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论文题目：Evolution Talks: Flagellation Pattern and Fitness in Bacteria

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Abstract: Flagella on many prokaryotes are long filamentous appendages that propel cells to move from detrimental to favorable environments. *Shewanella oneidensis*, a rod-shaped Gram-negative bacterium, evolved monotrichous polar flagellation that plays an important role in ecophysiological adaptation. However, it is not clear whether such flagellation pattern confers the bacterium a fitness gain in dynamic and static environments than other types. Here, an evenly mixed culture of the wild-type and four mutants with distinct flagellation patterns, including aflagellated Δ*flaA*Δ*flaB*, monotrichous lateral Δ*flhF*, lophotrichous Δ*flhG*, and peritrichous Δ*flhF*Δ*flhG*, was used to test fitness of these flagellation patterns in three different conditions. On semi-solid plates, only the wild-type strain spread. In static liquid medium, population of pellicle (cell community formed at air-liquid interface) was dominated by the wild-type strain (94.4%) after 18 h incubation, but underneath Δ*flaA*Δ*flaB* and Δ*flhF* exhibited almost the same proportions as the wild-type strain. Interestingly, Δ*flhF* grew faster than other four strains including the wild-type in the condition of shaking liquid culture. By revealing that monotrichous polar flagellum that the wild-type strain has evolved is the optimum for growth fitness of *S. oneidensis* than that of any other mutants in the static environment but not in the dynamic environments, these findings provide insights into impacts of flagellation patterns on bacterial adaptation to environments.

Key words: flagellation patterns, *Shewanella oneidensis*, fitness.

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Evolution Talks: Flagellation Pattern and Fitness in Bacteria

Introduction:

For many bacteria, the ability to actively move from detrimental to favorable niches confers an important advantage for survival (1, 2). The surface appendages that prokaryotic cells have evolved for locomotion vary, including flagella, pili, and mycoplasma “legs” (3). A very effective means is by flagella, semi-rigid filamentous external structures through the cell wall, which can propel cells in liquid as fast as 60 μm/s in many *Vibrio* and *Pseudomonas* species (4-7). More than 80% of known bacterial species are motile by means of flagella that provide swimming and swarming motilities and also play a central role in adhesion, biofilm (cell community) formation, and host invasion (8，9). According to previous studies, three flagellar families were classified based on the flagellar location on a cell: peritrichous, polar, and lateral (10).

Bacterial species often differ distinctively in flagellation patterns. According to the number and location of flagella, the organelle is categorized into a variety of types of flagellar arrangement, including monotrichous polar flagellation (i.e. *Vibrio cholerae, Pseudomonas aeruginosa, Idiomarina loihiensis*), atrichous (i.e. *Staphylococcus aureus*), lophotrichous (i.e. *Vibrio fischeri* and

Spirillum spp.), lateral flagellation (i.e. *Selenomonas ruminatium*), peritrichous (i.e. *Escherichia coli* and *Proteus vulgaris*) (11, 12). For monotrichous polar bacteria, flagellum rotates counterclockwise (viewed from outside the cell) during forward movement. When flagellum rotates in a reversing direction, the cell stops and tumbles. Peritrichously flagellated bacteria use a similar way: they form a counterclockwise-rotating bundle of flagella to move forward. Clockwise rotation of flagella disrupts the bundle and the cell tumbles.

The *Shewanella* genus has expanded during the last two decades as an important family of Gram-negative facultative γ-proteobacteria (13). Recently, *S. oneidensis*, the representative species of *Shewanella*, has become a study model for respiration diversity, metabolic network, and biofilm formation (13). In nature, the *Shewanella* species are motile by a polar flagellum in general. *S. oneidensis*, can move at a high speed by using the thrust that provided by its polar flagellum. Additionally, the flagellum of *Shewanella* species has been reported to be involved in formation of biofilms and pellicles (14, 15). Interestingly, some *Shewanella* species have an accessory peritrichous lateral flagellar system that is conditionally synthesized (16).

