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Characterization of Lateral Flagella of *Selenomonas ruminantium*▽

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Selenomonas ruminantium **produces a tuft of flagella near the midpoint of the cell body and swims by rotating the cell body along the cell's long axis. The flagellum is composed of a single kind of flagellin, which is heavily glycosylated. The hook length of *S. ruminantium* is almost double that of *Salmonella*.**

Selenomonas ruminantium is a Gram-negative anaerobic bacterium, often found in the rumens of domestic animals (8, 10). According to Leifson's *Atlas of Bacterial Flagellation* (13), *S. ruminantium* flagella originate "as a tuft from the concave side of the organism." In bacteria in general, there are three flagellar families, classified according to the flagellar location on a cell: peritrichous, polar, and lateral (2, 7, 13). Thus, *S. ruminantium* cells are described as having several lateral flagella. Other species that have lateral (or subpolar) flagella include *Vibrio alginolyticus* (12), *Vibrio parahaemolyticus* (15, 16), *Bradyrhizobium japonicum* (9), *Photobacterium profundum* (6), and *Rhodobacter sphaeroides* (3, 18). In fact, these bacterial species have two flagellar systems, which can produce either polar or lateral flagella, and the bacterial cells can express either of these flagellum types or both of them, depending on growth conditions (16). Here, we report the structural analysis of the lateral flagella of *S. ruminantium* subsp. *lactilytica* TAM6421 (NBRC 103574). This is the first analysis of flagellar proteins in a solely laterally flagellate bacterium.

Flagellation is suppressed in the presence of glucose. Cells were anaerobically grown at 37°C overnight as previously described (27) and observed with a phase-contrast microscope. When grown in tryptone-yeast extract-lactate (TYL) medium, which contains lactate as the sole carbon source, cells were actively motile, whereas cells grown in tryptone-yeast extract-glucose (TYG) medium, which contains glucose, were immotile. Cells grown in TYL medium supplemented with glucose or TYG medium supplemented with lactate were also immotile (data not shown). Moreover, electron microscopic observation, as previously described (7), of negatively stained cells from these cultures showed that motile cells had several flagella but that immotile cells had none (Fig. 1). These data suggest that flagellar gene expression in *S. ruminantium* cells might be under catabolite repression by glucose, as is known to be the case for other bacteria (1, 21, 23). In the bovine rumen, cellulose is degraded by various rumen bacteria into sugars, which are subsequently turned to lactate by other bacteria, including *Streptococcus bovis* (4, 19). Thus, *S. ruminantium* cells in the rumen likely live under conditions similar to those in TYL medium and may be motile.

Swimming patterns and speeds. *S. ruminantium* cells appeared to swim shakily by rotating the cell body along the cell's long axis, in contrast with *R. sphaeroides* (a bacterium with subpolar flagella [18] that is the nearest appropriate comparison), which swims by rotating in the direction perpendicular to the cell's long axis (3). Swimming speeds of cells were measured by using recorded images as previously described (14). The average speed of actively swimming cells was about 16 μm/s, which is much slower than the 29 μm/s for *Salmonella enterica* serovar Typhimurium cells of a similar size (Table 1).

Number and position of flagella on a cell. The number of flagella on cells of *S. ruminantium* was measured by electron microscopy (Fig. 1). The average flagellar number per cell for 20 cells was about six (Table 1). Sites of flagella on the cell surfaces were measured using electron microscopic images of cells that were osmotically shocked to reveal the flagellar bases (Fig. 2A). The position is expressed as a percentage of the cell length, by setting the distance from a pole to the midpoint of the cell body as 50% (Fig. 2B). As seen in the histogram (Fig. 2C, left), *S. ruminantium* flagella protruded from points all clustered around the midpoint. In comparison, each single *Rhodobacter sphaeroides* WS8 flagellum protruded randomly on the side of the cell body (Fig. 2C, right). These data indicate that the *R. sphaeroides* flagella, albeit known to be subpolar (3), might actually be single flagella with a random arrangement, such as individual examples of peritrichous flagella, and that *S. ruminantium* flagella might be authentic lateral flagella; these could be accurately regulated to grow at certain positions on the side of the cell body in a manner similar to that of polar flagella, which grow from poles of the cell body. It has been known that two genes, the *flhF* and *flhG* genes, are essential for controlling the position and number of polar flagella for *P. aeruginosa* (5), *V. alginolyticus* (12), and *V. parahaemolyticus* (16). Interestingly, both genes are also found in *S. ruminantium* but are not found in *R. sphaeroides*. How can lateral flagella be distinguished from peritrichous flagella? Peritrichous flagella connote flagella growing randomly around the cell body. Judging from the scattered distribution of flagellar positions, so-called lateral flagella, such as those of *V. alginolyticus*, may be peritrichous flagella (L. L. McCarter, private communication).

