

ANTIGENIC AND FUNCTIONAL DIFFERENCES BETWEEN THE POLAR
AND LATERAL FLAGELLA OF AZOSPIRILLUM BRASILENSE

by

Patrick G. Hall

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APPROVED:

N. R. Krieg, Chairman

James M. Conroy

John L. Neal

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(ABSTRACT)

Cells of the genus Azospirillum possess a single polar flagellum when grown in broth; when grown on solid media they also possess numerous lateral flagella of shorter wavelength (0.7 μm vs. 1.2 μm) and thinner diameter (13.5 nm vs. 18 nm). The antigenic and functional differences between these two types of flagella were studied for A. brasilense ATCC 29145 (type strain). An indirect immunoperoxidase stain was used in conjunction with electron microscopy to demonstrate that an antigenic difference that exists between these two types of flagella in this strain. No evidence could be obtained that the polar flagellum was ensheathed as it is in members of the genus Vibrio.

A. brasilense was found to swarm on nutrient broth medium solidified with 0.75% agar ("swarm medium" or SM), and also on medium solidified with carrageenan. Swarming also occurred on a dialysis membrane overlying SM, indicating that the nature of the solid surface may not be important. The effects of various physical and chemical factors on swarming were determined. A temperature of 30°C rather than the optimal growth temperature of 37°C

was stimulatory to swarming. Certain chemical agents, *p*-nitrophenylglycerol, EDTA, sodium deoxycholate, sodium taurocholate, and Na_2SO_4 , at appropriate concentrations could inhibit swarming without preventing growth. Based on the use of mutants lacking either the polar flagellum or the lateral flagella, the lateral flagella were found to be responsible for swarming, whereas the polar flagellum was responsible for free-swimming motility.

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LIST OF ABBREVIATIONS USED

- ALF - Antibodies against lateral flagella
- APF - Antibodies against polar flagella
- APLF - Antibodies against both polar and lateral flagella
- ATCC - American Type Culture Collection, Rockville, Maryland
- EDTA - Ethylenediamine tetraacetic acid
- FPBS - Formalinized phosphate-buffered saline
- IgG - Immunoglobulin, class G
- i.m. - intramuscularly
- JEOL - Japan Electron Optics Laboratory Co., Ltd., Tokyo
- LF - Lateral flagella
- Mol% G + C - Moles percent guanine + cytosine
- MPSS - Modified peptone-succinate-salts
- PBS - Phosphate-buffered saline
- PF - Polar flagella
- SM - Swarm medium
- Tris - Tris(hydroxymethyl)aminomethane buffer

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INTRODUCTION

The genus Azospirillum consists of motile, plump, slightly curved to straight rods having a diameter of 1.0 μm and ranging in length from 2.1 to 3.8 μm . The organisms fix nitrogen under microaerobic conditions. They occur free-living in soil or as nitrogen-fixing symbionts in the roots of a great variety of plants.

When grown in broth these bacteria possess a single polar flagellum; however, when cultured on solid media they also form numerous lateral flagella of smaller diameter and shorter wavelength than that of the polar flagellum. This flagellar arrangement (polar + lateral) is known to occur in only a few other unrelated genera. In some of these genera the lateral flagella have been shown to be antigenically distinct from the polar flagella; moreover, the ability of some of the genera to swarm on agar media (i.e., to spread across the agar surface as a thin film) has been correlated with the presence of the lateral flagella.

Because the antigenic and functional properties of the flagella of Azospirillum brasilense are not known, the objectives of this research are to determine: (i) whether the polar and lateral flagella of the type strain are antigenically distinct; (ii) whether the type strain can swarm on appropriate solid media; (iii) whether the lateral flagella are responsible for such swarming, by obtaining and testing mutants lacking one or the other type of flagella; and (iv) whether various chemical and physical factors can influence the ability of the organisms to swarm.

REVIEW OF THE LITERATURE

Taxonomy and General Properties of Azospirilla. The genus Azospirillum presently consists of two species, A. lipoferum and A. brasilense (25). A review of the biological properties of the genus has been given recently by Krieg and Döbereiner (11). The organisms are aerobes capable of growing on salts of certain organic acids: malate, succinate, lactate, or pyruvate. Fructose and other monosaccharides, but no disaccharides, may also serve as carbon and energy sources. The organisms are able to fix dinitrogen under microaerobic conditions in association with the roots of various plants, and this is perhaps the major physiological feature of the genus. Table 1 lists some of the general properties of the genus Azospirillum, and Table 2 indicates those features that are used to differentiate the two species of the genus.

Distribution of Azospirillum spp. is worldwide and as many as 10^7 cells per g of rhizosphere soil have been reported. Their occurrence may be more frequent in tropical rather than temperate regions (6), but little difference has been reported by some researchers (26). The organisms are of potential value to agriculture since they are associated with the roots of a variety of C3 and C4 plants (6). They have been found within the mucigel layer, where they become firmly attached to root hairs (27). The bacteria occur not only on the surface of roots but also in the outer cortex, inner cortex, and in the stele of infected plants (27). Infection starts in root branches and spreads longitudinally into main roots. Azospirilla are usually located within the interstitial spaces of