The flagellar assembly in *S. oneidensis* has been extensively

studied and many mutants with distinct flagellation patterns are generated and characterized (17, 18). These include an aflagellated strain (Δ*flaA*Δ*flaB*) and strains with a monotrichous lateral flagellum (Δ*flhF*), with lophotrichous flagella (Δ*flhG*), and with peritrichous flagella (Δ*flhF*Δ*flhG*). While both FlaA and FlaB are flagellins, FlhF and FlhG act in a coordinate manner to control the number and location of flagella (17). With respect to motility, the Δ*flaA*Δ*flaB* and Δ*flhG* strains were non-motile, whereas the Δ*flhF* and Δ*flhF*Δ*flhG* strains retained some, approximately 30% and 10% relative to that of the wild-type, respectively (17). These mutants open the door to understanding of the mechanism through which the polar flagellum is advantageous of other flagellation patterns and thus is fixed in evolution.

In the present study, the wild-type, and four above-mentioned mutants were used to test their growth fitness in the three different conditions. It is clear that the wild-type strain has great advantages over other four mutants in semi-solid conditions and pellicles (biofilm formed at air-liquid interface), where motility is critical. In the conditions of agitating or underneath the pellicles, strains having a flagellum outcompete those either flagellum-free or with multiple flagella.

Materials and Methods:

Bacterial strains and culture conditions

All bacterial strains used in this study were listed in Table 1. *S. oneidensis* strains were grown in lysogeny broth (LB) medium under the aerobic condition at 30°C. In the test of attachment ability, liquid LB medium was placed at level shaker which was set to 150 rpm.

Table 1. Strains used in this study

Strain	Description	Reference or source
S. oneidensis		
MR-1	Wild-type	ATCC 700550
HG3237-8	Δ*flaA*Δ*flaB* derived from MR-1	18
HG3211	Δ*flhG* derived from MR-1	11
HG3212	Δ*flhF* derived from MR-1	11
HG3212-1	Δ*flhF*Δ*flhG* derived from MR-1	11

Growth competition of *S. oneidensis* strains

A single colony of freshly prepared cultures on LB plates for each of five strains under test was used to prepare the starting cultures in 3 mL LB. Then 3 mL fresh LB was inoculated by 1% of overnight cultures. Growth of *S. oneidensis* strains in liquid LB medium was measured by recording optical densities at 600 nm (OD_{600}) under aerobic conditions. Two hundred microliters of each culture of mid-log phase (~0.4 of OD_{600}) were transferred to a new tube, giving to the mixed culture, which was used for subsequent experiments. Motility test was carried out by spotting 2.5 μl of

mid-log phase cultures on semi-solid LB plates (agar concentration, 0.25%, w/v). To facilitate comparison, Δ*flaA*Δ*flaB* strain (non-motile) was always included at the center of the same plate. Photograph was taken 18 h after incubation at 30°C unless otherwise noted.

Analysis of population composition

To investigate influences of flagellation patterns on fitness of *S. oneidensis*, an aliquot of the evenly mixed culture of the five strains were subjected to growth competition experiments under various conditions. After the experiments, the samples from the resultant cultures were collected, properly diluted and applied onto fresh solid LB plates (agar concentration, 1.5% w/v, on which all test strains grew indistinguishably) to form separated colonies. Thirty-six separate colonies chosen randomly were transferred onto semi-solid LB plates to test their motility. Colonies with over 2 cm in diameter were the wild-type strain and colonies with over 0.5 cm in diameter were the Δ*flhF* strain according to the previous report (17).

The non-motile colonies which were unable to be identified were further tested by polymerase chain reaction (PCR). The cultures were used as templates (all cultures had been preconditioned at

100°C for 5 min for disrupting cells and releasing genomic DNA), *FlhFG* was amplified by PCR. In PCR, amplification was primed by pairs of oligonucleotides (Primer-F: 5'-CCTAATCTCAGAGT-GATTTC-3'; and Primer-R: 5'-GGTAATCTGGCAAGCAAGTG-3') derived from the *FlhFG* sequence. The PCR program consisted of following steps: denaturing at 94°C for 10 min, 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min 10 s, and polymerization at 72°C for 10 min. Products from the PCR were then analyzed by 1% agarose gel electrophoresis. The products with approximately 2 Kb, 1 Kb and 0.2 Kb indicate the strain for Δ*flaA*Δ*flaB*, Δ*flhG* and Δ*flhF*Δ*flhG*, respectively.

