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Spreading versus biomass production by colonies of the fish pathogen *Flavobacterium psychrophilum*: role of the nutrient concentration

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Summary. Colonies of the fish pathogen *Flavobacterium psychrophilum* have gliding motility in media with low agar concentrations. Although gliding motility, particularly in *Flavobacterium johnsoniae*, has been well-studied, little is known about its regulation by environmental factors. The work described here shows that the ability of *F. psychrophilum* to spread over surfaces depends on nutrient availability. In fact, as the nutrient contents of the medium decreased, spreading was favored and the diameter of the colonies increased. Macroscopy examination revealed modifications in colony morphology as nutrient depletion increased: from a dense and defined colony to the formation of microcolonies inside a general colony structure. Additionally, colony expansion dynamics and population density across the colony radius varied inversely with bacterial biomass production. Motility was an immediate response when bacteria were transferred from a rich to a more diluted medium. Our results suggest that, when nutrients are limiting, *F. psychrophilum* activates a specific growth mode that enables it to colonize surfaces by means of gliding motility. The use of diluted media allowed the differentiation, among previously isolated *F. psychrophilum* non-gliding mutants, of those completely unable to glide and those with only partially impaired gliding ability. [**Int Microbiol** 2009; 12(4):207-214]

Keywords: *Flavobacterium psychrophilum* · colony spreading · nutrient concentration · biomass production

Introduction

Bacteria have at least six different types of motility, some of which are linked to intricate communication networks that trigger a collective response to changes in environmental conditions. This behavior results in the organization of colonies that function essentially as multicellular organisms [17]. The evolutionary maintenance of motility despite its high energetic cost indicates its importance for the survival of bacteria in changing environments. Gliding motility is displayed by, among others, members of *Bacteroides* and *Myxobacteria* [26]. Although this bacterial translocation system is still poorly defined, early studies on *Cytophaga* spp. and *Flavobacterium johnsoniae* (formerly *Cytophaga johnsonae*) established several of its basic characteristics, such as the effect of environmental conditions [29], speed, and direction [21]; the relation between motility and the presence of high-molecular-weight polysaccharides on the cell surface [11,12]; and the general effect of nutrients on motility [12, 34]. Recently, cell surface appendages probably involved in this type of motility have been identified [23]. In addition, non-spreading mutants have been isolated and characterized [4] and a set of 11 non-gliding mutants of *F. johnsoniae* has been genetically engineered [18,26,27]. Analyses of both the genes interrupted in these non-spreading mutants (*gld* genes)

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and their respective products have resulted in a proposed model of translocation by gliding [18,26]. Most of the *gld* genes are also found in *F. psychrophilum* [7], a fastidious bacterium responsible for cold-water disease in salmonids [2,31]. Disease outbreaks occur at temperatures below 14°C and cause important economic losses to fish farmers. However, knowledge of the mechanisms involved in the infection process and in the survival of *F. psychrophilum* outside the fish host is quite limited.

Bacteria such as *F. psychrophilum* that are able to alternate between a saprophytic and a free-living state must have systems allowing them to adapt quickly to these extremely different conditions; particularly the change from limited nutrient availability in the free-living state to the nutritionally rich environment encountered during infection and disease development [10]. Despite these adaptations, motility and chemotactic responses are essential for survival and must therefore be retained. However, as motility is an energy-dependent process, most bacteria do not move in media with low nutrient contents [8,15,36]. The exceptions are species such as *Vibrio parahaemolyticus* [28], which are able to move in nutrient-depleted media; myxobacteria, during fruiting body formation in response to nutrient exhaustion [35]; and *Bacillus subtilis* and *Bacillus cereus*, in which motility is induced under minimal nutrient conditions [6,9,16]. In some fish pathogenic bacteria, such as *Vibrio (Listonella*) *anguillarum* [19,20] and *Moritella viscosa* [37], motility is also influenced by the specific type of nutrients, as well as by temperature and salinity. Additionally, motility is involved in the infectivity of *V. anguillarum* [32], *Vibrio fischeri* [13], and *Vibrio vulnificus* [22].