TABLE 1. Properties of the genus Azospirillum^a

Property	Descriptive information
Cell shape and size	Plump vibrioid to straight rods, ca. 1 μm in diameter.
Intracellular granules	Poly- β -hydroxybutyrate.
Flagella	When grown in broth, cells have a single polar flagellum. When grown on solid media, cells also form numerous lateral flagella that are thinner and that have a shorter wavelength.
Optimum growth temperature	35-37°C.
Colony morphology on potato agar	Light or dark pink in color, nonslimy and often wrinkled.
Metabolism	Aerobic. Respiratory; able to use O_2 or NO_3^- as the terminal electron acceptor. Some strains also have a weakly fermentative metabolism.
Nitrogen fixation	N_2 fixed only under microaerobic conditions.
Dissimilatory NO_3^- reduction	NO_3^- reduced to NO_2^- or to N_2O and N_2 when oxygen levels are low.
Oxidase reaction	Positive.
Carbon and energy sources	Salts of organic acids, especially malate, succinate, lactate or pyruvate. Fructose and a few other monosaccharides can be used, but disaccharides are not used. Some strains can grow on methane. Some strains can grow autotrophically on H_2 .
Habitat	Free-living in soil or associated with the roots grasses, cereal grains and tuber plants as symbiotic N_2 -fixers.
Mol% G + C of DNA	69-71.

^aData from Tarrand et al. (25) and from Malik and Schlegel (16).

TABLE 2. Characteristics differentiating the two species of the genus
Azospirillum^a

Characteristic	<u>A. lipoferum</u>	<u>A. brasilense</u>
Hydrogen autotrophy	+	-
Biotin required for growth	+	-
Glucose or alpha-ketoglutarate used as carbon sources for N ₂ fixation	+	-
Glucose media acidified	+	-
Acid produced from glucose or fructose under anaerobic conditions	+	-
In N ₂ -deficient semisolid malate medium, large nonmotile pleomorphic cells occur in 24-48 h	+	-

^aData from Tarrand et al. (25) and from Malik and Schlegel (16).

the middle lamella of root tissues. They have also been found intracellularly, but entry is probably gained only when dead plant cell walls autolyze. How the bacteria penetrate the root along the interstitial spaces is not known.

Morphology of Azospirilla. Azospirilla are straight to slightly curved, plump rods, often with pointed ends, ranging from 2.1 to 3.8 μm in length and ca. 1 μm in diameter (25). Large pleomorphic forms may occur under alkaline conditions in old cultures or at high oxygen tensions (14, 18). These "cystlike" forms may function in survival since they are resistant to desiccation. The cells are Gram-variable and intracellular poly- β -hydroxybutyrate granules are usually present, being most abundant under nitrogen-fixing conditions (25). A polar membrane located beneath the cytoplasmic membrane in the region of the polar flagellum has been found; this type of membrane is characteristic of helical organisms such as aquaspirilla, campylobacters, oceanospirilla, and phototrophic spirilla (11).

A single polar flagellum is produced when cells are grown in broth. However, when the cells are grown on solid media numerous lateral flagella of shorter wavelength are also produced (25). This "mixed flagellation" is relatively rare among bacteria and has been demonstrated in only five other genera. Another odd occurrence is the formation of a spiral structure surrounding the base of the polar flagellar filament; this structure occurs only in A. lipoferum (17).

Other Bacterial Genera Having "Mixed Flagellation". Vibrio is the most extensively investigated genus with regard to mixed flagellation. Most species of Vibrio have a marine habitat and require Na^+ for growth, and even those

that do not require this cation (e.g., V. cholerae) are greatly stimulated by its presence (19). When grown on solid NaCl-containing media many Vibrio species produce unsheathed lateral flagella in addition to a sheathed polar flagellum. The lateral flagella have a diameter of 14-15 nm and a wavelength of 0.9 μm . Some strains of Vibrio have >100 lateral flagella per cell. The polar flagellum has a wavelength of 1.4-1.8 μm and a diameter of 24-30 nm. It is composed of a core 14-16 nm thick surrounded by a sheath that is continuous with the outer wall membrane (30).

The flagellins of the polar and lateral flagella are antigenically distinct. Shinoda et al. (22) separately isolated and purified the flagellins of the polar and lateral flagella of V. parahaemolyticus by hydroxylapatite column chromatography and raised antibodies against each. Ouchterlony gel diffusion tests of the purified flagellins vs. specific antisera showed that antigenic differences existed between the flagellins. Moreover, by using antibodies conjugated with ferritin, these authors demonstrated that anti-polar-flagellin and anti-lateral-flagellin specifically stained the polar and lateral flagella, respectively.

Many marine strains of Vibrio are able to swarm on solid media (1). Various chemical factors have been shown to influence this swarming and also the flagellation of V. alginolyticus (4). On agar media containing >4.0% (w/v) NaCl, an inverse relationship between the number of lateral flagella and the NaCl concentration was found. On both agar media and in broth containing 6-9% (w/v) NaCl, cells with tufts of 2-4 sheathed and unsheathed polar flagella were seen. The use of 7.3-9.8% Na_2SO_4 in agar media drastically reduced the

numbers of lateral flagella. Ethylenediamine tetraacetic acid (EDTA) did not affect flagellar arrangement. Lateral flagella were produced in liquid media containing 0.1-0.3% boric acid or 0.05-0.10% aluminum hydroxide.

Among the surface-active agents tested with Vibrio spp., Tween 80 (polysorbitan monooleate) and the bile salt sodium taurocholate did not affect flagellation. Bile salts and sodium deoxycholate reduced the number of polar and lateral flagella. Sodium dodecyl sulfate (sodium lauryl sulfate) and Teepol reduced the number of lateral flagella but increased the number of polar flagella; Teepol (a mixture of the sodium salts of various fatty alcohols) also caused deterioration of the sheaths of the polar flagella.