Results:

The wild-type strain shows significant growth advantage on semi-solid plates

In *S. oneidensis*, four flagellar mutants, aflagellated Δ*flaA*Δ*flaB*, monotrichous lateral Δ*flhF*, lophotrichous Δ*flhG*, and peritrichous Δ*flhF*Δ*flhG*, were identified and characterized (11, 17, 18). To investigate influences of flagellation patterns on fitness of *S. oneidensis* on semi-solid plates, 2.5 μL evenly mixed cultures of these five strains were spotted. After 24 h incubation, the droplet grew to 3 cm in diameter (Fig. 1A). To examine the proportion of

each strain in the droplet, the samples were collected at 3 different locations by a pipette tip, center, middle and edge of the droplet (Fig. 1A), diluted and applied onto fresh solid LB plates (agar concentration, 1.5% w/v, on which all test strains grew indistinguishably) to form separated colonies. In total, 36 separate colonies from each plate were randomly transferred onto semi-solid LB plates as shown Fig. 1B (center), Fig. 1C (middle) and Fig. 1D (edge).

Thirty-three of 36 colonies from the center were the wild-type based on over 2 cm in diameter on semi-solid LB plates (Fig. 1B). The remaining three included one for the Δ*flhF* strain (purple arrow, 0.5 cm in diameter) according to its motility in a previous report (17), one for the Δ*flaA*Δ*flaB* strain (red arrow) and one for the Δ*flhF*Δ*flhG* strain (black arrow), which were determined by PCR (Fig. 1E). In the case of middle and edge samples, all of 36 colonies were the wild-type as they grew to over 2 cm in diameter on semi-solid LB plates (Fig. 1C and 1D).

The distribution of each strain in the single droplet was summarized in Fig. 1F. From the center of the droplet, although the wild-type strain predominates, other strains were found. In contrast, all colonies were identified to be the wild-type from both middle and edge sites of the single droplet. These results, all

together, indicate that the monotrichous polar flagellum of the wild-type strain offers an overwhelming advantage in competition when motility matters.

