Designation of *Spirillum volutans* Ehrenberg 1832 as Type Species of the Genus *Spirillum* Ehrenberg 1832 and Designation of the Neotype Strain of *S. volutans*

Request for an Opinion

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In 1832 Ehrenberg transferred *Vibrio spirillum* Müller and *V. undula* Müller to a new genus, *Spirillum*. Müller's two species were renamed *Spirillum volutans* (Müller) Ehrenberg and *S. undula* (Müller) Ehrenberg, respectively. A comparison of Müller's description of *V. spirillum* with Ehrenberg's description of *S. volutans*, however, strongly indicates that the organisms described were not the same; therefore the name *S. volutans* should be related to the organism described by Ehrenberg. It is therefore requested that the Judicial Commission delete Müller's name from the author citation for *S. volutans* (i.e., *S. volutans* Ehrenberg, not *S. volutans* [Müller] Ehrenberg). Although Ehrenberg included *S. undula* and *S. volutans* in his new genus, he did not designate a type species. Stiles (1905) was apparently the first to designate the type species (*S. volutans*), but the later designation of *S. undula* as the type by Vuillemin (1913) is the designation that has been generally recognized. The acceptance of *S. undula* as the type species is not only contrary to the rules of nomenclature [Rule 9c(3)] but also unfortunate because the name is a source of confusion and error. It is therefore requested that the Judicial Commission issue an opinion establishing *S. volutans* Ehrenberg as the type species of *Spirillum* Ehrenberg and placing *S. undula* on the list of rejected names. Wells' strain (ATCC 19554) is designated as the neotype strain of *S. volutans*.

In 1832, Ehrenberg (7) transferred *Vibrio spirillum* Müller and *V. undula* Müller to a new genus, which he named *Spirillum*. The species were named *Spirillum volutans* and *S. undula*, respectively, by Ehrenberg. Both of these names and the generic name *Spirillum* were validly published and are legitimate. Ehrenberg did not designate the type species of the genus, and Stiles (16) appears to have been the first to designate the type species (*S. volutans*) for *Spirillum*. Invoking the zoological code of nomenclature, Stiles maintained that, since

Ehrenberg had used Müller's specific epithet *spirillum* as his new generic name, *Spirillum volutans* (i.e., *Vibrio spirillum*) should be the type species by absolute tautonomy. Stiles further recommended that Müller's specific epithet be retained, and that *S. volutans* be correctly named *Spirillum spirillum*; however, this is not in accord with Rule 25(c) of the International Code of Nomenclature of Bacteria (6). Therefore, Ehrenberg's specific epithet *volutans* should stand. However, the later designation by Vuillemin (18) of *S. undula* as the type species is generally followed (1, 2), contrary to the rules [Rule 9c(3)] of nomenclature (6). The intent of this paper is to clarify the problem of the type species of the genus *Spirillum* and to designate a neotype strain for

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this species, Ehrenberg's original strains not being extant.

MATERIALS AND METHODS

Bacterial strains. Pringsheim's strain (ATCC 19553) of *S. volutans* was isolated by Rittenberg and Rittenberg (14) from a mixed culture originally derived from the cooling tower water of a beet sugar refinery in England. The mixed culture was obtained from E. G. Pringsheim in 1951. A second strain, ATCC 19554 (Wells' strain), was isolated from pond water by Wells and Krieg (19).

Basal medium. Peptone-succinate-salts medium (PSS) had the following composition (grams per liter): peptone (Difco), 10.0; succinic acid, 1.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.002; and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.002. The pH was adjusted to 6.8 with KOH.

Characterization tests. *S. volutans* was cultured in 75-ml volumes contained in 250-ml side-arm filtering flasks flushed with a mixture of 30% air and 70% nitrogen. The inoculum for tubed test media was 0.04 ml from the second of two 24-h serial transfers. PSS medium in either liquid or semisolid (0.15% or 0.7% agar) form was supplemented with appropriate substrates for physiological tests except where noted. Tests were performed and interpreted by the methods of Cowan and Steel (5) except where noted. Plates of PSS medium containing 0.7% agar were heavily inoculated to give confluent growth. The catalase test was performed by adding 4 to 5 drops of 3% H_2O_2 to semisolid cultures, with the appearance of a froth within 30 min indicating activity; sterile controls were used. All cultures in liquid medium or on plates were incubated in an atmosphere of 30% air-70% nitrogen. For measurement of cell dimensions, 18-h PSS broth cultures were examined by phase-contrast microscopy (Leitz Ortholux microscope with Heine condenser and Pv 90x/1.15 N.A. objective). The infrared absorption method of Rouf and Stokes (15) was used to detect the presence of poly- β -hydroxybutyrate (PHB) in chloroform extracts of the bacteria.