In all cases where chemical factors reduced the formation of lateral flagella on Vibrio cells, there was also a decrease in the ability of the organisms to swarm. This suggested that swarming might be a function of the lateral flagella, as later proposed by Shinoda and Okamoto (21) based on data obtained using mutants lacking one or the other flagellar type. They mutagenized cells of V. parahaemolyticus with nitrosoguanidine and plated them on either 0.3% or 0.7% agar media containing NaCl. Motile cells spread out from the point of inoculation, whereas nonmotile cells did not. Cells remaining at the point of inoculation were transferred to fresh agar plates. By repeating this procedure, nonmotile mutants became concentrated at the center of the plates. The concentrated cells were streaked onto NaCl-supplemented nutrient agar. The colonies that formed were transferred to plates containing either 0.3% or 0.7% agar and nonmotile strains were selected. By using these mutants, which lacked one or the other flagellar type, the authors concluded

that the lateral flagella were locomotor organelles on solid media and that the polar flagella functioned as locomotor organelles only in broth.

The effects of some physical factors on flagellation and swarming of V. alginolyticus have also been investigated (5). Cells from static or mildly agitated broth cultures exhibited no difference in flagellation and culture age had no effect on flagellar arrangement. When cells cultured on agar media were incubated at a temperature of 17 to 37°C many lateral flagella and occasionally polar flagella were observed; cells also swarmed within this temperature range. At 40 and 42°C the number of lateral flagella was greatly reduced and swarming did not occur.

As the agar concentration of the medium was decreased, fewer lateral and more polar flagella were observed. Furthermore, when membrane filters were placed on the surface of agar- or gelatin-solidified media or on broth media, the cells produced lateral flagella. It was concluded by the authors that "a certain degree of rigidity of the substratum is a condition for the development of lateral flagella". However, they also concluded that unknown factors besides the lateral flagella are required for swarming.

Members of the genus Chromobacterium possess a single polar flagellum and usually one to four lateral flagella of a shorter wavelength than that of the polar flagellum. Sneath (23) stated that antigenic differences existed between the two types of flagella but no specific references regarding the data for this conclusion are available. One species, C. fluviatile, may swarm since its colonies have been described as "flat and spreading".

Bacteria of the genus Pseudomonas typically form a single polar flagellum; in only one species, P. stizolobii, this has been shown to be sheathed (9). Some species -- P. stutzeri and P. mendocina -- also produce lateral flagella of short wavelength which are more easily shed from the cells than is the polar flagellum (7). The formation of the lateral flagella is favored by growth on solid media. P. testosteroni forms both polar and lateral flagella when cultured at low temperatures (3). There are no reports of swarming in the genus Pseudomonas.

In the genus Aeromonas most species are motile by a single polar flagellum having a wavelength of 1.7 μm . Lateral flagella of shorter wavelength have been reported to occur on young cells growing on solid media (8), but it is not known whether aeromonads can swarm or if the two types of flagella are antigenically distinct.

The genus Janthinobacterium consists of one species, J. lividum, which is motile by a single polar flagellum and one to four lateral flagella (24). No reports are available to indicate whether the organism swarms or what the antigenic relationship is between the two flagellar types.

Swarming Behavior of Bacteria. Swarming has been defined by Henrichsen (10) as "a kind of surface translocation produced through the action of flagella but different from swimming. The micromorphological pattern is highly organized in whirls and bands. The movement is continuous and regularly follows the long axis of the cells which are predominantly aggregated in bundles during the movement." Two members of the genus Proteus, P. mirabilis

and P. vulgaris, are regarded as the classic examples of swarming bacteria. Therefore, this discussion will concentrate on swarming by Proteus spp.

Swarming of Proteus is typically seen as alternating periods of migration and concentration of cells. This appears as concentric rings radiating from the point of inoculation. In a review of the swarming phenomenon in Proteus, Williams and Schwarzhoff (29) divided it into three events: the development of swarm cells, migration of these cells across the surface of the agar medium, and finally the division of the cells into shorter forms.

Swarm cells are 0.7 μm wide and 20-80 μm long. They are more heavily flagellated than resting cells which are 0.8 μm in diameter and 2-4 μm in length (29). Although the formation of these cells is not fully understood, Kvittingen (12, 13) concluded that they were a stage in the normal life cycle of Proteus.

Lominski and Lendrum (15) indicated that toxic metabolites accumulating in the agar medium acted as negative chemotactic stimulants to swimmers as well as influencing their development. However, Williams et al. (29) concluded that swarming is a nonchemotactic event and may simply be the result of the ability of the organisms to move across the surface of a solid medium.

Nothing is known about the process of consolidation, but it is thought that since swarm cell formation is essentially the reverse of consolidation, an understanding of swarm cell formation may be helpful in understanding the nature of consolidation (29).

Vibrio parahaemolyticus forms lateral flagella and swarms on solid media. Swarm cells similar to those of Proteus are produced but, unlike Proteus, swarming does not produce concentric rings of consolidation (1). Swarm cells are heavily flagellated and may be up to six times the length of resting cells (2).

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The Polar and Lateral Flagella of Azospirillum brasilense are
Antigenically Distinct

PATRICK G. HALL AND NOEL R. KRIEG^{*}

Microbiology Section, Department of Biology, Virginia Polytechnic
Institute and State University, Blacksburg, Virginia 24061

ABSTRACT

An indirect immunoperoxidase stain was used to demonstrate by electron microscopy that an antigenic difference exists between the polar flagellum and the lateral flagella of Azospirillum brasilense ATCC 29145. No evidence could be obtained that the polar flagellum was ensheathed as it is in Vibrio spp. The diameter and wavelength of the two types of flagella are reported.

