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Titanospirillum velox: A huge, speedy, sulfur-storing spirillum from Ebro Delta microbial mats

(Adrianus Pijper/bacterial motility/polar organelle/sulfur globules)

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ABSTRACT **A long (20–30 μm), wide (3–5 μm) microbial-mat bacterium from the Ebro Delta (Tarragona, Spain) was grown in mixed culture and videographed live. Intracellular elemental sulfur globules and unique cell termini were observed in scanning-electron-microprobe and transmission-electron micrographs. A polar organelle underlies bundles of greater than 60 flagella at each indented terminus. These Gram-negative bacteria bend, flex, and swim in a spiral fashion; they translate at speeds greater than 10 body lengths per second. The large size of the spirillum permits direct observation of cell motility in single individual bacteria. After desiccation (i.e., absence of standing water for at least 24 h), large populations developed in mat samples remoistened with sea water. Ultrastructural observations reveal abundant large sulfur globules irregularly distributed in the cytoplasm. A multilayered cell wall, pliable and elastic yet rigid, distends around the sulfur globules. Details of the wall, multiflagellated termini, and large cytoplasmic sulfur globules indicate that these fast-moving spirilla are distinctive enough to warrant a genus and species designation: *Titanospirillum velox* genus nov., sp. nov. The same collection techniques at a similar habitat in the United States (Plum Island, northeast Essex County, Massachusetts) also yielded large populations of the bacterium among purple phototrophic and other inhabitants of sulfurous microbial-mat muds. The months-long survival of *T. velox* from Spain and from the United States in closed jars filled with mud taken from both localities leads us to infer that this large spirillum has a cosmopolitan distribution.**

Microbial mats, among the oldest ecosystems on Earth, have covered the shallow benthos with their layered communities since at least the start of the Proterozoic Eon; they probably prevailed in the Archean Eon over 3 billion years ago as well (1). At marine margins, ancient bacteria most likely interacted—as they do today—via sulfur oxidation-reduction cycles. Here, we report a recently discovered sulfur-storing microbial-mat bacterium. This fast-swimming heterotroph is so large that it is recognizable by morphology alone; direct observation of movement and desiccation of single individual cells is possible. We collected samples from the Alfacs Peninsula in the Ebro Delta, Tarragona, northeastern Spain, in November 1997, when the surface of the *Microcoleus*-dominated microbial mat (2) lacked standing water (Fig. 1*a*). Glass jars were filled to the brim with mud. Sulfide-rich black mud underlies the photosynthetic layer, which is composed primarily of filamentous cyanobacterial and purple sulfur phototrophs.

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MATERIALS AND METHODS

In previous studies, only coastal laminated microbial mat with well developed *Microcoleus chthonoplastes* cyanobacterial layers with underlying purple phototrophs yielded the large viviparous variable-diameter spirochete *Spirosymplokos deltaeiberi* (3). Because spirochete populations develop when pieces of *Microcoleus* mat are inoculated into certain anoxic media (3), the jars were sealed tightly to minimize oxygen exposure. Samples for growth of spirochetes (4), amitochondriate protists, and amoebomastigotes [e.g., *Paratetramitus jugosus* with its chromidia (tiny reproductive propagules; ref. 5)] were removed from the jar. After 4 weeks, to compensate for the dry surface, we remoistened the contents with the addition of approximately 5 ml of filtered sea water; 2 days after rewetting, three or four types of motile protists were seen with the ×4 objective in darkfield (Nikon Diaphot inverted microscope). Closer observation at higher magnifications (×40 and ×100) revealed the misidentification of one type of "protist." Here, we describe these morphologically distinctive rapid swimmers that we cultured in the laboratory (Fig. 1 *b–f*).

We performed scanning-electron microscopy (including microprobe) and transmission-electron microscopy (both negative-stain and thin-section) by using standard techniques to support observations of live cells (3).

