely related roseobacter *S. pomeroyi* (25). The factors controlling fla2 gene expression are currently unknown (30), and yet the evident synteny between the two loci

hints that similar molecular mechanisms work to control the expression of the respective flagellar genes in both *R. sphaeroides* and TM1040. Further, since many other roseobacters possess homologs of the TM1040 flagellar genes, it is likely that the molecular mechanisms underlying the regulation of flagellar gene expression discovered in TM1040 are common to other members of the *Roseobacter* clade.

The marked similarity between the 35.4-kb flagellar locus of TM1040 and the fla2 locus of *R. sphaeroides* is nearly perfect, with the major difference being an insertion of four genes (*flgF2*, *flbT*, *flaF*, and *fliC3*) in the TM1040 locus. These four are found on pRS241A3 in *R. sphaeroides* (30), suggesting that some time in the distant past an event took place in the progenitor of these two *Alphaproteobacteria* that integrated these genes into the 35.4-kb flagellar locus of the bacterium that would become TM1040. The addition of four ORFs—TM2967 to TM2970—whose products may have been involved in such an integration is evident in the 35.4-kb flagellar locus immediately adjacent to *flgF2*, *flbT*, *flaF*, and *fliC3* (Fig. 2A). The genome of TM1040 has several prophage genomes (12), three of which form phage particles, therefore, one of these may have played a role in moving these genes into TM1040. The integration of the flagellar genes, which include the primary flagellin-encoding gene (*fliC3*), as well as the putative regulatory genes *flbT* and *flaF*, may have improved the survival of TM1040, since loss of plasmid-borne copies of these genes could result in a loss of motility.

The 35.4-kb flagellar locus has other interesting features. Unlike the flagellar regulons of enteric bacteria, the 35.4-kb flagellar locus of TM1040 lacks ORFs encoding MCPs, Che proteins, flagellar chaperone proteins (for example, FliD), and flagellar sigma and antisigma factors. Underscoring this difference, transposon mutagenesis screens did not find genes encoding Che proteins or MCPs, especially since several biosynthetic genes were identified that had multiple independent insertion of the transposon, suggesting that the mutagenesis was saturating. The failure to find these mutants suggests that any one mutation in a given *che* or *mcp* gene can be compensated by the expression of other *che* or *mcp* genes, respectively. Such compensatory mechanisms have been observed between the multiple *che* genes of *R. sphaeroides* (32, 34). In previous work, we identified multiple copies of genes encoding MCPs, as well as multiple instances of *cheR*, *cheW*, *cheY*, and *cheD*, underscoring the possibility that the multiple copies of these genes have overlapping functions that can compensate for mutations. Also, unlike the organization in enteric bacteria, none of the *mcp* or *che* genes are associated with the 35.4-kb flagellar locus bearing the majority of flagellar biosynthetic genes (25). The physiological significance of separating the *mcp* and *che* genes away from the biosynthetic genes is unknown but may allow the cell greater control over the expression of *mcp* and *che* genes independent from the flagellar biosynthetic genes and suggests that TM1040 and other roseobacters might have alternative uses for the products of *mcp* and *che* genes that transcend motility.

Although the locus harbors two genes encoding regulators of motility, *flbT* and *flaF*, we conclude, on the basis of a lack of identifiable domains, that no other regulatory proteins are present in the 35.4-kb locus. The respective genes encoding CckA and CtrA are located in two distinct loci remote from the 35.4-kb flagellar locus (22).

Mutations in *cckA*, *ctrA*, or *flaF* result in a Mot^- phenotype implying that regulation of flagellar biosynthesis in TM1040 is similar to *C. crescentus*. This thought is bolstered by the presence of a homolog of *flbT* and identification of *flaB*, a gene whose deduced protein sequence has weak homology to ChpT, which acts as a histidine phosphotransferase in a phosphorelay between CckA and CtrA in *C. crescentus* (9). This suggests that FlaB is also a histidine phosphotransferase that may play a role in shuttling phosphoryl groups between CckA and CtrA. However, it is important to emphasize that mutations in these genes are lethal in *C. crescentus* but do not affect the growth of TM1040. Thus, the respective function of these genes in the *Roseobacter* clade bacteria is likely to be different from the function in *Caulobacter*.

Although there are obvious similarities between the 35.4-kb flagellar locus of TM1040 and *R. sphaeroides* fla2, the present results underscore potential differences between these two species as well, particularly in the putative mechanism regulating transcription of the *fliC* genes in roseobacters. *R. sphaeroides* uses σ^{54}-dependent promoters to express flagellar genes encoding the hook and basal body proteins (31), with two σ^{54} enhancer binding proteins, FleQ and FleT, playing a central role in the regulation of flagellin genes (31). The genome of TM1040 lacks homologs to RpoN, FleQ, and FleT, nor does it have genes encoding FliA or FlgM, both of which are controlled by FleQ-FleT. Adding to this evidence is the highly conserved upstream regulatory region 5′ to the start codon of *fliC1*, *fliC3*, *fliC4*, and *fliC5* (Fig. 3 and see Fig. S1 in the supplemental material) that has only limited homology to an obvious σ^{54} consensus sequence. Since flagellin protein profiles (Fig. 4) indicate that the flagellum is composed of two or more flagellin species, we conclude that *fliC1*, *fliC3*, *fliC4*, and *fliC5* are transcribed by a similar mechanism that is σ^{54} independent.