The factors involved in gliding by *Flavobacterium* have not been studied in detail [2,3,27], and little is known about the requirements and mechanisms enabling bacterial adaptation to different environmental conditions. The aim of this study was to determine the influence of nutrient availability on the gliding behavior of *F. psychrophilum* and to characterize the changes in colony morphology in response to nutrient supply. Based on these data, we propose a culture medium more suitable for research on gliding motility in *F. psychrophilum*.

Materials and methods

Bacterial strains and culture conditions. The parental strain *F. psychrophilum* THC02-90 [5] and the mutants FP523 [1] and FP26 (B. Álvarez, PhD thesis, 2006) were used throughout the study and maintained in nutrient broth (NB) (Pronadisa, Spain) or nutrient agar (NA), to which 15 g Bacto-Agar (Difco)/l was added. The strains were grown in NB in a rotary incubator at 250 rpm and 20°C. To study the effect of nutrient availability, the following dilutions of NB, with 7.5 g Bacto-Agar/l, were used: 1 (concentration recommended by manufacturer), 1/2, 1/4, 1/6, and 1/8, referred to as 1NA, 1/2NA, 1/4NA, 1/6NA, and 1/8NA, respectively. Solid Enriched Anacker Ordal Serum (EAOS) [30] was also tested. Prior to inoculation, plates containing the various media were dried for 15 min at room temperature in a biological safety cabinet. In the standard inoculation, 8 µl of a freshly prepared overnight liquid culture grown in NB at 20°C up to an OD_{525} of 1.0–1.2 (corresponding to the mid-exponential growth phase) was spotted onto the center of a plate. The plates were incubated at 20°C and spreading was evaluated during a period of 120 h by measuring the colony diameter (in cm) every 24 h. Images of the colonies were acquired with a Nikon coolpix990 camera. A maximum of 120 h of incubation was established since longer incubations were likely to lead to further expansion of cells at the edge of the colonies such that it would have been difficult to evaluate colony morphology.

Measurement of bacterial growth and population density. Since a proportion of *F. psychrophilum* cultures consists of viable non-cultivable cells [30], the number of cells present in liquid culture would not have correlated to the corresponding bacterial count following serial dilution on plates. Thus, instead, bacterial growth was measured by determining the relative biomass. Each colony was washed off the agar surface with 5 ml of distilled water, and 1 ml of this cell suspension was used to record the OD_{525} using a Kontron Instruments Unikon 930 spectrophotometer. The relative population density across the radius of a colony was determined by using a hollow plastic ring to remove agar plugs of 5 mm diameter every 5 mm from the center to the edge of the colony. The relative amount of cells on each plug was determined by the addition of 1 ml of distilled water and, after slight agitation with a Vortex mixer and 1 min of sedimentation, the OD_{525} of the resulting cell suspension was determined. Agar plugs taken outside the colony were processed simultaneously and used as blanks.

Influence of environmental factors on colony spreading. The influence of the inoculum origin, with regard to the nutrient concentration of the growth medium, on colony spreading was analyzed as follows: THC02-90 cells were grown overnight at 20°C in NB or 1/6NB. Eight µl of a cell suspension with $OD_{525} = 0.5$ were spotted onto the center of plates containing 1NA or 1/6NA followed by incubation at 20°C. Colony images were taken 24 h later. To determine the relationship between *F. psycrophilum* motility and nutrient concentration, a set of serial dilutions of NA containing 0.75% agar was prepared.

To evaluate colony spreading in previously isolated Tn*4351* mutants, shown to be non-gliding when growing on 1/2 NA [1], inocula of each mutant were prepared as described above, and 8-µl aliquots of each were spotted onto 1/2 NA and 1/6 NA. After 120 h of incubation at 20°C, colony spreading was examined visually.

Confocal laser scanning microscopy of gliding colonies. Thin layers of the cultures were made by spotting aliquots of overnight THC02-90 mid-exponential-phase cultures onto 5 ml of 1/2NA and 1/6NA medium previously placed on glass microscope slides. The cultures were incubated for 120 h at 20°C in a humid chamber. The resulting colonies were then stained with SYTO 9 (Molecular Probes, Eugene, Oregon, USA) and visualized with a Leica TCS-SP2-AOBS confocal laser scanning microscope at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistical analyses. Three independent experiments were carried out and the means and standard deviation (SD) were determined. Measurement of bacterial growth and population densities were assessed for statistical significance. Differences between diameters and population density of the colonies at different NA dilutions were examined by ANOVA. Differences between relative population densities throughout the colony were assessed using a two-tailed Student's test. In both cases, $p < 0.05$ was considered significant.

