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Morphology as a Basis for Taxonomy of Large Spirochetes Symbiotic in Wood-Eating Cockroaches and Termites: Pillotina gen. nov., nom. rev.; Pillotina Calotermitidis sp. nov., nom. rev.; Diplocalyx gen. nov., nom. rev.; Diplocalyx calotermitidis sp. nov., nom. rev.; Hollandina gen. nov., nom.

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Morphology as a Basis for Taxonomy of Large Spirochetes Symbiotic in Wood-Eating Cockroaches and Termites: *Pillotina* gen. nov., nom. rev.; *Pillotina calotermitidis* sp. nov., nom. rev.; *Diplocalyx* gen. nov., nom. rev.; *Diplocalyx calotermitidis* sp. nov., nom. rev.; *Hollandina* gen. nov., nom. rev.; *Hollandina pterotermitidis* sp. nov., nom. rev.; and *Clevelandina reticulitermitidis* gen. nov., sp. nov.

DAVID BERMUDES, DAVID CHASE, AND LYNN MARGULIS

The purposes of this paper are (i) to present a framework for the morphometric analysis of large uncultivable spirochetes that are symbiotic in wood-eating cockroaches and termites; (ii) to revive, in accordance with the rules of the *International Code of Nomenclature of Bacteria*, the names of three genera (*Pillotina*, *Diplocalyx*, and *Hollandina*) and three species (*Pillotina calotermitidis*, *Diplocalyx calotermitidis*, and *Hollandina pterotermitidis*) for the same organisms to which the names were originally applied, because these names were not included on the 1980 Approved Lists of Bacterial Names; and (iii) to formally propose the name *Clevelandina reticulitermitidis* for a new genus and species of spirochetes from the termite *Reticulitermes tibialis*. None of these genera and species has been cultivated either axenically or in mixed culture; hence, all are based on type-descriptive material.

Large spirochetes living under the anaerobic or microaerophilic conditions in intestines of wood-eating cockroaches and termites have never been cultivated (31). A new genus and species, "*Pillotina calotermitidis*," was proposed in 1967 by Hollande and Gharagozlou (10) for a spirochete from the termite *Calotermes praecox*. Another new genus and species, "*Diplocalyx calotermitidis*," was proposed for a separate morphotype from the hindgut of the termite *Calotermes flavicollis* by Gharagozlou (8) in 1968. A third genus and species, "*Hollandina pterotermitidis*" from the hindgut of the termite *Pterotermes occidentis*, was described by To et al. in 1978 (31). The genus "*Clevelandina*" has been informally proposed previously (26). Reports on these types of spirochetes have been reviewed by Breznak (6).

The work of Hollande and Gharagozlou is of particular interest because hollow tubules, 25 nm in diameter, were reported to be in the protoplasmic cylinder. Immunocytochemical investigations demonstrate the presence of antitubulin immunoreactivity in these large spirochetes (25). Further investigations of small cultivable spirochetes have resulted in the isolation of tubulinlike proteins (1). However, not all large spirochete morphotypes appear to have cytoplasmic tubules. Characterization of spirochetes with or without tubules requires distinctions among morphotypes.

We have examined the following four species of subterranean and dry-wood-eating termites by electron microscopy: *Pterotermes occidentis*, *Reticulitermes hesperus*, *Reticulitermes tibialis*, and *Incisitermes minor*. Our observations provide a framework for the morphometric analysis of uncultivable spirochetes that are symbiotic in wood-eating termites and cockroaches.

In this paper we revive the genera "*Pillotina*," "*Diplocalyx*," and "*Hollandina*" and the species "*Pillotina calotermitidis*," "*Diplocalyx calotermitidis*," and "*Hollandina pterotermitidis*," in accordance with the rules of the *International Code of Nomenclature of Bacteria* (24), for the same organisms to which the names were originally applied, because they were not included on the 1980 Approved Lists of Bacterial Names (30). We formally propose the name "*Clevelandina reticulitermitidis*" for the type species of the new spirochete genus "*Clevelandina*" from the termite *Reticulitermes tibialis*.