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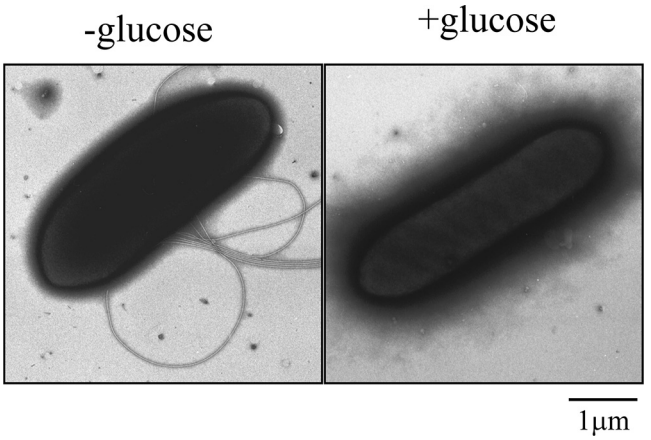

FIG. 1. Electron microscopic images of *Selenomonas ruminantium*. Cells were grown in the absence of glucose (−glucose, left) or in the presence of glucose (+glucose, right).

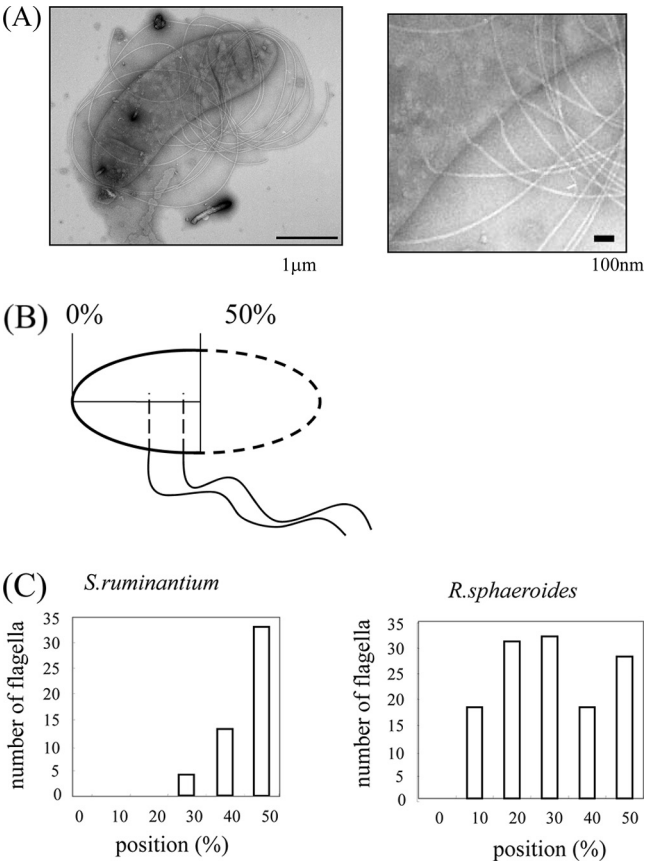

FIG. 2. Distribution of flagella on a cell. (A) Electron microscopic image of a cell that was osmotically shocked and stained with 1% phosphotungstic acid (PTA). The flagellar form is coiled (left). Enlarged image of the left panel showing flagellar basal bodies in the membranes (right). (B) Schematic presentation showing how the positions of flagella on a cell were measured. (C) Diagram of the distributions of sites of flagellar protrusion on a cell for *S. ruminantium* (left) and *R. sphaeroides* (right). The total numbers of flagella counted were 50 and 127, respectively.