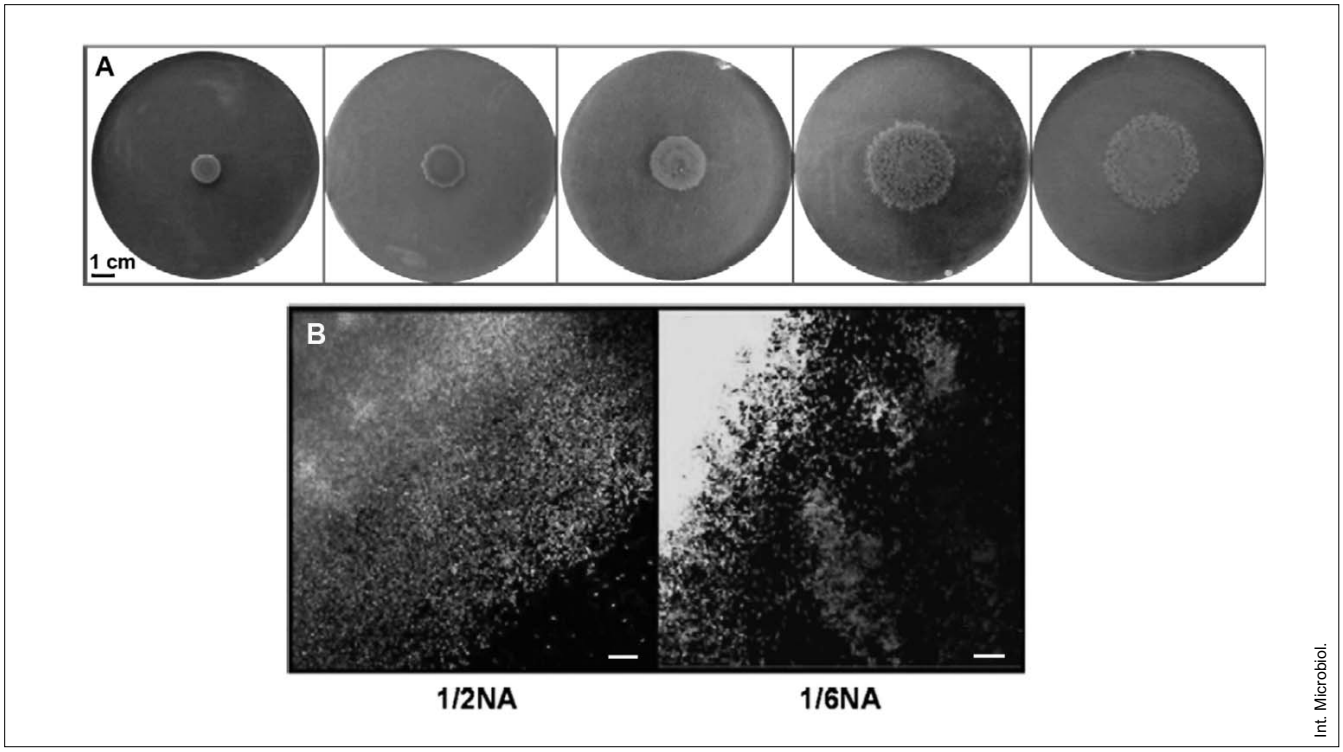

Fig. 1. Effect of nutrient concentration on surface colonization by *Flavobacterium psychrophilum* THC02-90. (**A**) Diameter and morphology of colonies on serially diluted NA. (**B**) Confocal laser microscopy detail of the bacterial cell distribution at the periphery of the colonies in 1/2NA and 1/6NA. Bar = 10 μm.