With these points in mind, TM1040 appears to use some of the molecular components of *Caulobacter* and some of the molecular components of *Rhodobacter* in novel ways to control the expression of its flagellar biosynthetic genes. In this regard, TM1040 may be thought of as a "value added" chimera of *C. crescentus* and *R. sphaeroides*, with unique molecular mechanisms that have evolved to permit its survival in the marine environment and in its symbiosis with phytoplankton. Emphasizing these unique molecular mechanisms are the proteins encoded by *flaC* and *flaD*, respectively.

The phenotype of *flaC* cells is pleiotropic, showing defects in biofilm formation and significant decreases in pigment and antibiotic production, hallmarks of cells in the sessile phase. The pleiotropic nature of FlaC implicates it as a regulator involved in multiple functions of the cell, perhaps acting at early stages in a regulatory hierarchy to control the biphasic swim-or-stick switch. FlaC has homology to CenR, a protein that functions to control cell envelope biogenesis and structure in *C. crescentus* (36). Unlike mutations in *cenR* that result in a lethal phenotype, *flaC* defects show no effects on cell growth. This may reflect differences in the lifestyles of *Silicibacter* and *Caulobacter* or that FlaC and CenR have different functions.

Motility of TM1040 is growth phase dependent and relatively short-lived, peaking around the mid-exponential phase

and then quickly diminishing. The evidence present here suggests that FlaC is part of the motility timing mechanism, e.g., defects in FlaC result in cells that are constitutively motile because they no longer know when to turn off flagellum synthesis and/or motility. This hypothesis provides an explanation for the increase in the percentage of motile cells when *flaC* strains are grown in broths, why *flaC* cells have the same number of flagella as wild-type cells, and why more flagellin protein (on a per-cell basis) is present in *flaC* cultures. It also helps to explain why in *trans* expression of *flaC* from a plasmid gives inconsistent and partial complementation: motility requires the correct dosage of FlaC at the correct time during cell growth. When *flaC* is constitutively transcribed and overexpressed, as is the case when *flaC*$^+$ is expressed on a multicopy plasmid, FlaC is produced at an inappropriate time and dosage leading to aberrant cell physiology.

Although *flaC* cells swim well in liquid media and have wild-type chemotaxis behavior (see Fig. S2 in the supplemental material), they swim poorly through semisolid nutrient agars, a characteristic that can be partially complemented by *in trans* *flaC* (Fig. 7). Why do *flaC* cells migrate poorly through semisolid agar, when they have no apparent problems swimming in liquids? Migration of TM1040 through the semisolid agar matrix requires flagellar swimming, chemotaxis behavior, and a FlaC-dependent function, which we argue controls whether the cell is in the motile phase or the sessile phase. This hypothesis implies that FlaC is involved in controlling more than motility and suggests that FlaC must be made at the right time and amount during migration in a viscous environment if the cells are to migrate outward from the point of inoculum. Our data do not provide insights into why the sessile stage would be required for progressive migration through the semisolid agar, but perhaps the bacteria need to temporarily stick to the agar matrix, swim, and then stick again, moving from agar fiber to agar fiber much like a trapeze artist moves from one trapeze bar to another. Alternative, the cells may have to enter sessile phase before they can start a new cycle of migration in the viscous milieu—akin to pushing a reset button.

If it is required for bacterial migration through viscous environments, then FlaC is also likely to be important in symbiosis. The outer surface of phytoplankton is covered with glycoproteins and other macromolecules that create a viscous layer or microniche in which TM1040 becomes embedded during attachment to its host. We would predict that under this condition in the marine environment, FlaC-dependent migration would aid the bacteria in establishing and maintaining the interaction with its algal or dinoflagellate host. The function of *flaC* is under investigation in our laboratory.

While the primary focus of the current study is *flaC*, we think it significant that *flaD* cells synthesize flagella but are nonmotile, i.e., they have paralyzed flagella, a phenotype in common with mutations in *motA1* and *motB2* (Table 2). In enteric bacteria, MotA and MotB are integral membrane proteins that form the stator of the flagellar rotary motor (19). We speculate that the paralyzed flagellar phenotype observed in mutations in *flaD*, *motA1*, and *motB1* indicates that the products of these genes are involved in the same function: rotation of the flagellar motor. The amino acid sequence of FlaD suggests that the protein functions as a transcriptional regulator and is not a component of the flagellar motor. We therefore believe that FlaD regulates expression of *motA1* and/or *motB1* and perhaps other genes, e.g., *flaA*, *fliL* (5, 7), and *pflI*, that produced paralyzed flagella when mutated. Since motor proteins are often late gene products that are synthesized in the last stage of flagellar biosynthesis along with filament (*fliC3*, in TM1040) and MCPs (2), FlaD most likely controls transcription of only the motor proteins, while other transcriptional (or posttranscriptional) regulators are involved in controlling flagellin and chemoreceptor expression. The presence of FlbT and FlaF in the 35.4-kb flagellar locus supports the latter hypothesis, since these proteins function posttranscriptionally to control flagellin expression (20).

We are gaining a better understanding of the molecular mechanisms used by bacteria of the ecologically significant *Roseobacter* clade to survive, adapt, and compete successfully in the marine environment. Although our knowledge is limited, it has become clear that bacterial motility and chemotaxis behavior are important factors in the initial stages of symbiosis between TM1040 and the phytoplankton that are its hosts. In this context, the present study provides insights into the genes required for swimming motility and offers exciting clues on how the bacteria may control the swim-or-stick switch, allowing the cells to transition from a motile to a sessile lifestyle. Understanding the factors that control expression of the genes that are the hallmarks of each life phase is important in order to get to a clearer picture of how the swim-or-stick lifestyle affects marine bacterial community structure and composition, both in biofilms and during bacterial interactions with phytoplankton hosts.

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