Mixed flagellation, i.e., the presence of both polar and lateral flagella on the same cell, occurs in only a few bacterial genera such as Vibrio (5), Chromobacterium (7), Pseudomonas (3), and Aeromonas (4). The antigenic relationship between the two flagellar types has been investigated in only two genera. Shinoda et al. (6) used ferritin-conjugated antibodies to demonstrate the antigenic difference between the sheathed polar flagellum and unsheathed lateral flagella of Vibrio parahaemolyticus by electron microscopy. Sneath (7) indicated that the polar and lateral flagella of Chromobacterium were antigenically distinct.

Azospirillum species produce a single polar flagellum when grown in broth, and also numerous lateral flagella of thinner diameter and shorter wavelength when grown on solid media (8); see Fig. 1A. The difference in diameter and wavelength suggested that the two types of flagella might be antigenically different, and in this paper we report evidence for such a difference.

Azospirillum brasilense ATCC 29145 (type strain) was used throughout the study. Observations of negatively stained cells treated by the methods of Fuerst and Hayward (2) failed to indicate the presence of a sheath on the polar flagellum even though its diameter (18 nm) was greater than that of the lateral flagella (13.5 nm). The wavelength of the polar and lateral flagella were 1.2 μm and 0.7 μm , respectively. Antiserum against both lateral and polar flagella was prepared by the following procedure: cells were grown in Roux bottles on nutrient agar at 30°C for 48 h and suspended in formalinized phosphate-buffered saline to a density of 300 Klett units (blue filter). This suspension was emulsified with an equal volume of Freund's complete adjuvant

and 2-ml portions were administered i.m. into each hind leg of a 3 kg New Zealand white rabbit. A serum sample from this animal taken before immunization exhibited no agglutination titer against the cells. Immune serum against both polar and lateral flagella (APLF) was obtained after 4 weeks and agglutinated the cells to a titer of 1:5,120 at 25°C. The globulin fraction of the serum was obtained by ammonium sulfate fractionation and was exhaustively adsorbed with broth-grown cells of A. brasilense (which have only polar flagella) at 25°C, thereby leaving the antibodies for lateral flagella (ALF) in the supernatant. Antibodies specific for polar flagella (APF) were obtained by a similarly adsorbing the globulin fraction with broth-grown cells, washing the cells in cold saline, and then heating the cells to 58°C for 1 h to elute the adsorbed antibodies from the polar flagella.

Flagella were stained by an immunoperoxidase procedure previously used in studies of the flagella of various strains of Legionella (1). Cells grown on nutrient agar at 30°C for 48 h were adsorbed to parlodion-coated, carbon-stabilized grids for 2 min. The grids were washed with phosphate buffered saline (PBS), floated on human serum for 20 min to reduce nonspecific staining, washed for 20 min in three changes of PBS, floated on APLF, APF, or ALF for 20 min, and again washed with PBS. They were then treated with a 1:1,000 dilution of horseradish peroxidase-conjugated goat antirabbit IgG (Sigma Chemical Co., St. Louis, MO) for 20 min. After washing the grids with PBS, the stain was developed by treatment with a mixture of 0.03% diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H₂O₂ in 0.5 M Tris buffer (pH 7.6) for 10 min. The grids were finally washed with Tris buffer and examined with a JEOL 6A electron microscope.

Fig. 1B illustrates that APLF stained both the polar flagellum and the lateral flagella. In contrast, ALF stained only the lateral flagella (Fig. 1C) and APF stained only the polar flagellum (Fig. 1D). We conclude that the two flagellar types are indeed antigenically distinct. Although the immunoperoxidase technique has been used previously to differentiate the flagella of different strains of bacteria, this study represents the first application of the method for differentiating two types of flagella occurring on the same organism.