Results

Nutrient depletion and gliding behavior in *Flavobacterium psychrophilum*. Colonies of the *F. psychrophilum* parental strain THC02-90 displayed a more obvious gliding phenotype as the nutrient concentration of the medium decreased (Fig. 1A). In fact, the more diluted the medium, the greater the diameter of the colony. For example, after 120 h of incubation at 20°C, the colony size on 1/8 NA was twice that on 1NA (Table 1). These results were not exclusive for NA; they were also obtained when dilutions of other complex media, such as EAOS, were used (data not shown).

Amongst all assayed media, bacterial growth, as measured by optical density (Table 2), was maximum on 1NA and decreased proportionally as more diluted media were used. However, the relative determination of cell numbers in samples collected at several distances from the colony center (0.5, 1.0, and 1.5 cm) after 72, 96, and 120 h of incubation on 1/2 and 1/6NA showed that, under any nutrient condition,

Table 1. Effect of the nutrient concentration on the colony expansion (in cm) over time

Time (h)	1NA	1/2NA	1/4NA	1/6NA	1/8NA
24	1.10 ± 0.00	1.35 ± 0.07	1.30 ± 0.14	1.43 ± 0.04	1.55 ± 0.07
48	1.25 ± 0.07	1.65 ± 0.21	1.85 ± 0.21	1.90 ± 0.00	2.05 ± 0.21
72	1.29 ± 0.28	1.91 ± 0.33	1.93 ± 0.14	2.25 ± 0.34	2.50 ± 0.40
96	1.43 ± 0.13	2.35 ± 0.13	2.40 ± 0.14	2.83 ± 0.12	2.85 ± 0.07
120	1.47 ± 0.32	2.57 ± 0.15	2.60 ± 0.10	2.95 ± 0.58	3.15 ± 0.35

Numbers are the mean ± SD of three independent experiments ($P < 0.05$).

Table 2. Effect of the nutrient concentration on the relative colony biomass production

	OD_{525}				
Time (h)	1NA	1/2NA	1/4NA	1/6NA	1/8NA
24	0.36 ± 0.01	0.31 ± 0.01	0.19 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
48	0.77 ± 0.04	0.57 ± 0.13	0.44 ± 0.03	0.27 ± 0.02	0.24 ± 0.03
72	1.22 ± 0.05	1.08 ± 0.04	0.51 ± 0.13	0.39 ± 0.01	0.37 ± 0.09
96	1.47 ± 0.08	1.37 ± 0.06	1.08 ± 0.01	0.52 ± 0.01	0.38 ± 0.03
120	1.67 ± 0.06	1.57 ± 0.10	1.11 ± 0.14	0.66 ± 0.03	0.41 ± 0.01

Numbers are the mean ± SD of three independent experiments ($P < 0.05$).

population density decreased gradually from the center to the edge of the colony (Table 3). In addition, population densities across the colony radius decreased when nutrient concentrations were low. Together with the growth values, this result suggested that, at high nutrient concentrations, the bacteria preferentially grew, whereas with decreasing nutrient concentrations there was a proportional increase in colony diameter and a decrease in population density. Note that *F. psychrophilum* was able to divide under all the nutritional conditions tested.

Changes in colony morphology in relation to gliding. As nutrients became increasingly depleted, the morphology of the colony changed, as did the organization of the cells within the developing colony (Fig. 1A). A dense and compact colony developed in the presence of 1NA to 1/4NA dilutions, whereas at lower nutrient availability (1/6NA and 1/8NA) microcolonies separated from each other within the general colony structure (Fig. 1A). Analysis of colonies by confocal microscopy showed that, when grown on 1/2NA, cells moved away from the edge of the colony, forming a regular-shaped area with a population density lower than that at the colony edge. On 1/6NA plates, groups of many cells were found far removed from the colony edge (Fig. 1B). Based on the above-described observations, these cells at a distance were expected to begin to divide.

Nutrient concentration during growth of the inoculum and gliding behavior. Cells of *F. psychrophilum* grown overnight on NB or 1/6NB were used as inocula for plates containing 1NA or 1/6NA. Cells grown on NA after transfer from 1/6NB were observed to enter a lag phase compared to cells from NB cultures (Fig. 2A,B). By contrast, spreading on 1/6NA was the same and independent of the origin of the inoculum (NB or 1/6NB) (Fig. 2C,D). Thus, the shift from low to high nutrient conditions resulted in delayed growth, whereas gliding was not influenced by prior nutrient conditions.