For determination of deoxyribonucleic acid (DNA) base composition, cells were suspended in a medium containing 0.15 M NaCl and 0.01 M ethylenediaminetetraacetic acid (pH 8.0). The cells were lysed with sodium dodecyl sulfate, and the protein was extracted twice with liquefied phenol. The DNA was purified by the method of Marmur and Doty (11) and adjusted to 50 $\mu\text{g}/\text{ml}$ with 0.5 $\times$ SSC (1 $\times$ = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Melting point (T_m) determinations were made by a variation of the method of Marmur and Doty: the starting temperature was 60 C, and the temperature was increased linearly at 0.5 C/min over the entire range. An automatic recording Gilford model 2400 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) and *Escherichia coli* reference DNA were used.

RESULTS AND DISCUSSION

Müller (13) described *Vibrio spirillum* as follows (translated from the Latin):

Vibrio spirillum

A thread-shaped *Vibrio*, with windings smoothed off into an acute angle.

A singular animalcule sporting a spiral *vasculum* [translator's note: a small vessel]; we justly wonder about the function of spiral *vascula* in the animal and vegetable economy. What should we do, if we should meet among living creatures an animal that looks like such an organ? But in this [animalcule] the coils or bendings can hardly be introduced or removed, but are rigid, insofar as this could be observed, wherever the animalcules remain in motion.

Anyhow, it is definitely a pearly animalcule with approximately equal windings, even if these are rounded into acute angles, numbering from four to twelve at the outside, the smaller animalcules having the lower numbers. It is just about the smallest of all animalcules, being narrower than *Monas termo* and *Vibrio lineola*; determined by the longitude of its body and the amplitude of its windings, it comes into view [only] in the sharpest focus. Frequently, however, with the slightest change of location it eludes the keenness of the best augmented eye.

Rectilinear motion is achieved by slowly vibrating the posterior and anterior part, or by twisting perpendicularly, usually while stretched out straight, occasionally while curved into an arc; but the windings are always rigid without any discernible motion.

Ehrenberg (8) described *Spirillum volutans* in 1838 as follows (translated from the German):

Spirillum volutans, large cylinder-spiral.

A *Spirillum* consisting of fibers that are very tortuous, rather stout and rather long, distinctly articulated, and pearly, with three or four or more divisions.

The discoverer or first cataloguer of this animalcule was Köhler in Leipzig in 1777, then Herrmann in Strassburg described it from an infusion of vegetable garbage; he says, in 1784, that he made the observations eighteen years earlier. He sent his drawing to Müller for classification and thus his animalcule got from Müller the name *Vibrio undula*. The drawing is sketched from a too-small enlargement; it clearly shows, however, through multiple screw-windings, the form of *Spirillum volutans*. Müller himself became acquainted with this form as early as 1782 in an infusion of *Sonchus arvensis*, but he had encountered still longer screws, which induced him to designate the shorter ones from Herrmann as *V. undula*. Not until the later work on Infusoria was it noticed that in the case of *V. undula* there were forms of this species with multiple spiral windings. Bory found his species in a water glass in which the dregs of frog eggs had rotted; he then investigated them. He also found the same in other infusions of animal and human substances. I myself have always observed this animalcule in ill-smelling infusions with a white film. On the trip with von Humboldt in Petersburg in 1829, I saw it in water in which meat was decaying. Recently, on the 10th of June and 27th of July 1835, I observed it to be quite numerous in an infusion of vegetable rot. In general, this larger species is rarer than the smaller.