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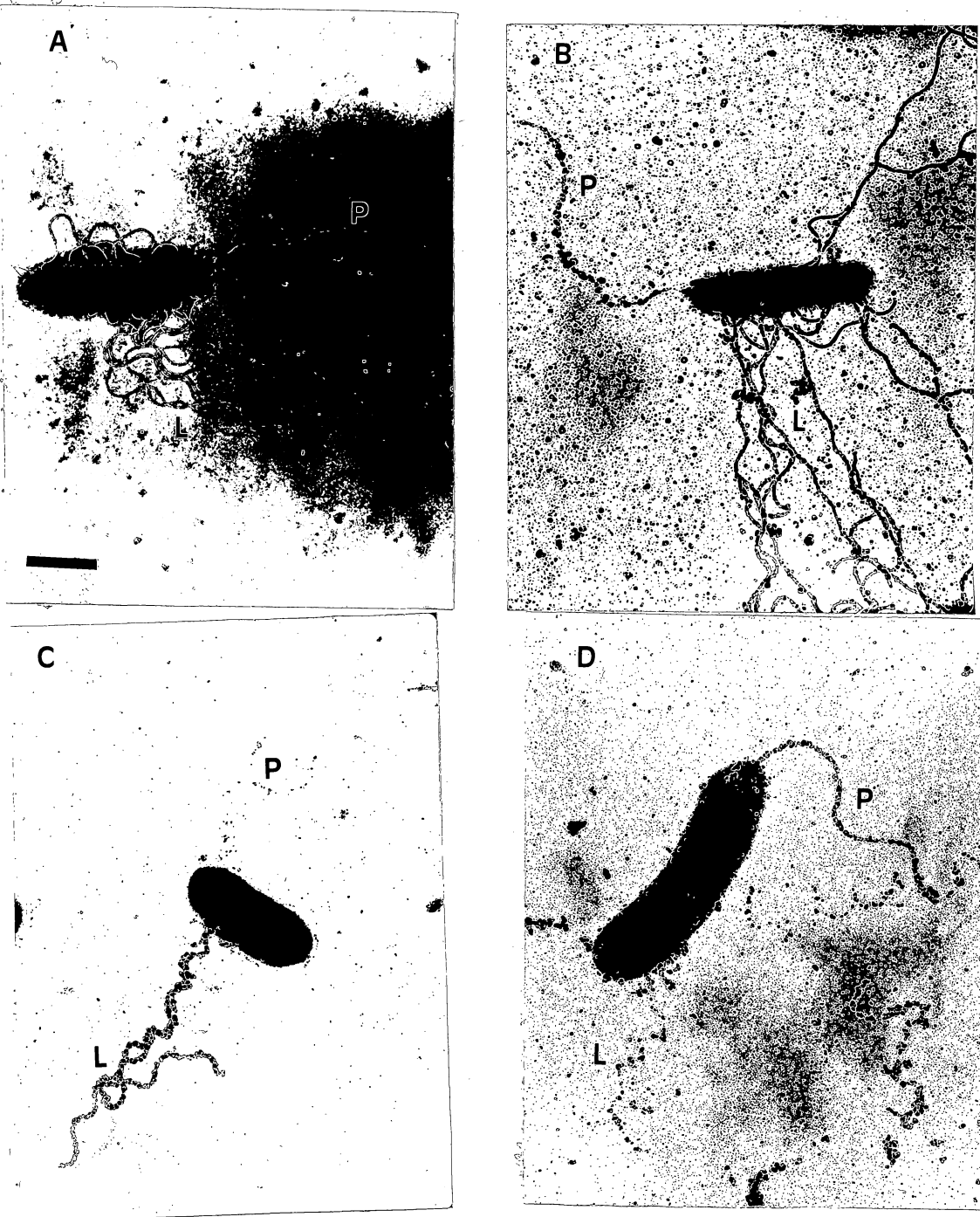


FIG. 1. Electron micrographs showing flagella of *Azospirillum brasilense*. (A) Agar-grown cells, negatively stained with phosphotungstate, showing polar flagellum and lateral flagella. (B - D) Flagella stained by indirect immunoperoxidase method. (B) Polar and lateral flagella stained with APLF. (C) Lateral flagella stained with ALF. (D) Polar flagellum stained with APF. P = polar flagellum; L = lateral flagella. Bar = 1 μ m.

The Influence of Physical and Chemical Agents on the Swarming of
Azospirillum brasilense

PATRICK G. HALL AND NOEL R. KRIEG*

Microbiology Section, Department of Biology, Virginia Polytechnic
Institute and State University, Blacksburg, Virginia 24061

ABSTRACT

Azospirillum brasilense ATCC 29145, which possesses both a polar flagellum and also lateral flagella, swarms on certain solid media. The effects of various physical and chemical factors on swarming were determined. Based on mutants lacking either polar or lateral flagella, the lateral flagella were found to be responsible for swarming on solid media whereas the polar flagellum was responsible for swimming motility in liquid media.

On modified peptone-succinate salts (MPSS) agar Azospirillum spp. form discrete colonies but the cells possess a "mixed flagellation"; i.e., they possess not only a single polar flagellum but also numerous lateral flagella that are thinner and have a shorter wavelength than the polar flagellum (7). Such mixed flagellation has been reported in only a few bacterial genera such as Vibrio (1, 2, 5), Pseudomonas (4), and Chromobacterium (6). In Vibrio alginolyticus the lateral flagella are responsible for swarming on solid media (1); therefore it seemed likely that A. brasilense might also be capable of swarming under certain conditions.

Stock cultures of A. brasilense ATCC 29145 (type strain) were maintained on semisolid malate medium (7) at 30°C with monthly transfer. For testing swarming ability, plates of solid media were dried at room temperature for 24 h, inoculated in the center with a 2.5-mm (i.d.) loop from a 24-h-old MPSS broth culture grown at 37°C, and incubated in a humid atmosphere at 30°C for 24-48 h. Swarming, i.e., the formation of a thin, flat, spreading type of growth, was found to occur on nutrient broth (Difco) containing 0.75% agar (hereafter termed "swarm medium" or SM) (see Fig. 2). The swarming differed from that exhibited by Proteus spp. in that periodic cycles of swarming that produce concentric zones or "waves" of growth on the agar did not occur.

The effect of agar concentration and temperature of incubation on swarming is indicated in Table 3. The spreading occurring at agar concentrations below 0.4% is attributed to an ability to swim through the media rather than true swarming activity. Swarming occurred optimally at agar concentrations from 0.4 to 1.0%; above 0.75% swarming became increasingly inhibited with increasing agar concentration and on plates containing 1.5% agar

TABLE 3. Effect of agar concentration and incubation temperature on swarming of A brasilense ATCC 29145

Temperature, °C	Agar concentration, %	Diameter of swarm zone, mm ^a
30	0.10	100 ± 0
	0.20	62 ± 2
	0.30	36 ± 1
	0.40	17 ± 2
	0.50	22 ± 2
	0.75	45 ± 6
	1.00	17 ± 0.6
	1.25	NS ^b
	1.50	NS ^b
37	0.10	100 ± 0
	0.20	64 ± 2
	0.30	48 ± 6
	0.40	27 ± 0
	0.50	11 ± 1
	0.75	21 ± 9
	1.00	NS ^b
	1.25	NS ^b
	1.50	NS ^b

^aEach value represents the mean from three replicate plates followed by the standard deviation.