Selection of non-gliding and partially gliding mutants. The ability of previously isolated *F. psychrophilum* transposon Tn*4351* mutants—which do not glide on

Table 3. Effect of the nutrient concentration on the relative colony density over time

	Distance from colony center (cm)					
	1/2NA			1/6NA		
Time (h)	0.5	1	1.5	0.5	1	1.5
72	0.73 ± 0.02	0.41 ± 0.08	0.37 ± 0.01	0.27 ± 0.03	0.22 ± 0.03	0.11 ± 0.05
96	0.75 ± 0.02	0.54 ± 0.07	0.27 ± 0.03	0.27 ± 0.03	0.25 ± 0.03	0.17 ± 0.01
120	0.68 ± 0.03	0.55 ± 0.09	0.30 ± 0.09	0.29 ± 0.01	0.24 ± 0.04	0.19 ± 0.01

Numbers are the mean ± SD of three independent experiments ($P < 0.05$).

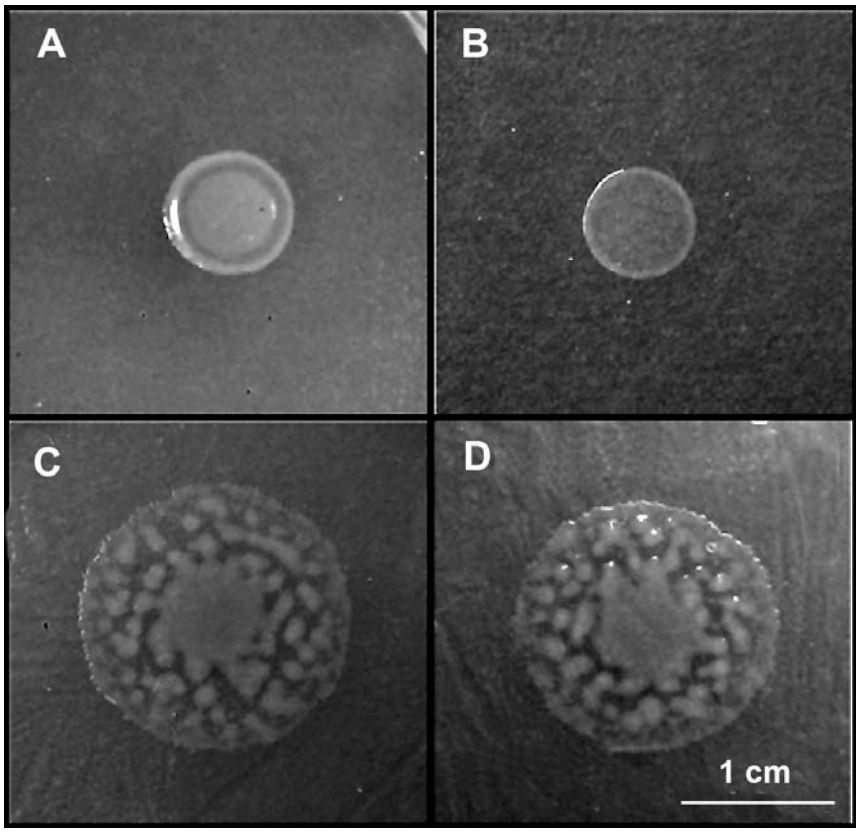

Fig. 2. Effect of the origin of the inoculum on *Flavobacterium psychrophilum* THC02-90 colony spreading. Growth and spreading of the colonies were observed on 1NA (upper panels) and 1/6NA (lower panels). **A** and **C** correspond to plates inoculated from cultures grown in NB, and **B** and **D** to inocula from 1/6NB.