MATERIALS AND METHODS

Sources of termite spirochetes. Termites were collected as indicated in Table 1. They were maintained in a laboratory, and the guts were removed for light and electron microscopic observations (31, 33).

Light microscopy. For observation of living material, freshly dissected guts were placed upon a glass microscope slide, covered with 1 or 2 drops of Trager solution A or Trager solution U (34), and pierced with a dissection needle. A cover glass was then sealed over the preparation with petroleum jelly.

Electron microscopy. For preparation of thin sections, entire termite guts were fixed with 3% glutaraldehyde in 0.025 M phosphate buffer at pH 6.8, postfixed with 1% osmium tetroxide in the same buffer, dehydrated in acetone, and embedded in Spurr resin by using the methods of Bloodgood (3) and Bloodgood and Fitzharris (4) as modified by To et al. (31) (2 to 5% tannic acid was added to the fixative for some of the samples and the impregnation time was extended to 24 h). Thin sections were stained first with 2% uranyl acetate and then with 2% lead citrate.

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‡ Deceased.

TABLE 1. Sources and identification of North American termites used in this study

Isopteran insect	Locality	Collected by:	Identified by:
Subterranean termites (Reticulitermitidae)			
Reticulitermes hesperus	San Diego, Calif.	L. Margulis	D. Chase
Reticulitermes tibialis	Joshua Tree National Monument, Nev.	D. Chase	D. Chase
Dry-wood-eating termites (Kalotermitidae)			
Pterotermes occidentis	Tucson, Ariz.	L. Margulis	W. Nutting
Incisitermes (Kalotermes) minor	Los Angeles, Calif.	D. Chase	D. Chase
Incisitermes (Kalotermes) snyderi	Naples, Fla.	S. F. Bermudes	P. Luykx

Morphometrics. A total of 15 morphological criteria were measured (Table 2 and Fig. 1). The averages reported below represent measurments of all material from the original publications and from 3 to 20 examples of each morphotypes from the termites which we examined. Diameters (criterion 1) in oblong cells were measured at the narrowest and widest points enclosed by the cell membrane. In crenulated cells, measurements were made at the tips of the crenulations. The number of flagella (criterion 2) is reported as the number inserted at each end, approximated by the median number observed. The number of flagella observed could range from zero (beyond the most distal insertion) to twice the number of flagella inserted at one end, where, toward the center, flagella overlapped with the flagella from the other end. Previous descriptions (8, 10, 26, 31) have reported the maximum number observed in cross sections. The sillon (10) or groove (criterion 3) is an invagination of the outer membrane toward the inner membrane (Fig. 1B). This structure provides a reference point for the description of other structures. Crenulations (criterion 4) are conspicuous folds in the outer membrane (Fig. 1B). Criteria 5 through 7 deal with the presence of coatings on the inner and outer membranes of the gram-negative cell walls (i.e., the glycocalyx or sheath); the use of a ratio results in partial compensation for slightly oblique sections. When such surface layers are absent, the ratio of their thickness to the thickness of the membrane itself is zero. Absolute values are also given below. Spirochetes and protoplasmic cylinder cross sections of oblong shape were measured at both their widest (Fig. 1C) and narrowest points. The method for determining the ratio of the protoplasmic cylinder diameter to the overall cell diameter (criterion 8) is illustrated in Fig. 1C. By using a ratio, comparisons could be made among spirochetes of different diameters. Criterion 9, the angle subtended by the flagella, is a measurement of the distribution of the flagella in the periplasm (Fig. 1D). Flagellar bundles (criterion 10) were deemed to be present if the angle subtended by the flagella was less than 180° and in areas where the flagella were not present, the outer membrane was in close apposition to the inner membrane (Fig. 1E). In such cases, a concavity was formed by the outer membrane near the junction between the flagellar bundle and the protoplasmic cylinder. An angle was formed by three points. Two points were formed by the intersection of a straight line with the outermost region of the outer membrane (Fig. 1E, line A, points 1 and 2). A third point was formed by the intersection of a line perpendicular to line A (line B) with the outer membrane at a distance maximal from line A. The angle of concavity was measured as the angle formed by the intersection of a line between points 1 and 3 (line C) and a line between points 2 and 3 (line D). The angle was less than 180° when a bundle was present. Cytoplasmic tubules (criterion 14) were small, hollow, walled structures as observed by electron microscopy. The polar organelle (synonym, polar membrane) (criterion 15) was an electron-dense lamina located on the inside of the inner membrane toward the distal end of the cell (Fig. 1B).