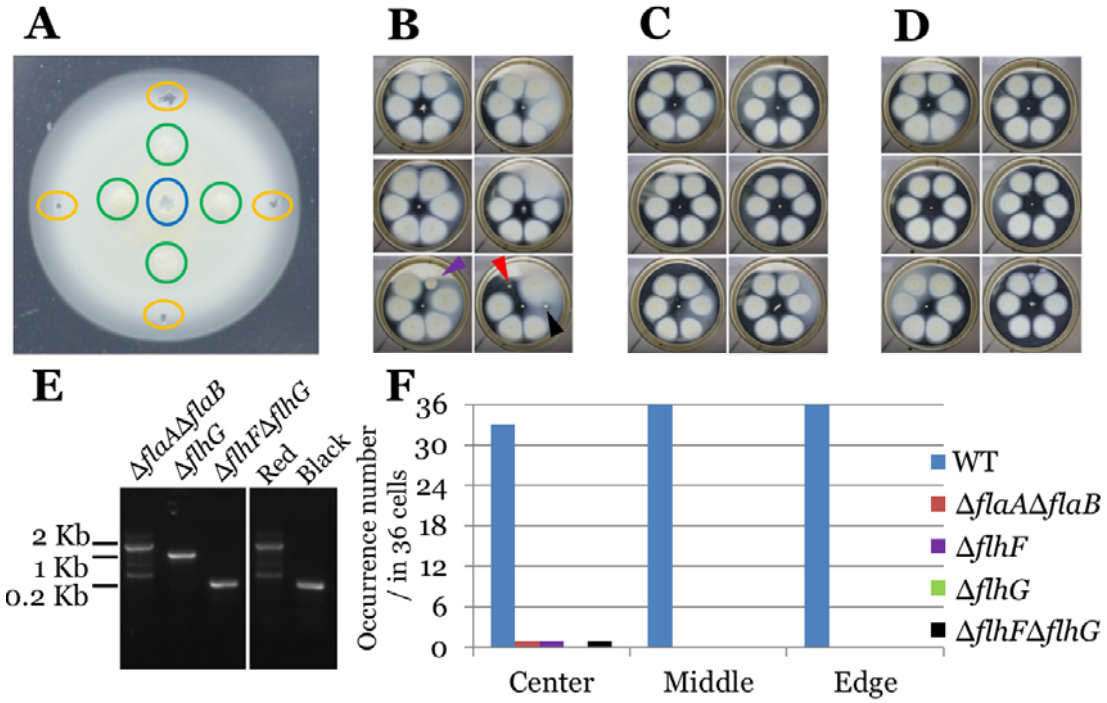

Fig. 1 Comparison of growth and mobility of the five strains of *S. oneidensis* on semi-solid plates. A: A single droplet from the evenly mixed cultures of five strains on LB plate with an agar concentration of 0.25% (w/v). Each strain distribution in the droplet was examined in center (blue), middle (green) and edge (yellow) for next step. The samples in middle and edge were repeated 4 times, respectively. **B-D**: Thirty-six colonies from center (**B**), middle (**C**) and edge (**D**) in the single droplet (Fig. 1A). Thirty-three colonies with over 2 cm in diameter were identified to be the wild-type strain (WT) and one colony (purple arrow) with 0.5 cm in diameter was identified to be the Δ*flhF* mutant strain according to the previous report (17). Two unidentified strain of colonies were indicated by red and black arrows. **E**: Strain identification of the two colonies (red and black arrows) by PCR. DNA markers are indicated on left. **F**: The distribution of each strain in the center, middle and edge of the single droplet. Each strain was presented by different colours.

The wild-type strain outcompetes others in pellicles formed in static liquid medium.

Fifty microliters evenly mixed cultures of five strains under test were inoculated into 2 mL LB liquid medium and incubated 18 h under static condition. To examine the distribution of each strain in the static liquid medium, the samples were collected in the pellicle and underneath of the medium, processed as mentioned above, and results were shown in Fig. 2A (pellicle) and Fig. 2B (underneath, free-living).

Thirty-four colonies from the pellicle were the wild-type strain based on over 2 cm in diameter on semi-solid LB plates and two colonies (purple arrow, over 0.5 cm in diameter) were the Δ*flhF* strain. In contrast, only 13 colonies from the underneath were the wild-type strain and 12 colonies were the Δ*flhF* strain (purple arrow in Fig. 2B). Furthermore, the remaining 11 colonies (colony number, 1-11 in Fig. 2B) were the Δ*flaA*Δ*flaB* strain, which were determined by PCR (Fig. 2C).

Based on these results, the distribution of each strain in the population of pellicle and free-living cells in static liquid medium was showed in Fig. 2D. In the pellicle, the wild-type strain was found to present 34 in total 36 colonies, and the Δ*flhF* mutant strain appeared 2 in total 36 colonies. However, strains of the wild-type, Δ*flaA*Δ*flaB* and Δ*flhF* shared approximately equal percentages in the underneath population. The results indicate that

monotrichous polar flagella of the wild-type strain exhibits much stronger ability than other mutant strains to move to surface of static liquid medium where O_2 is rich for *S. oneidensis* growth. Under the pellicle, the wild-type did not show the growth advantage comparing with Δ*flaA*Δ*flaB* and Δ*flhF*. Notably, in both cases, mutants with multiple flagella were not found, implicating that this flagellation pattern, either lophotrichous Δ*flhG* or peritrichous Δ*flhF*Δ*flhG*, substantially reduces fitness in static growing conditions.