According to Köhler's first and by far best investigations the larger form could be rarer because the small ones do not often develop so far, but I have observed the small ones so frequently and have seen them by the millions, that this development could hardly have escaped notice, since one sees the larger ones more easily than the small. Since these rigid *Spirilla* collectively retain their shape and size on drying, I have been tempted to consider them armored animals, but masses of them burnt on a platinum plate certainly show no silicious armor, and no residue with any shape to it. I have therefore let the thought drop for now.—Müller's species name has been elevated to genus name.—Length of the spiral: 1/192 to 1/48 Linie. Thickness about 1/1200 Linie.

Despite Ehrenberg's elevation of Müller's specific epithet *spirillum* to the generic level, it seems apparent that Müller's organism was not the same as that described by Ehrenberg. A Linie is equivalent to 0.080 inch, or 2,032 μm , and therefore the dimensions for *S. volutans* in micrometers would be 10.6 to 42.3 for length and 1.7 for width, indicating a very large bacterium. Müller's statement that *V. spirillum* "is just about the smallest of all animalcules, being narrower than *Monas termo* and *Vibrio lineola*" seems to be at variance with Ehrenberg's description in this regard. Moreover, from the final sentence of Müller's description referring to the motility of *V. spirillum*, it would seem that a flexible organism (spirochete) was being described, rather than the inflexible organism described by Ehrenberg. Furthermore, although no magnifications were given, the drawing of *V. spirillum* by Müller, when compared to his drawings of other spirilla, does not suggest the organism of large diameter and great length described by Ehrenberg. Williams (20) also noted that "a direct comparison of Müller's drawing of *Vibrio spirillum* with Ehrenberg's drawing of *S. volutans* leaves little doubt that the two organisms were not the same. Müller described *V. spirillum* as being thread-like while the dimensions given by Ehrenberg for *S. volutans* could never be so construed." Consequently the Judicial Commission is requested to delete Müller's name from the author citation for *S. volutans* and to consider Ehrenberg's description as the original description of this organism.

Ehrenberg's description of *S. volutans* is definitive because of the magnitude of the dimensions and the large, deep curves that were depicted in his drawings (8) and also in the later drawings by Cohn (3). Another spirillum of large diameter, *Spirillum tenue*, was also described by Ehrenberg (8); although the cell diameter was given as 1/1000 Linie (2.0 μm) and the usual length as 1/96 Linie (22 μm), the

organism did not possess the deep curves associated with *S. volutans*. In the drawing by Cohn (3), the diameter of *S. tenue* appears to be smaller than that of either *S. volutans* or *S. undula*; this diameter is given as 0.7 μm in the 7th edition of *Bergey's Manual* (1). Although Ehrenberg's description of *S. volutans* is definitive, that for *S. undula* is not. The dimensions of the latter were given as: length, 1/168 to 1/96 Linie (12.1–21.2 μm); width, 1/1680 Linie (1.2 μm). This description could apply to any of several moderately large spirilla, such as *S. giesbergeri* (23), *S. metamorphum* (17), *S. anulus* (23), etc. Müller's descriptions of *Vibrio undula* (*S. undula*) (12, 13) are even less definitive.

The lack of definitive early descriptions of *S. undula* has led to confusion for later investigators of the genus *Spirillum*. Williams (21) stated that "it is even doubtful that any two investigators of the genus *Spirillum* have given the name *S. undula* to identical organisms." In our opinion, it would be impossible for any investigator to determine that a culture bearing the label "*S. undula*" would be the same organism as that seen by Müller or Ehrenberg. In contrast, *S. volutans* can be easily distinguished from other spirilla by Ehrenberg's description. Rittenberg and Rittenberg (14) have stated that "because of its size, shape, distinctive motility and the existence of the excellent drawings of both Ehrenberg and Cohn, a modern investigator should have no difficulty in concluding that he is observing the same creature as was observed by these early microbiologists." Indeed, in Cohn's (3) drawings of *S. volutans*, the characteristic polar fascicles of flagella are clearly depicted. Nearly every student in introductory microbiology who has examined hay infusion has seen *S. volutans*, but it was not until 1962 that Rittenberg and Rittenberg succeeded in isolating the organism (14), and it was not until 1965 that Wells and Krieg demonstrated (19) that the organism is an obligate microaerophile.