RESULTS AND DISCUSSION

Morphological characteristics. The major criteria (criteria 1 through 10) for distinguishing spirochete morphotypes are listed in Table 2. Criteria 11 through 15 provide less impor-

TABLE 2. Morphological criteria for assigning spirochetes to genera

Criterion		Figure illustrating criterion	Overall range in termite and cockroach spirochetes
No.	Description		
1	Diameter	1A or C	0.4–1.5 μm
2	Number of flagella	1B	15–70
3	Presence of sillon	1B	+,−[a]
4	Presence of crenulations	1B	+,−
5	Ratio of thickness of outer coat of outer membrane to outer membrane thickness	1B	0–5
6	Ratio of thickness of inner coat of outer membrane to outer membrane thickness	1B	0–12
7	Ratio of thickness of outer coat of inner membrane to inner membrane thickness	1B	0–8
8	Ratio of diameter of protoplasmic cylinder to cell diameter	1C	0.5–0.9
9	Angle of protoplasmic cylinder subtended by flagella	1D	50–350°
10	Presence of flagellar bundle	1E	+,−
11	Length	1A	10–>100 μm[b]
12	Amplitude	1A	0.3–0.6 μm[b]
13	Wavelength	1A	1.5–3.0 μm[b]
14	Presence of cytoplasmic tubules	1B	+,−[c]
15	Presence of polar organelle	1B	+,−

[a] +, Present; −, absent.
[b] Not correlated with species.
[c] The diameter of the tubules ranges from 15 to 25 nm.