RESULTS

The bacterium, a bipolar lophotrichously flagellated spirillum (Fig. 1 *e–g*), measures 19.1 ± 1.3 μm ($n = 10$) in length by 3.9 ± 0.1 μm ($n = 10$) in diameter. All large spirilla bear conspicuous cytoplasmic granules (Fig. 1 *c* and *d*). Tests (Nile Blue A stain) to identify these granules as usual storage products (poly-β-hydroxybutyrate or other poly-β-hydroxyalkanoates) were negative. The possibility that the granules were nucleoids (DNA material) visible with optical microscopy was precluded by DNA fluorescence staining. As determined by 4′,6-diamidino-2-phenylindole staining for DNA and by UV microscopic observations at λ = 420 nm, the ribbon-shaped nucleoid extends the length of the cell and shows no tendency to clump or form granules. The hypothesis that the granules are polyphosphate ("volutin" as in *Spirillum volutans*) was excluded by both electron-microprobe analysis and electron-microscopic image. No phosphate peak was detected by the probe, and no polyphosphate granules under the electron beam fragmented to form "wheels with spokes" (6). Yellowish spots in live cells resolved in electron micrographs as membrane-bound vacuoles (Fig. 2*b*, *c*, and *e*); most were empty, but some were filled. The size (0.5 ± 0.2 μm; $n = 10$), yellowish glossy appearance, spherical morphology, membrane-bound ghosts, and their exclusion as nucleoids or bacterial polyalkanoate storage granules led us to hypothesize an elemental

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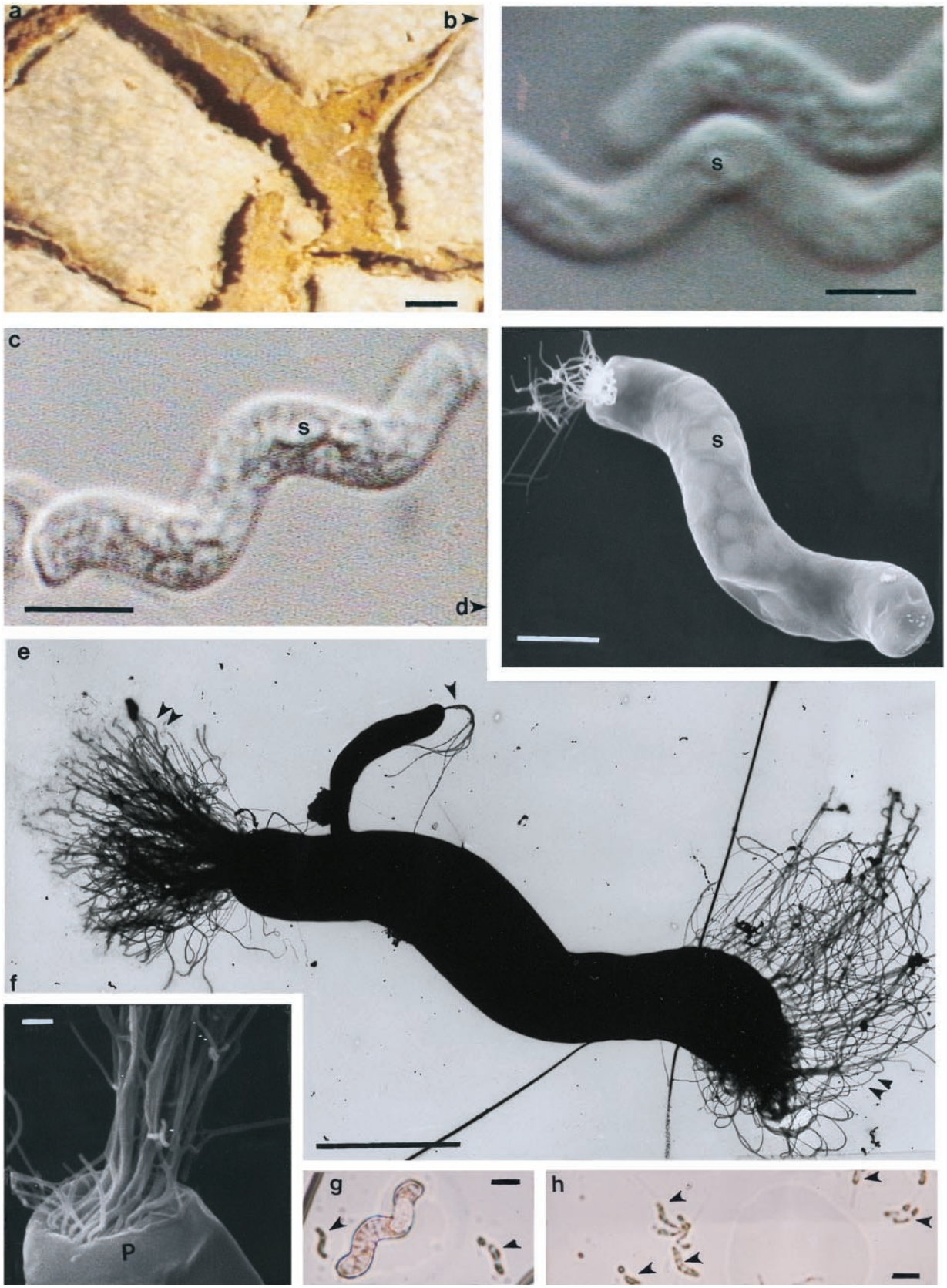