Despite the differences in source and time of isolation, Wells' and Pringsheim's strains of *S. volutans* appear to be nearly identical in the characteristics so far studied. A description of these strains follows.

Morphology. Cells observed by phase microscopy in 18-h PSS broth cultures were 1.4 to 1.7 μm in diameter; wavelength, 16 to 28 μm ; diameter of helix, 5 to 8 μm ; length of helix, 14 to 60 μm with from less than one to a maximum of five turns. Using mixed cultures grown in Pringsheim's soil medium (14), Williams and Rittenberg (23) reported that Prings-

heim's strain had a cell diameter of 1.5 to 2.0 μm and a cell length of 17 to 33 μm , that the majority of cells were S-shaped (although cells having three or more turns were not uncommon), and that in young cultures the cells were predominantly half-curves. Williams and Rittenberg provided photomicrographs of stained cells of Pringsheim's strain. Wells and Krieg (19) provided phase-contrast photomicrographs of cells of Wells' strain, as well as photographs of the "volutin" granules (which in fact contain PHB) and of the microaerophilic bands formed in wet mounts. They reported that cells cultured in nutrient broth possessed a cell diameter of 1.5 to 1.8 μm , wavelength of 16 to 24 μm , and a cell length of 16 to 24 μm . McElroy et al. (10) provided photomicrographs of Wells' strain stained with fluorescent antibody. Figure 1 shows the appearance of Wells' strain by dark field microscopy, and Figure 2 depicts its bipolar fascicles of flagella as seen by electron microscopy. The coordinated behavior of the polar fascicles of this strain, the dual-tail and dual-head uncoordination caused by various chemical agents, and the microaerotactic behavior of the organism were described and photographed by Krieg et al. (9). The polar fascicles of Pringsheim's strain can be seen in the electron micrographs by Williams (22).

Both strains are gram negative.

Isolation. Isolation of both strains was accomplished by the capillary tube procedure of Rittenberg and Rittenberg (14). Ordinary isolation methods are uniformly ineffective for isolation of this species.

Cultural characteristics. Both strains were obligately microaerophilic, requiring an atmosphere of 1 to 9% oxygen for growth (19). No growth occurred anaerobically, even in the presence of nitrate. Growth in nutrient broth was scanty. Abundant cloudy growth was produced in PSS broth, but higher turbidities and a faster growth rate occurred when the peptone concentration was decreased to 0.5%. It was important to use KOH rather than NaOH for adjusting the pH of media, since the strains are inhibited by Na^+ at low concentrations. If PSS broth was inoculated very heavily, or if semisolid PSS medium was used, it was unnecessary to decrease the level of oxygen within the culture vessel.

Neither strain was able to grow on plates of PSS medium containing 1.5% agar, even with low oxygen levels. Light confluent growth of both strains occurred in 3 to 4 days on plates of PSS medium containing 0.7% agar when heavily inoculated and incubated under decreased oxygen in a moist atmosphere. Separated colonies of Wells' strain have been obtained in PSS medium containing 0.7% agar by dilution

FIG. 1. Living cells of Wells' strain of *Spirillum volutans* seen by dark field microscopy. The coils have been flattened by the pressure of the cover slip, and some of the polar flagellar fascicles have become split into subbundles. Magnification, $\times 1,225$.

FIG. 2. The polar flagellar fascicles of Wells' strain of *Spirillum volutans* seen by electron microscopy. Shadowed with tungsten oxide. A, $\times 9,200$; B, $\times 23,100$.

plating (Fig. 3) after 3 to 4 days. When the subsurface, pinpoint, irregular colonies were examined at a magnification of $\times 100$, the individual spirilla could be seen but they were nonmotile. If a colony was picked from the

agar and suspended in a drop of water, the cells were then seen to be actively motile. The larger surface colonies (Fig. 3) were not present unless plates were incubated under decreased oxygen.

Using a modified PSS broth, McElroy et al.

FIG. 3. A, Colonies of Wells' strain of *Spirillum volutans* from a dilution plating in semisolid (0.7% agar) PSS medium at 4 days, with incubation under 30% air-70% nitrogen. B, Portion of same plate at $\times 2.6$. The larger colonies are surface colonies.

(10) reported an average generation time of 4.2 h for Wells' strain.