For both flagellar systems, no extra genes corresponding to the *flhF* and *flhG* genes have been found.

Polymorphism of flagellar shapes. By electron microscopy, *S. ruminantium* flagella at a neutral pH appeared in a coiled form, wrapping the cell body (Fig. 1). To understand how cells with coiled flagella are able to swim, we examined the polymorphic transition of isolated flagella under a dark-field microscope as previously described (7). To characterize polymorphic forms more accurately, we measured the helical parameters: the helical pitch (P), the diameter (D), and the handedness. At pHs between 5 and 8 in the absence of salt, the coiled form (~0-μm P, 1.93-μm D, left-handed) was predominant (Fig. 3A, middle). At pHs higher than 8, the left-handed normal helical form (4.7-μm P, 1.93-μm D, left-handed) appeared at all salt concentrations (Fig. 3A, right). At pHs lower than 5, there appeared a right-handed form, which has a set of P and D similar to that of the normal form helix (Fig. 3A, left). We named this form large curly (4.84-μm P, 1.86-μm D, right-handed) according to a convention used in previous flagellar studies (7). At higher salt concentrations in the neutral pH range (5.0 to 8.0), a mixture of the large curly and coiled forms appeared (Fig. 3B). At pHs lower than 3, flagella were depolymerized and disappeared. These helical parameters suggest that *S. ruminantium* flagella belong to an exceptional group, which is so far dominated by *Alphaproteobacteria* (7). It should be noted that *S. ruminantium* belongs not to the *Proteobacteria* but rather to the *Bacteroidetes*. In the rumen, where the pH is between 5.5 and 7.0, flagella would take on the coiled form. The coiled form might be converted to the normal form when rotational force is applied to the flagella, similar to the change seen for the flagella of *R. sphaeroides* (3).

Flagellin. Flagellar filaments were sheared off cells by vigorous pipetting, purified by differential centrifugations, and analyzed by SDS-12.5% polyacrylamide gel electrophoresis (Fig. 4A). The filament was found to be composed of a single flagellin with an apparent molecular size of 75 kDa. The N-terminal 10-amino-acid (aa) sequence of *S. ruminantium* FliC1 was analyzed as previously described (9). The sequence was AMVVXNNMSA. It should be noted that the first methionine

TABLE 1. Summary of physical properties of *S. ruminantium* flagella

Parameter	Measured value ± SD for flagella of:		Reference
	S. ruminantium	*S.* Typhimurium	
Avg swimming speed	16 ± 6 μm/s (n = 19 cells)	29 ± 5 μm/s	This study
Avg no. of flagella/cell	6 ± 1.4 (n = 20 cells)		This study
Avg hook length	105 ± 12 nm (n = 319 hooks)	55 ± 6 nm	20
Size of FliK	817 aa	405 aa	20

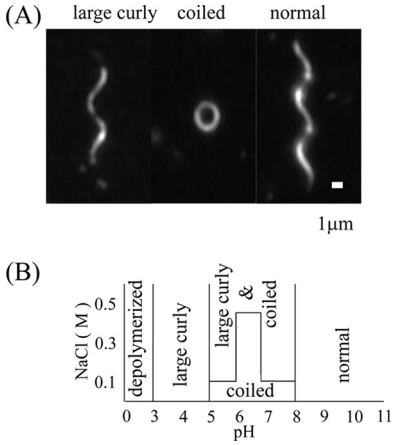

FIG. 3. Flagellar polymorphic transition. (A) Dark-field microscopic images of polymorphs observed under various pH and salt conditions. The upper side of the helix was focused to show the handedness. (B) Schematic phase diagram of polymorphs observed at different pHs and NaCl concentrations.