^bNS = no swarming occurred.

the colonies were nonspreading and discrete. At an agar concentration of 0.75% an incubation temperature of 30°C supported a greater degree of swarming than at 37°C. Swarming was not solely dependent on the presence of agar, however; nutrient broth solidified with 0.5% carrageenan (Sigma Type I) also supported swarming. Moreover, swarming also occurred on the surface of pieces of sterile dialysis membrane which overlaid the swarm medium.

Table 4 indicates the effect of several chemical agents on swarming when added to SM. The surface active agents sodium deoxycholate and sodium taurocholate at 0.1% and 0.1%, respectively, inhibited swarming but did not inhibit growth or formation of lateral flagella. These results were consistent with those reported by De Boer et al. (2), who found that the swarming of Vibrio alginolyticus was inhibited by these agents. Boric acid inhibited swarming at 0.05 and 0.10% but greatly elongated cells with lateral flagella were formed. In contrast to results obtained by De Boer et al. with V. alginolyticus, the formation of lateral flagella was not stimulated in broth-grown cultures by either boric acid or aluminum hydroxide. NaCl and Na₂SO₄ inhibited swarming and the formation of lateral flagella at 2.0% and 3.0%, respectively; at 4.0% Na₂SO₄ allowed only faint growth. At 2.0-3.0% KCl inhibited swarming but not the formation of lateral flagella. p-Nitrophenylglycerol has been reported to inhibit swarming of Proteus by preventing the formation of swarm cells (8); it inhibited swarming of A. brasiliense at 0.006-0.010% (Table 4); however, greatly elongated cells with lateral flagella were formed resembling swarm cells of Proteus. At a concentration of 0.17% (0.45 mM), ethylenediamine tetraacetic acid (EDTA) also strongly inhibited swarming but not growth or formation of lateral

TABLE 4. Effect of various chemical agents on swarming of Azospirillum brasilense ATCC 29145

Agent	Concentration, %	Diameter of swarm zone, mm ^a	Presence of lateral flagella
None	--	35 ± 11 ^b	+
Sodium deoxycholate	0.01	44 ± 5.0	+
	0.05	37 ± 3.4	
	0.10	16 ± 2.6	
Sodium taurocholate	0.25	28 ± 1.1	+
	0.5	12 ± 1.7	
	1.0	NS ^c	
H ₃ BO ₃	0.05	NS ^c	+ ^d
	0.10	NS ^c	
	0.20	NG ^e	
NaCl	2.0	NS ^c	-
	3.0	NG ^e	
KCl	2.0	NS ^c	+
	3.0	NS ^c	
	4.0	NG ^e	
Na ₂ SO ₄	2.0	NS ^c	-
	3.0	NS ^c	
	4.0	NS ^c	
p-Nitrophenylglycerol	0.002	48 ± 3.5	+ ^d
	0.006	11 ± 1.9	
	0.010	NS ^c	

^aValues represent the mean from five replicate plates followed by the standard deviation.

^bValues for the control represent the mean from 15 replicates.

^cNS = no swarming occurred.

^dGreatly elongated cells occurred, similar to the swarm cells of Proteus.

^eNG = No growth.

flagella, suggesting that metal ions might be involved in the swarming. Consequently, attempts were made to reverse this inhibition by addition of metal ions at various concentrations: Mg^{2+} , 0.05 to 1.0 M; Ca^{2+} , 0.05 to 2.0 M; and a nitric acid digest (3) of SM containing the metal ion components, 0.1 to 0.9%. However, in no instance did supplementation of the SM-EDTA medium with these components cause reversal of inhibition of swarming, and the reason for the action of EDTA remains as yet unknown.

Mutants were employed in order to define the role of polar or lateral flagella in swarming. A mutant having only polar flagella (PF) was obtained by mutagenesis with ethyl methane sulfonate, and a mutant having only lateral flagella (LF) was obtained by mutagenesis with ultraviolet light; selection was done according to the methods of Shinoda and Okamoto (5). The PF mutant was motile in broth but incapable of swarming on SM. In contrast, the LF mutant was nonmotile in broth but could swarm on SM.

We conclude that the lateral flagella of A. brasilense are the organelles responsible for swarming but not for swimming motility, whereas the polar flagellum is responsible for swimming motility but not swarming. We have been unable to induce formation of lateral flagella in liquid media, and a solid surface is apparently required for lateral flagella production. Chemical agents such as p-nitrophenylglycerol, sodium deoxycholate and sodium taurocholate can inhibit swarming but not growth of the organisms or formation of lateral flagella.