FIG. 1. (*a*) Mat surface at the Ebro Delta field site (3) showing lack of standing water. (Bar = 10 cm.) (*b*) Two spirilla cells (S, sulfur globule) shown by differential interference contrast (Nomarski). (Bar = 5 μm.) (*c*) Phase contrast microscopy of live spirillum cells. (Bar = 5 μm.) (*d*) Bipolar lophotrichous large spirillum in which only one pole has retained flagella. Sulfur globules are visible through the cell wall (scanning electron micrograph). (Bar = 5 μm.) (*e*) Negative-stain transmission electron micrograph of an entire bipolar lophotrichous large spirillum showing flagella "braids" (double arrowheads) compared with standard-sized spirilla (single arrowhead). (Bar = 5 μm.) (*f*) This scanning-electron micrograph of a cell terminus shows one vaulted end with residual flagella. The indentation coated by the polar organelle (P; see Fig. 2) is implied. (Bar = 0.5 μm.) (*g*) This Gram-stain brightfield preparation compares the two size classes, huge and standard, of Gram-negative spirilla. (Bar = 5 μm.) (*h*) Standard-sized spirillum Gram stain. The lighter spots are probably sulfur globules. (Bar = 5 μm.)

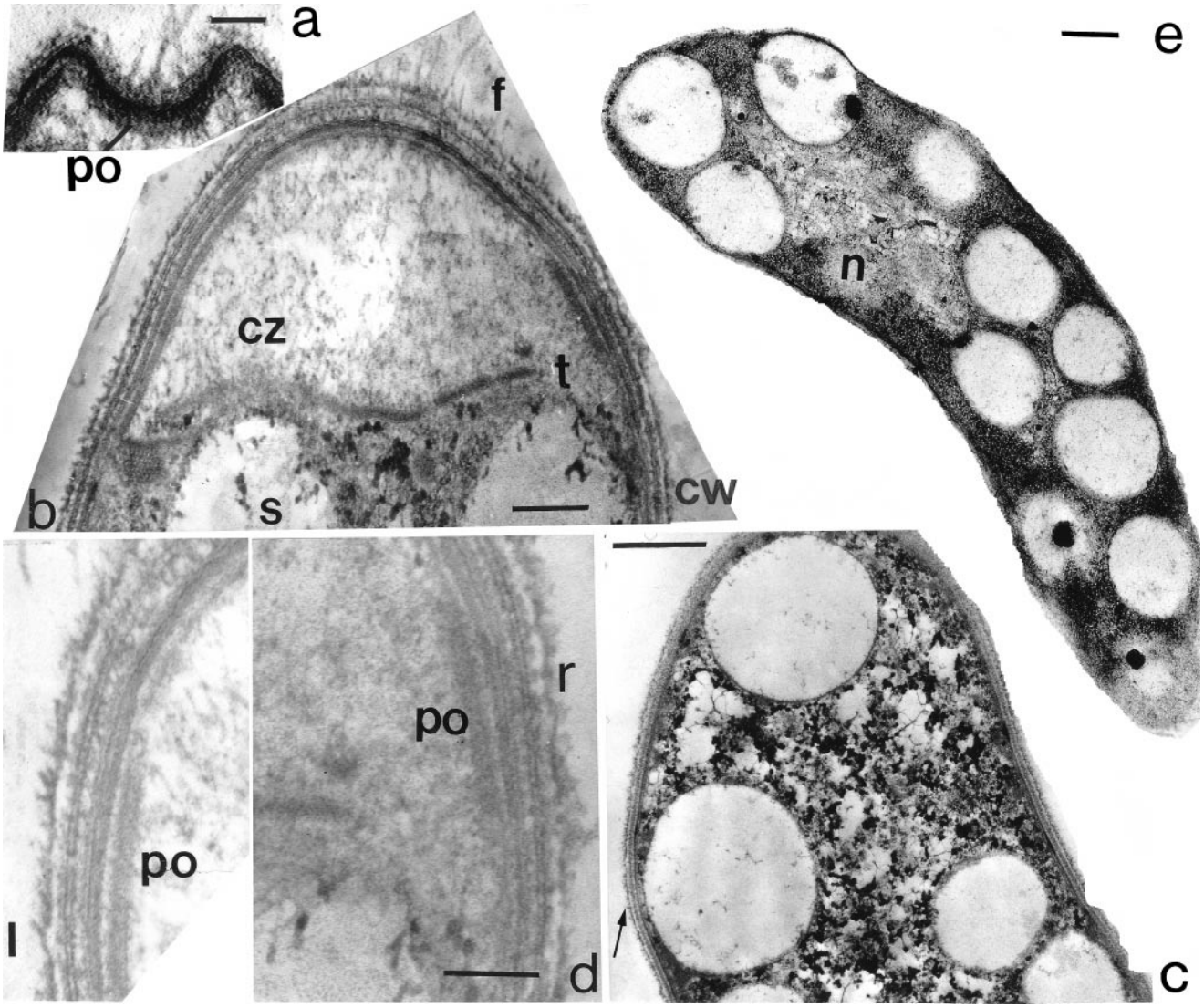