was cleaved, as is often the case for flagellar proteins. The 5th residue (X) was not read, probably because of a chemical modification of lysine (see below). We also analyzed tryptic fragments by nanoscale liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS; Matrix Science K.K., Tokyo, Japan) and obtained a fragment that read VAEGAVSSTVD ILK. Both sequences agreed with that of FliC1 deduced from the *fliC1* gene (chromosome 2577; 441 aa) annotated as one of the 11 flagellin genes on the *S. ruminantium* genome (Fig. 5). The other flagellins, FliC2 to FliC11, vary in size from 285 aa to 954 aa. Although the terminal regions of these flagellins showed a typical conserved pattern for flagellin, the central region was as diverse as that of *S.* Typhimurium. Since flagellins other than FliC1 were not detected in mature filaments, they may be minor components in the filament below the detection level of SDS-PAGE or may not be expressed at all.

The predicted molecular size of FliC1 is about 46 kDa. On the other hand, the apparent molecular size of FliC1 when examined by SDS-PAGE was 71 kDa, which would make it one of the largest flagellins studied so far (most are ≤60 kDa) (2). This unusually large difference in molecular sizes between predicted and observed flagellin might be derived from chemical modification of the protein. There are several flagellins known to be glycosylated in other species (24). We have tested this possibility for *S. ruminantium* flagellin by the periodic acid-Schiff (PAS) staining method with SDS-PAGE followed by blotting onto polyvinylidene difluoride paper. The flagellin band of *S. ruminantium* was stained pink as strongly as that of *Azospirillum brasilense* (a kind gift from Gladys Alexandre, University of Tennessee), which was chosen as a positive control (17), while the *S*. Typhimurium flagellin was not stained, as would be expected (Fig. 4B). Thus, the *S. ruminantium* flagellin is glycosylated.

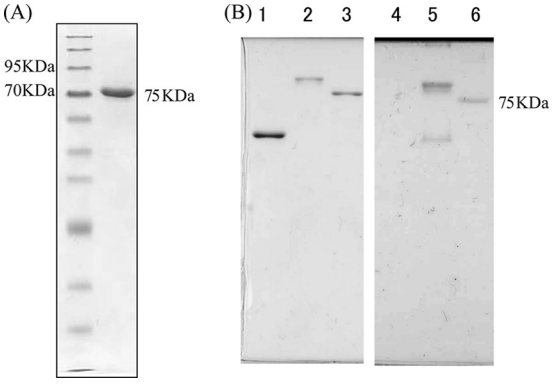

FIG. 4. The *S. ruminantium* flagellin is glycosylated. (A) SDS gel pattern of an *S. ruminantium* flagellin. First lane, molecular size marker; second lane, single band of flagellin. (B) Detection of glycosylation of an *S. ruminantium* flagellin (lanes 3 and 6). *Salmonella* SJW1103 flagellin was used as a negative control (lanes 1 and 4), and *Azospirillum* flagellin was used as a positive control (lanes 2 and 5). Coomassie brilliant blue (CBB) staining (left) and PAS staining (right) of the purified flagellins are shown.

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        10         20         30         40         50         60
MAMVVKNNMS AISTLNTLNK NSSALSKSLE KVSSGMKING AADDASGYAI SERMRVQIRG
        70         80         90        100        110        120
LDQSNANTQN GNAMMKVAEG AVSSTVDILK TLKEKVINAA NDTNTDSDRQ TIQKELDQSI
       130        140        150        160        170        180
DQIDDNANIT YNGKYLVDGS HNNKTTNTTT ALTNESMDAA TTKTSALTGL KNRNGESLNI
       190        200        210        220        230        240
HSTDNVTISY VHEGKTYSTT FQVGSESLAT AVAKTAYNGD KSLTGALSFN ESGATSKIGL
       250        260        270        280        290        300
DGSGNTVYTA DKGCALTLTA KTTGVAGQIS GFTISITTNK GAINKSANAV LDNFTESIRA
       310        320        330        340        350        360
QNKSEDNAMV FQVGTKANQA IKVGLTDMRS TALGLKGADG ETLSVSTQVK ANAAINVLDN
       370        380        390        400        410        420
AIQKALDQQT TIGSVESRLS YTSSNLTTAS ENVQSSESTI RDADMAKEMT NYTKNNVLLQ
       430        440
AAQSMLAQAN QSSSSVLSLL Q
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FIG. 5. Amino acid sequence deduced from the *S. ruminantium fliC1* gene. The underlined sequences were obtained by an analysis of purified FliC.