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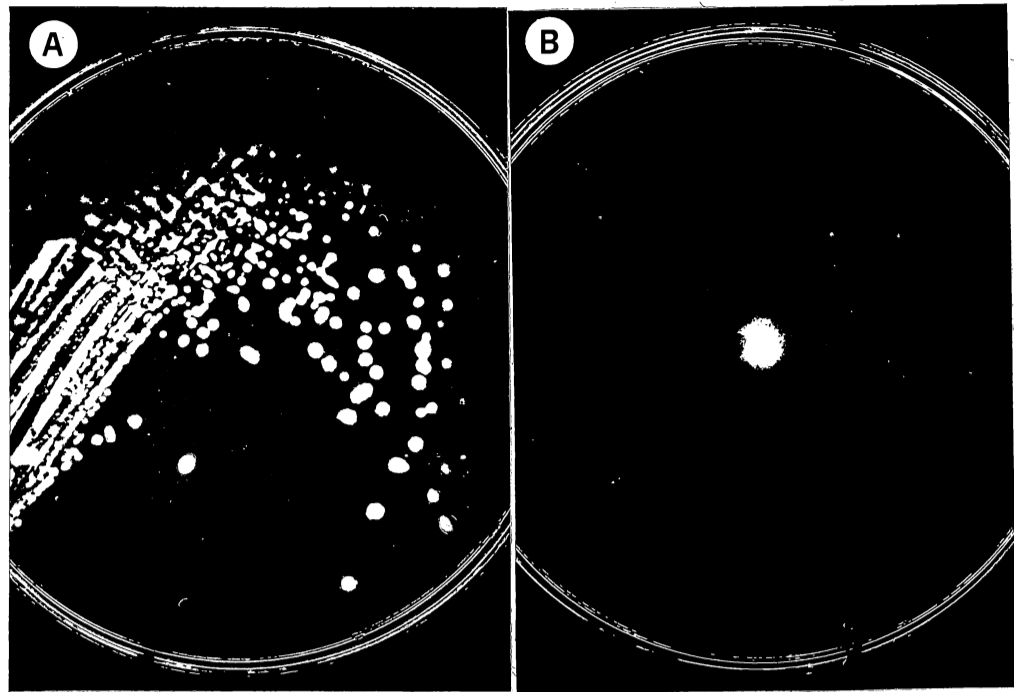


FIG. 2. Colonies of *Azospirillum brasilense*. (A) Typical discrete colonies on nutrient agar (1.5% agar). (B) Typical swarm colony formed on SM (0.75% agar). 0.8 x.

SUMMARY AND CONCLUSIONS

This has been an investigation of the form and function of the polar and lateral flagella of Azospirillum brasilense ATCC 29145. It was determined that the lateral flagella are 13.5 nm in diameter with a wavelength of 0.7 μm , and that the polar flagellum is 18 nm in diameter and has a wavelength of 1.2 μm . Despite repeated efforts, it was not possible to demonstrate the presence of a sheath on the polar flagellum. An indirect immunoperoxidase strain used in conjunction with electron microscopy indicated that the two flagellar types were antigenically different.

The organism was shown to be capable of swarming on solid media. The use of mutants indicated that the lateral flagella are required for swarming on solid media and that the polar flagellum was required for motility in broth.

The production of the lateral flagella is governed by at least two factors. One factor is the presence of a solid surface. Not only were lateral flagella produced (and swarming seen) on media solidified with agar but also on media in which carrageenan was used as the solidifying agent. Lateral flagella and swarming were also observed when cells were placed on a sterile membrane overlying agar media so that the cells were not in direct contact with the medium. Two surface active agents -- sodium deoxycholate and sodium taurocholate -- inhibited swarming to various degrees depending on their concentration. These data suggest that interaction with a solid surface is required for production of lateral flagella.

A chemical factor influencing swarming was indicated by the inhibitory action of the metal-chelating agent EDTA. Attempts to reverse the inhibition by addition of Ca^{2+} , Mg^{2+} , and a metal solution (acid digest of the swarm medium) were unsuccessful. Consequently, the chemical factor remains unknown.

Factors other than those listed may also affect swarming. For example, incubation at 37°C rather than 30°C decreased swarming. Moreover, boric acid, Na^{+} , and Cl^{-} , all of which normally inhibit growth at high concentrations, suppressed swarming but not growth when used at lower concentrations. Unlike Vibrio alginolyticus, A. brasilense did not produce lateral flagella in broth containing boric acid or aluminum hydroxide. An agent which suppresses swarming in Proteus, p-nitrophenylglycerol, also prevented swarming in A. brasilense but did not inhibit growth.

APPENDIX

Additional Swarm Data. The effect of various chemical agents on the swarming of A. brasilense is indicated in Tables 5 - 9.

In Table 5, it can be seen that addition of 0.00013 to 0.00052% CaCl_2 , 0.05 to 0.1% KNO_3 , and 0.01 to 0.10% MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$, oxgall, or bile salts inhibited swarming. In no instance could any of these agents be said to have enhanced swarming. AlOH generally depressed swarming when used at concentrations of 0.01 to 0.10%; the increase in zone diameter seen at a concentration of 0.20% may be the result of the particulate nature of AlOH . Tween 80 at concentrations of 0.10 to 1.0% had no appreciable effect on swarming. EDTA progressively reduced swarming (but not growth) when used at concentrations from 0.038 to 0.171%; at 0.19% neither swarming nor growth occurred.

Table 6 indicates the effect of adding metal ions to swarm medium containing 0.45 mM EDTA. Since EDTA chelates divalent cations, it was hoped that the inhibitory effect on EDTA on swarming might be reversed by addition of such ions in excess of the chelating capacity of the EDTA; however, addition of Ca^{2+} or Mg^{2+} did not effect a reversal of the inhibition.