FIG. 2. Transmission electron micrographs of *Titanospirillum velox*. (*a*) The polar organelle (po) underlies the indented terminus. (Bar = 1 μm.) (*b*) Complex cell wall (cw), flagella (f), and clear zone (cz) at the cell terminus subtended by thickening (t) and empty sulfur-globule (s) vacuoles. (Bar = 0.5 μm.) (*c*) Distended cell wall around a peripheral membrane-bound sulfur-globule vacuole (arrow). (Bar = 1 μm.) (*d*) The left (l) and right (r) polar organelles lie proximal to at least nine layers of wall material at the cell termini. (Bar = 0.25 μm.) (*e*) Sulfur-globule vacuoles distributed irregularly in the cytoplasm are especially abundant at the cell periphery distal to the nucleoid (n). (Bar = 1 μm.)

sulfur composition. The intracellular holes left by the granules after processing for electron microscopy are reminiscent of sulfur globules typical of *Chromatium* or *Thiospirillum* phototrophic purple bacteria (7).

The culture sample, fixed in 2% (vol/vol) glutaraldehyde, was dispensed in drops onto glass slides for dehydration, carbon coating, and viewing. X-ray diffraction/scanning-electron-microscope scans indicated that, although carbon, phosphorus, silicon, and nitrogen were uniformly distributed in the cells, sulfur varied along their length. The sulfur peak over the terminus of one well preserved cell was compared directly to that over the subterminus with inclusions. Because the subterminus was approximately three times richer in sulfur, we conclude that at least some of the cytoplasmic granules are sulfur-rich globules.