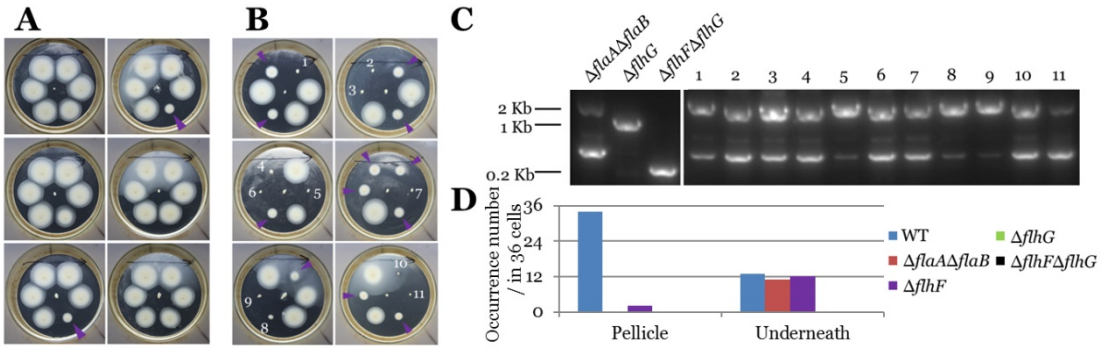

Fig. 2 Comparison of growth and mobility of the five strains of *S. oneidensis* in static liquid medium. Thirty-six colonies from the pellicle (**A**) and underneath (**B**) in static liquid medium. Thirty-four colonies in **A** and 13 colonies in **B** with over 2 cm in diameter were identified to be the strain of the wild-type (WT). The colonies (purple arrow) with over 0.5 cm in diameter were identified to be the Δ*flhF* mutant strain according to the previous report (17). Eleven colonies (number 1-11) were not identified strains. **E**: Strain identification of the eleven colonies by PCR. DNA markers are indicated on left. **F**: The distribution of each strain in the pellicle and underneath in static liquid medium. Each strain was presented by different colours.

Monotrichous flagellation confers *S. oneidensis* a fitness gain in shaking liquid culture.

One hundred microliters evenly mixed cultures of five strains

under test were inoculated into 10 ml LB liquid medium in a 250 mL flask and incubated to 0.7 of OD_{600} with shaking at 250 rpm. To examine the distribution of each strain in the shaking liquid medium, the samples were collected and processed as before.

As shown in Fig. 3A, while 9 colonies were the wild-type strain (over 2 cm in diameter on semi-solid LB plates), 13 colonies (over 0.5 cm in diameter) were Δ*flhF*. Besides, 7, 5, and 2 colonies were Δ*flhG*, Δ*flaA*Δ*flaB* and Δ*flhF*Δ*flhG* strains respectively, which were determined by PCR (Fig. 3B).

Based on the results, the distribution of each strain in the shaking liquid medium was summarized in Fig. 3C. Apparently, strains with monotrichous flagellation, either polar (the wild-type) or lateral (Δ*flhF*) had an advantage over others in growth of shaking cultures. However, compared to other conditions adopted in this study as discussed above, superiority of such flagellation pattern is relatively low, suggesting that the shaking minimizes the physiological impacts of flagella on *S. oneidensis* growth.