Flagellar basal structure. Intact flagella were purified and observed by electron microscopy (Fig. 6A). In the basal body, there were 4 rings, as is typical for Gram-negative species (2).

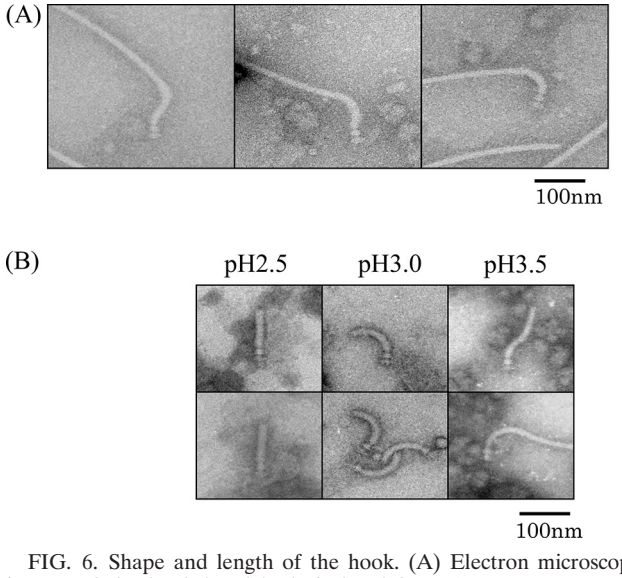

FIG. 6. Shape and length of the hook. (A) Electron microscopic images of the hook basal body isolated from *S. ruminantium*. The desalted samples were stained with 2% PTA at pH 7.0. (B) Polymorphs of the hook under different pH conditions.

Although *S. ruminantium* was previously categorized as a Gram-positive bacterium by analysis of 16S rRNA (22), our observation of the flagellar basal structure confirms that *S. ruminantium* has an outer membrane, a characteristic of Gram-negative bacteria (8, 26). Gram-positive outer membranes or mycomembranes were recently reported for the Gram-positive general *Corynebacterium* and *Mycobacterium* (28), but none of them have flagella.

Hook shape and length. The flagellar hook looked unusual, often showing an S shape by electron microscopy (Fig. 6A). Under different pH conditions (using Tris-glycine buffer), the hook underwent the following polymorphic transition (2): an S shape at and above pH 3.5, a curved shape (ordinary hook shape) at pH 3.0, and a straight form at pH 2.5 (Fig. 6B). Thus, under physiological conditions, the hook may take on an S shape rather than a simple curve. The average hook length was about 105 nm (Table 1), which is much longer than the 55-nm *Salmonella* hooks or the 74-nm *R. sphaeroides* hooks (11) and is probably the longest among naturally occurring hooks studied so far (20). The molecular size of FliK deduced from the *fliK* gene (chromosome 690), the hook-length control gene, was 817 amino acids, whereas that of *S.* Typhimurium FliK was 405 aa. This length of the hook regulator protein FliK may explain the lengthy hooks of *S. ruminantium*.

In peritrichous flagella of *Pseudomonas syringae*, glycosylation of flagellin was necessary for smooth swimming and for proper conversion of flagellar shapes (25). Heavy chemical modification of *S. ruminantium* flagellin, together with long hooks, might also be necessary for the smooth rotation of flagella in a bundle at the side of the concave cell body. These assumptions will be tested by making knockout mutants of related genes and point mutations of potential flagellin modification sites in the near future.

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