These large spirilla, unlike *Rhodospirillum* or *Thiospirillum*, are not phototrophs, because they maintain and grow no differently in light or darkness. They are sulfur-rich heterotrophs; however, because they are far larger, differ morphologically, and fail to grow on *Oceanospirillum* medium, they cannot be *Oceanospirillum*. Although these spirilla survive some desiccation and anoxia, they are aerobes. We grew them only by dropwise addition of an inoculum of a suspension to tubes with a large airspace, to which we added a 1-cm^3 piece of *Microcoleus* microbial-mat inoculum from the original Ebro Delta site and 1–2 ml of filtered, but not sterilized, natural sea water. Typically, in sets of 10 such tubes, enormous populations of small spirilla develop in 2 or 3 days (videographic documentation and Fig. 1 *g* and *h*). After 3–6 days, the huge spirillum appears among standard-size ones (Fig. 1*g*). Populations of other bacteria (e.g., small rods and spirilla) and protists (e.g., ciliates and diatoms) develop in all tubes. Only 20–30% of the tubes with numerous huge spirilla were studied in detail. After 3–4 weeks, the numbers of huge spirilla spontaneously declined. Later, unless transferred to fresh seawater, both spirilla morphotypes disappear. More than three times, standing water was absorbed, and the mud surface of the tube or jar containing numerous huge spirilla was dried out for at least 24 h. The dropwise addition of sea water, in a few days, led to the recovery of swimming. The spirillum maintained its morphology through the drying process. At least 3 days of desiccation tolerance and recovery by quick resorption of water was monitored microscopically in 3–4 individual cells. Both large and small spirilla survived for

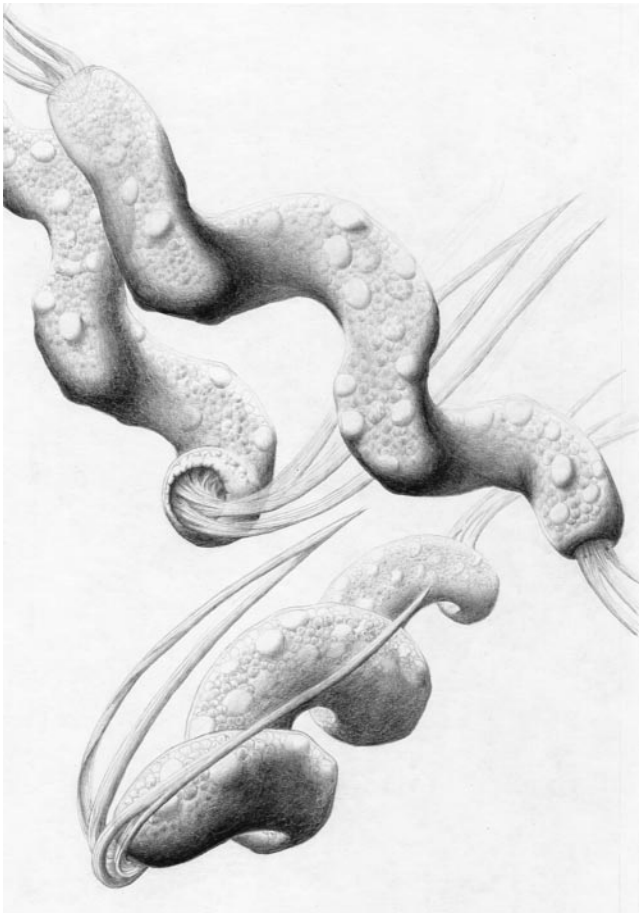

FIG. 3. Huge spirilla rendered from both life and micrographs.

months when undisturbed mat-mud samples filling closed jars were exposed to diurnal light cycles.

So many flagella, inserted subterminally, are present that they cannot be counted accurately even in favorable preparations (Fig. 1*e*). Only a few flagella are seen at one time in thin section (Fig. 2 *a* and *b*). Under each raised-rim indented cell terminus (Fig. 2*a*) is a polar organelle (i.e., polar "membrane"; Fig. 2 *b–d*), "a densely staining line of globular units 5–6 nm in diameter" (8), and a unique conspicuous space (Fig. 2 *a* and *b*). Bundles ("braids" in the older literature) of flagella emerge from these vaulted unique cell ends. Each bundle likely has more than 30 flagella rotating in unison. As observed by using brightfield, Nomarski, and phase-contrast optics, flagella bundles form and move such that swimming and stationary cells seem to bear only 2–4 thick flagella (Fig. 3).