NA or 1/2NA [1]—to recover at least part of their gliding behavior when grown on more diluted media was examined. Mutants FP523 [1] and FP26 (B. Álvarez, PhD thesis, 2006) were simultaneously grown on 1/2NA and 1/6NA. The behavior of mutant FP523, selected previously on 1/2NA as non-gliding, was the same when grown on 1/6NA (Fig. 3E,F). Conversely, mutant FP26 was able to glide on 1/6NA (Fig. 3D) and its colony morphology differed from that of the parental strain (Fig. 3B). Genetic analysis of FP26 showed that, in addition to the genomic Tn*4351* transposon insertion, it had another, non-Tn*4351*-dependent mutation. Therefore, it was not possible to identify the gene involved in the partial recovery of motility.

Discussion

Previous studies on gliding motility in *Flavobacterium* focused on characteristics such as speed and direction, [21] as well as on the mechanisms involved in movement [18,27]. Nevertheless, the patterns governing colony gliding are also of interest as their identification yields insight into the biology of this pathogen and, more specifically, its virulence. Here, nutrient depletion was shown to have profound effects on the spreading of *F. psychrophilum* colonies. Indeed, the inverse relation between nutrient concentration and colony spreading indicated that gliding is a thoroughly regulated process in which a decrease in the availability of nutrients seems to trigger a proportional gliding response in *F. psychrophilum* cells.

Early studies in *Cytophaga johnsonae* (presently, *Flavobacterium johnsoniae*) also indicate that abundant nutrients inhibit cell gliding [12]. However, in most cases, bacterial translocation mechanisms are inhibited under the opposite, i.e., limited, nutrient conditions because of their high energetic cost [14]. For instance, swarming and twitching, two forms of motility widely distributed among gram-negative bacteria, are inhibited at low nutrient concentrations [8,15, 24,36]. By contrast, the concentration of cells during fruiting body development, a social motility system in myxobacteria, is induced by starvation [35], and in *Sinorhizobium meliloti*, higher nutrient concentrations give rise to swarm colonies with smaller diameters [38]. Recently, a similar result has been described in *B. cereus*, whose translocation was found to be more efficient under low-nutrient conditions [16]. In the case of *F. psychrophilum*, it seems that, when the concentration of nutrients is low, most of the bacterium's energy is directed towards colony expansion rather than growth, at