Table 7 indicates another attempt to reverse the inhibition of swarming caused by EDTA: a nitric acid digest of the swarm medium was added to the swarm medium containing EDTA. The digest was prepared by adding 0.8 g of

TABLE 5. Effect of additional chemical agents on swarming of A. brasiliense ATCC 29145

Agent	Concentration, % (w/v)	Diameter of swarm zone, mm ^a
None	--	33 ± 12
AlOH	0.01	17 ± 4
	0.05	12 ± 3
	0.10	12 ± 2
	0.20	24 ± 8
MgSO ₄	0.01	51 ± 11
	0.05	55 ± 7
	0.10	46 ± 6
CaCl ₂	0.0013	50 ± 12
	0.0026	41 ± 9
	0.0052	46 ± 12
(NH ₄) ₂ SO ₄	0.01	50 ± 12
	0.05	41 ± 8
	0.10	39 ± 5
Tween 80	0.10	53 ± 10
	1.00	52 ± 2
Oxgall (Difco)	0.01	45 ± 3
	0.05	46 ± 2
	0.10	37 ± 4
Bile salts (Difco)	0.01	48 ± 12
	0.05	56 ± 1.2
	0.10	47 ± 5
KNO ₃	0.05	24 ± 8
	0.10	21 ± 3
	0.20	23 ± 4

(Continued.....)

^aValues represent the mean from 5 replicate plates followed by the standard deviation.

TABLE 5 (continued). Effect of additional chemical agents on swarming of A. brasilense ATCC 29145

Agent	Concentration, % (w/v)	Diameter of swarm zone, mm ^a
EDTA	0.038	19 ± 3
	0.057	16 ± 2
	0.076	16 ± 2
	0.095	14 ± 3
	0.114	12 ± 3
	0.13	12 ± 2
	0.15	10 ± 5
	0.171	NS ^b
	0.19	NS ^b

^aValues represent the mean from 5 replicate plates followed by the standard deviation.

^bNS = no swarming occurred.

TABLE 6. Effect of adding metal ions to swarm medium containing 0.45 mM (0.171%) EDTA on swarming of A. brasilense

Metal ion ^a	Concentration, mM	Occurrence of swarming ^b
None	--	-
Ca ²⁺	0.05	-
	0.10	-
	0.50	-
	1.00	-
	2.00	-
	3.00	-
	4.00	-
	5.00	-
Mg ²⁺	0.05	-
	0.10	-
	0.50	-
	1.00	-

^aSupplied as CaH₄(PO₄)₂·H₂O or MgSO₄.

^bFive replicate plates were tested.

TABLE 7. Effect of adding an acid digest on swarming of A. brasilense^a

Acid digest, ml/100 ml of medium	Occurrence of swarming ^b
None	-
0.1	-
0.2	-
0.3	-
0.4	-
0.5	-
0.6	-
0.7	-
0.8	-

^aAn acid digest of swarm medium (SM) was added to swarm medium containing 0.45 mM (0.171%) EDTA.

^bFive replicate plates were tested.

dry swarm medium to 10 ml of 2 N HNO₃ and incubating at 60°C for 24 h. Each 0.1 ml of extract was therefore equivalent to adding the metals contained in 8.0 mg of medium. The addition of up to 0.8 ml of the digest failed to reverse the inhibition of swarming, however.

As indicated in Table 8, the pH of the medium only mildly affected swarming. Since a pH of 7.0 produced the best swarming, it was decided that all other swarm experiments should be done at this pH.

Table 9 indicates that swarming could occur on media solidified with carrageenan. The behavior of the cells was similar to that on agar-solidified medium. Optimum swarming occurred at a carrageenan concentration of 0.5% and decreased as the carrageenan concentration increased. Media containing <0.5% carrageenan were not tested because they could not be adequately dried before use.

Antigenicity of the Polar Flagellum of the "Polar-only" Mutant. The polar flagellum of the "polar-only" mutant strain was found to be antigenically dissimilar from the polar flagellum of the wild-type strain of A. brasilense ATCC 29145. Electron microscope observation of mutant cells treated with APLF by the indirect immunoperoxidase procedure indicated no staining of the polar flagellum. Similarly, treatment with antiserum prepared against the "polar-only" mutant failed to result in staining of the polar flagellum (or lateral flagella) of the wild-type strain. Moreover, no titers could be obtained in agglutination tests when the above combinations of cells and antisera were used.

Titers of Anti-wild-type Serum vs. Wild-type cells 4 weeks after immunization. Table 10 indicates the results of this titration. The titrations

TABLE 8. Effect of pH on the swarming of A. brasilense

pH	Diameter of swarm zone, mm ^a
6.0	20 ± 4
6.5	21 ± 2
6.8	21 ± 2
7.0	23 ± 5
7.3	22 ± 3
7.5	20 ± 4
8.0	22 ± 3

^aValues represent the mean from 5 replicate plates followed by the standard deviation.

TABLE 9. Swarming of wild-type cells and lack of swarming of "polar-only" mutant cells on medium containing carrageenan as the solidifying agent

% Carrageenan	Diameter of swarm zone, mm ^a	
	Wild-type	"Polar-only"
0.50	23 ± 2	NS ^b
0.75	16 ± 5	NS ^b
1.00	13 ± 5	NS ^b
1.25	10 ± 2	NS ^b

^aValues represent the mean from 5 replicate plates followed by the standard deviation.

^bNS = no swarming occurred.

TABLE 10. Titers of anti-wild-type-serum, vs. wild-type cells 4 weeks after immunization

Growth medium	Type of flagellation	Titer
Nutrient agar	Polar and lateral	1:5,120
Nutrient broth	Polar only	1:80

were performed at 25°C for 5 h. A macroscopically visible clumping was taken as positive agglutination. Two-fold dilutions in formalinized phosphate-buffered saline (FPBS) of a 1:10 dilution of the serum were repeated in 10 tubes. One ml of a 300-Klett unit bacterial suspension in FPBS was added to each tube, yielding final serum dilutions of 1:20 to 1:10,240. A saline-cell control containing 1.0 ml of FBPS and 1.0 ml of cell suspension was used in all agglutination tests.

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