Jar samples of *Microcoleus*-dominated mat filled to the brim with well developed purple photosynthetic bacteria were collected from Plum Island, MA, in autumn 1998 and were left undisturbed and exposed to ambient temperatures until April 1999. The large spirillum developed huge population densities in the odiferous purple sulfurous samples observed with a Swift phase-contrast field microscope.

DISCUSSION

Because it thrived in samples from very similar microbial-mat habitats on both sides of the Atlantic, this spirillum is likely to be cosmopolitan in sulfur-rich microbial muds. Based on the distinctive morphology, we designate the following genus and species names.

***Titanospirillum* (*titan*, Gr. Gigantic; *spirillum*, Gr. Spiral-Shaped).** The cells are 20- to 40-μm-long, 3- to 6-μm-wide, spirillum-shaped heterotrophic bacteria that can be enriched in marine sulfur medium, store sulfur globules, and bear a tuft of >60 flagella at each terminus.

***Titanospirillum velox* (*velox*, Gr. Speedy).** The cells have properties of the genus. Cell wall layers complex with one terminal indentation at each cell pole, and underlying the cell wall are a polar organelle inside the membrane and flagella outside the membrane. Cells translate at 250 $\pm$ 10 μm/s and can survive in native microbial mat for at least 1 week of desiccation (removal of standing water) and 3 weeks of freezing ambient temperatures. Samples were collected from the Alfacs Peninsula, Ebro Delta, Tarragona, Spain.

The polar organelle is a proteinaceous, ribbon-like submembranous structure that spans portions of the periphery of different but always flagellated bacteria (8, 9). Polar organelles underlie the flagellated portion of the cell wall during the developmental cycle when flagella are present. Polar organelles are associated with ATPase activity in *Campylobacter* (8) and *Sphaerotilus natans* (9) and presumably function in "liberation of energy for the flagella" (9). These structures correlate with motility in bacteria such as *Aquaspirillum* (10) and spirochetes (11); whether they are involved in cell-wall flexibility may now be investigated directly in single bacteria by using cytological techniques.

Using his "homemade" solar-illuminated ("sunlight darkground") microscope, South African biologist Adrianus Pijper (1886–1964; ref. 12) wrote that spirilla "exhibit a gyratory and at the same time an undulatory movement" (13). He argued with microbiologists, notably W. van Iterson, about the mechanism of bacterial movement (13). Pijper claimed that bacilli (rods), vibrios, and spirilla are all spiral-shaped when swimming and that such bacterial movement requires undulation of a flexible cell body. He claimed "flagella are a nonessential product of motility." Cell-body movement, he thought, explained the existence of motile bacteria "without tails" and "the frequent failure in staining flagella of motile bacteria." Pijper noted that when "a rapidly swimming bacterium suddenly stopped or somersaulted and reversed its direction, for a short time the "tail" continued to point backward along the original direction of travel, even as the cell body turned and started off in a new direction" (13). Pijper's theories, in part because he misinterpreted the rotary activity of bacterial flagella, were summarily dismissed. Our observations of flexibility and distention of walls by sulfur globules in individual motile bacteria and correlated electron micrographs (Fig. 2 *c* and *e*) support one contentious idea of Pijper's: the bacterial cell wall does play some role in swimming. We must reconsider the "near oblivion into which Pijper's reputation eventually sank" (13). Rapid swimming with a total absence of flagella in cyanobacteria was demonstrated by Waterbury (14) as well as by Berg and colleagues (15). The Ebro Delta spirilla rotate (left and right) as they translate up to 238 $\pm$ 48 μm/s (n = 10). Single cells are large enough to be documented in an "idle" state: flagella bundles rotated, but the cell did not translate. The polar organelles, the pliability of the wall, and the dissociation of flagellar movement from cell translation mean that factors other than flagella rotation could be involved in movement (16). This large bacterium should be useful for chemical dissection of prokaryotic motility.

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