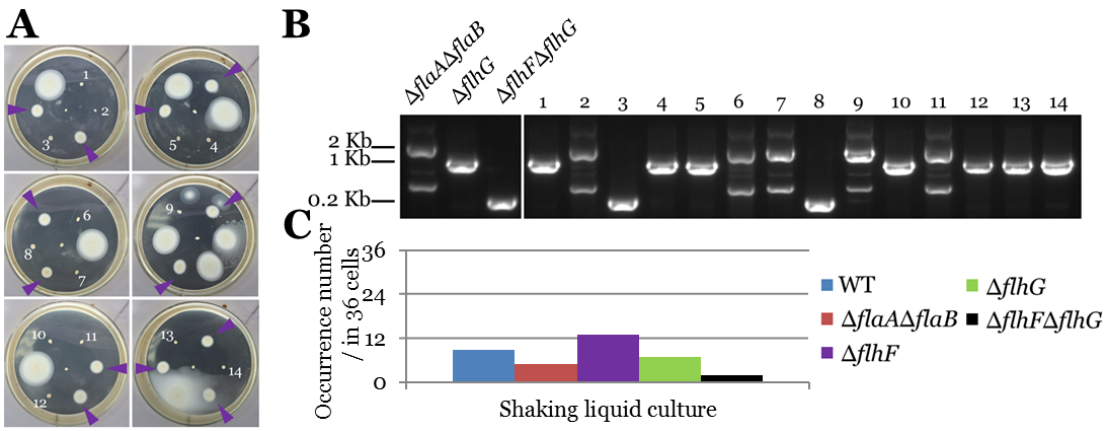

Fig. 3 Comparison of growth of the five strains of *S. oneidensis* in shaking liquid culture. A: Thirty-six colonies from the shaking liquid culture. Nine colonies with over 2 cm in diameter were identified to be the wild-type strain (WT). Thirteen colonies (purple arrow) with over 0.5 cm in diameter were identified to be the Δ*flhF* mutant strain according to the previous report (17). Fourteen colonies (number 1-14) were not identified strains. **B**: Strain identification of the 14 colonies by PCR. DNA markers are indicated on left. **F**: The distribution of each strain in shaking liquid culture. Each strain was presented by different colours.

Discussion

In nature, bacteria have evolved several types of flagellation pattern in respond to constant changes in environments. In the case of *S. oneidensis*, monotrichous polar flagellum is evolutionarily fixed, as in the wild-type. However, other types of flagellation pattern have become possible by using the genetic engineering approach. In this study, 4 mutant types of flagellation pattern, aflagellated (Δ*flaA*Δ*flaB*), monotrichous lateral (Δ*flhF*), lophotrichous (Δ*flhG*) and peritrichous (Δ*flhF*Δ*flhG*), of *S. oneidensis* were used to test their fitness in three different conditions. Based on the observed results, monotrichous polar flagellum (on the wild-type) enables *S. oneidensis* to grow

dominantly on semi-solid plates. As growth under this condition relies on motility, the data are in line with the motility characteristics of the strains under test: the wild-type is substantially superior to others in the feature. Similar results were obtained from pellicles in the static liquid environment. Oxygen on the surface of liquid is more abundant than that underneath, supporting fast growth for *S. oneidensis* (19, 20). To reach the surface, cells need effective locomotive ability. The finding that approximately 94% of the population of pellicle was the wild-type strain indicates that the polar flagellum is the best for moving away from the bottom, low-oxygen environments and reaching the surface, relatively favorable niches.

The wild-type (monotrichous polar), Δ*flhF* (monotrichous lateral) and Δ*flaA*Δ*flaB* (aflagellated) strains shared approximately equal percentages but strains of Δ*flhG* (lophotrichous) and Δ*flhF*Δ*flhG* (peritrichous) were not detected in the population under the pellicle, where oxygen is scarce. These data indicate that aflagellation and the monotrichous flagellation are better strategies than any other flagellar arrangements. Given that growth under pellicles is supported by respiration of non-oxygen electron acceptors, which is extremely low in efficacy. Thus, any extra energy cost such as multiple flagella could make a difference in

growth fitness.

In a vigorously agitated environment, the motility of bacteria is unlikely to be a significant factor for the growth. Indeed, strains with monotrichous flagellation (the wild-type and Δ*flhF*) still had an advantage over other strains under test. This observation implies that this flagellation pattern may somehow benefit *S. oneidensis* without costing considerable energy. The beneficial role of flagellum is supported by the finding that the aflagellated strain is inferior to strains of monotrichous flagellation. In contrast, cells with multiple flagella are less competitive because too much energy and resources were wasted on their synthesis. Notably, the peritrichous (the Δ*flhF*Δ*flhG* strain) is worst in fitness among the five strains, indicating that this flagellation pattern is least favored in all tested conditions in contrast to other types. Interestingly, Δ*flhF* showed modestly enhanced fitness over the wild-type. Although more work is needed, the fact that Δ*flhF* produces a flagellum shorter than the wild-type may offer an explanation (17). The shorter the flagellum is, the less the energy is used for its synthesis.

In summary, the data presented here illustrate intriguing and previously underappreciated impacts of flagellation patterns on ecophysiological fitness in bacteria. In nature, bacteria evolve best

strategies to survive and to proliferate in their respective niches. Apparently, multiple flagella, which are commonly found on bacteria associated with solid surfaces such as soil and intestinal tracts, are a burden to *S. oneidensis* cells. Instead, *S. oneidensis*, which lives in water bodies and sediments, adopts monotrichous polar flagellation. Such flagellation pattern is chosen by evolution because motility and energy cost are elegantly balanced.

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