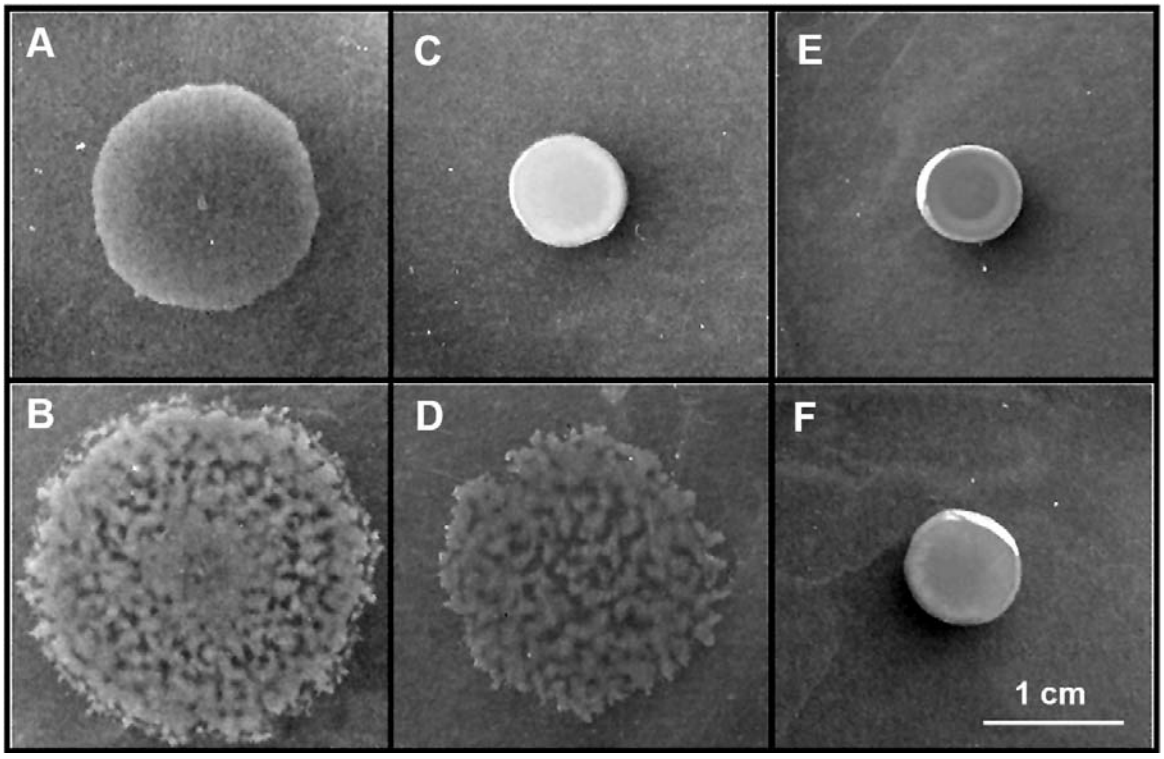

Fig. 3. Comparison between motility of *Flavobacterium psychrophilum* THC02-90 and previously selected non-gliding strains on 1/2 and 1/6 NA. THC02-90 and non-gliding mutant strains FP26 and FP523, previously selected on 1/2NA [1] were grown on plates containing 1/2NA (**A,C,E**) and 1/6NA (**B,D,F**).

least in the initial stage of development. These results suggest that spreading is a strategy to optimize biomass production in nutrient-poor environments, involving a first stage in which cells move and disperse and a second stage in which they start to divide.

Bacterial growth on solid nutrient media is controlled by the amount and the diffusion of nutrients. Therefore, on low nutrient plates, a nutrient gradient due to more rapid nutrient utilization could initiate the directional spreading of colonies towards higher nutrient conditions. Accordingly, it could be speculated that the goal of gliding behavior is to enhance the survival of bacteria in nutrient-stressed environments by facilitating the search for better nutrient conditions and/or maximizing nutrient utilization. Note that this response is fast and does not require an adaptation phase following the transfer of cells from a nutrient-rich to a nutrient-poor medium.

Lower concentrations of nutrients correlated with differences in colony morphology, as seen both macroscopically and microscopically. The following sequence of events can therefore be proposed. At high nutrient concentrations the cells would divide and the colony would grows predominantly as a dense structure. As nutrient concentrations were to decrease, the cells would dedicate higher amounts of energy to colony expansion, thus moving away from the edge of the colony, and then beginning to divide. When nutrients are very limited, the result is the appearance of numerous microcolonies within the major colony structure.

Changes of colony morphology in relation to nutrient availability have also been observed in other bacterial species. For example, *B. cereus* has a specific colony dendrite pattern that has been shown to be nutrient-dependent [16]; the colony structure of *Mycobacterium smegmatis* changes depending on media composition [25]; in *Proteus mirabilis*, nutrient depletion is related to colony morphology [33]; and *B. subtilis* develops a tendril-like growth structure depending on the nutrient composition of the medium [9].

These examples raise the question of the role of gliding in *F. psychrophilum*. Gliding might not be necessary during the infection process, but rather for surface colonization and nutrient access. Thus, nutrient availability in an eroded epithelium, within a wound, or inside the host fish should be high, and under these conditions the bacteria would be expected to grow but not glide. Instead, gliding is used when nutrient supplies are limiting, which forces the bacteria to move in order to seek better nutrient conditions. Gliding would therefore be a more appropriate mechanism during the

free-living state, when nutrients are usually scarcer than inside the host. Nevertheless, the ability to spread over surfaces may confer an evolutionary advantage to the bacterium in particular environments. The development of mutants in genes related to the regulation of the gliding response will further our knowledge about the role of this system in *F. psychrophilum*, both in its free-living state and during its interaction with the host fish. To date, however, attempts to obtain *F. psychrophilum* Tn*4351* insertional mutants related to spreading regulation have been unsuccessful. Based on our results, however, medium 1/6NA might allow the selection of non-gliding mutants. Moreover, a comparison of bacterial motility on 1/2NA vs. 1/6NA could be a useful strategy for selecting mutants with partial or limited gliding ability and for further studies on gliding behavior. Additionally, the ability of a particular nutrient to induce gliding could be assessed in chemotactic response experiments.

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