Physiological characteristics. A summary of the physiological characteristics of the two strains is presented in Table 1. The catalase test was weak, but a definite froth of bubbles appeared within 30 min compared to sterile controls.

In PSS broth, growth of Wells' strain was found to be inhibited by concentrations of added phosphate greater than 0.003 M. A similar phosphate toxicity was noted by Cole and Rittenberg (4) for Pringsheim's strain. These investigators also detected cytochromes *b* and *c* and determined levels of tricarboxylic acid cycle enzymes, cytochrome oxidase, nicotinamide adenine dinucleotide, reduced form, oxidase and catalase in this strain.

Neither strain of *S. volutans* has yet been cultivated in a defined medium, and the nutritional requirements remain to be determined. Abundant growth occurred, however, in a modified PSS broth in which 0.25% vitamin-free, salt-free, acid-hydrolyzed casein (Nutritional Biochemical Corp., Cleveland, Ohio) was

substituted for peptone. Attempts to substitute mixtures of amino acids for the casein hydrolysate have not yet been successful.

T_m values for the DNA of Wells' and Pringsheim's strains, respectively, were 84.9 C and 84.8 C, corresponding to a base composition of 38 mol % guanine plus cytosine for both strains.

Because *S. volutans* appears to have been the first species designated as the type species of *Spirillum*, according to the internationally accepted rules of nomenclature it should be so recognized irrespective of the fact that *S. undula* has for many years been regarded as the type. Furthermore, whereas *S. volutans* can presently be recognized from Ehrenberg's description (8), *S. undula* cannot. In fact, the name *S. undula* is a source of confusion and error. Consequently the Judicial Commission is requested to issue opinions establishing *S. volutans* Ehrenberg as the type species of *Spirillum* Ehrenberg and placing *S. undula* (Müller) Ehrenberg on the list of rejected names.

Wells' strain (ATCC 19554), described above,

TABLE 1. *Physiological characteristics of Spirillum volutans ATCC 19553 and 19554*

Test	Time when test performed (days)	Reaction
Catalase ^a	1	Weak
Phosphatase (0.01% phenolphthalein diphosphate) ^b	2	+
Sulfatase (0.01% phenolphthalein disulfate) ^b	2	—
Oxidase (moistened test disc inoculated with centrifuged culture) ^c	2	+
H ₂ S from 0.2% cysteine (detector strips) ^c	7	+
Urease ^d	1	—
Liquefaction of 12% gelatin ^c	4	—
Hydrolysis of 0.1% esculin ^c	4	—
Hydrolysis of casein (single-strength milk) ^b	14	—
Hydrolysis of 0.1% DNA (clear zone after acid addition) ^b	7	—
Hydrolysis of 0.1% ribonucleic acid (clear zone after acid addition) ^b	7	—
Indole production from 0.1% tryptophan ^c	2	—
Hydrolysis of 10% soluble starch ^b	14	—
Aerobic reduction of 0.1% KNO ₃ ^c	2	—
Aerobic reduction of 0.3% H ₂ SeO ₃ (by pink color) ^c	7	—
Visible growth with 1% bile ^c	14	—
Visible growth with 1% glycine ^c	14	—
Anaerobic growth with 0.1% KNO ₃ (sealed with petrolatum) ^a	7	—
Growth with 3% NaCl ^c	14	—
Growth in MRVP broth (Difco), 6% oxygen	7	—
Acid reactions from carbohydrates (38 compounds tested) ^e	1–21	—

^a Basal medium = PSS + 0.15% agar.

^b Basal medium = PSS + 0.7% agar.

^c Basal medium = PSS broth.

^d Cells suspended in distilled water to a dense, milky concentration; 0.5 ml added to 2.0 ml of the following medium: BES buffer, *N,N*-bis-(2-hydroxy-ethyl)-2-aminoethane sulfonic acid (0.1065%); urea, 2.0%; phenol red, 0.001%; pH 7.0. Controls without urea were used.

^e Basal medium = PSS broth lacking succinate and with peptone decreased to 0.2%; 0.0018% phenol red indicator added.

is here designated as the neotype strain of *Spirillum volutans* Ehrenberg.

REPRINT REQUESTS

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