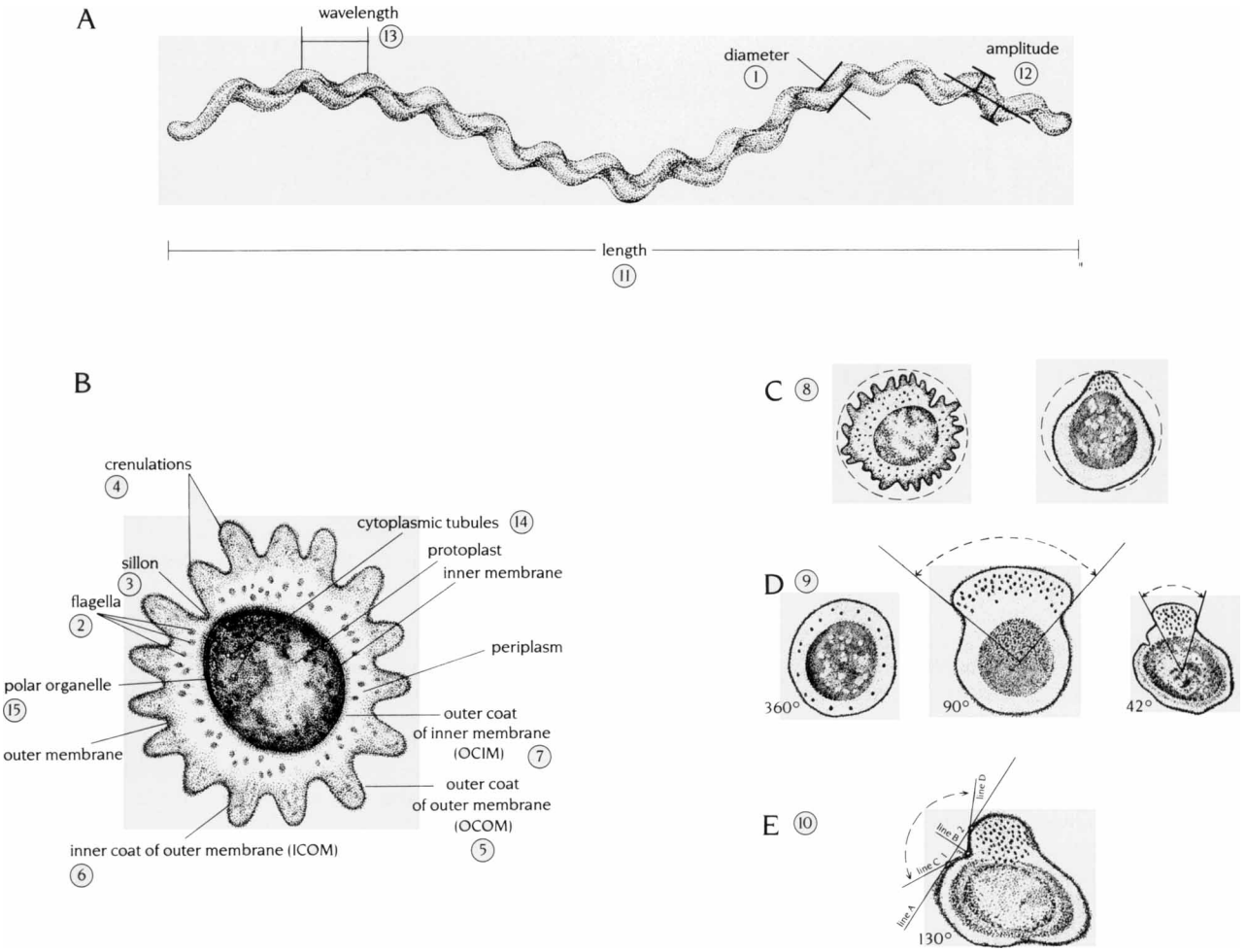


FIG. 1. Morphological criteria for distinguishing spirochetes. The numbers in circles refer to the criteria listed in Tables 2 and 3. (A) Criteria visible by light microscopy (drawing by S. Alexander). (B) Idealized transverse section of *Pillotina* membranes and cytoplasmic tubules (drawing by J. Kaczman). (C) Two transverse sections showing how the ratio between the cell diameter and the periplasmic cylinder diameter is measured in irregularly shaped spirochetes (drawing by C. Lyons). (D) Three transverse sections indicating how angles subtended by flagella are measured (drawing by C. Lyons). (E) Transverse section showing how the angle of concavity is determined (drawing by C. Lyons).

tant but still useful bases for distinction among the morphotypes which we observed.

Spirochetes from the hindguts of wood-eating cockroaches and termites may possess a sillon, crenulations, or elaborate coatings of their membranes. The flagella and flagellar insertions of the large spirochetes have the appearance of the flagella and flagellar insertions of typical gram-negative bacteria (Fig. 2). The number of flagella ranges up to approximately 70 but is consistent within morphotypes. Some spirochetes tend to have flagella equally distributed around the protoplasmic cylinders, whereas in other spirochetes the flagella are confined to a limited portion of the circumference. Spirochetes with a low value for the angle subtended by the flagella tend to have their outer membrane tightly apposed to the inner membrane in regions where the flagella are not present. In those spirochetes with a high value (up to 350°) the outer membrane is not apposed, and the flagella are distributed throughout the periplasmic space. The thickness of the outer and inner membranes of the spirochetes which we examined ranged between 6.0 and 7.0 nm (average, 6.6 nm). Coatings on the outer and inner membranes very greatly in thickness among morphotypes but are relatively constant within individual morphotypes.

Length, amplitude, and wavelength are apparently consistent features of spirochetes observed by light microscopy. However, all of the termites examined harbor more than a single morphotype. The presence of morphotypes overlapping in diameter prevents the direct correlation of morphotype diameters observed by transmission electron microscopy with morphotype diameters observed by light microscopy. Because overall length, wavelength, and amplitude are not observable in individual transmission electron micrographs, diameter is the only morphological feature that is observable by both forms of microscopy. Thus, the spirochete features observed by transmission electron microscopy cannot yet be reliably correlated with any of the other morphological features observed by light microscopy. Because of this unresolved problem we disregarded previous reports on the length, amplitude, and wavelength (9, 10, 26, 31) of these spirochetes.

The presence of cytoplasmic tubules is more significant

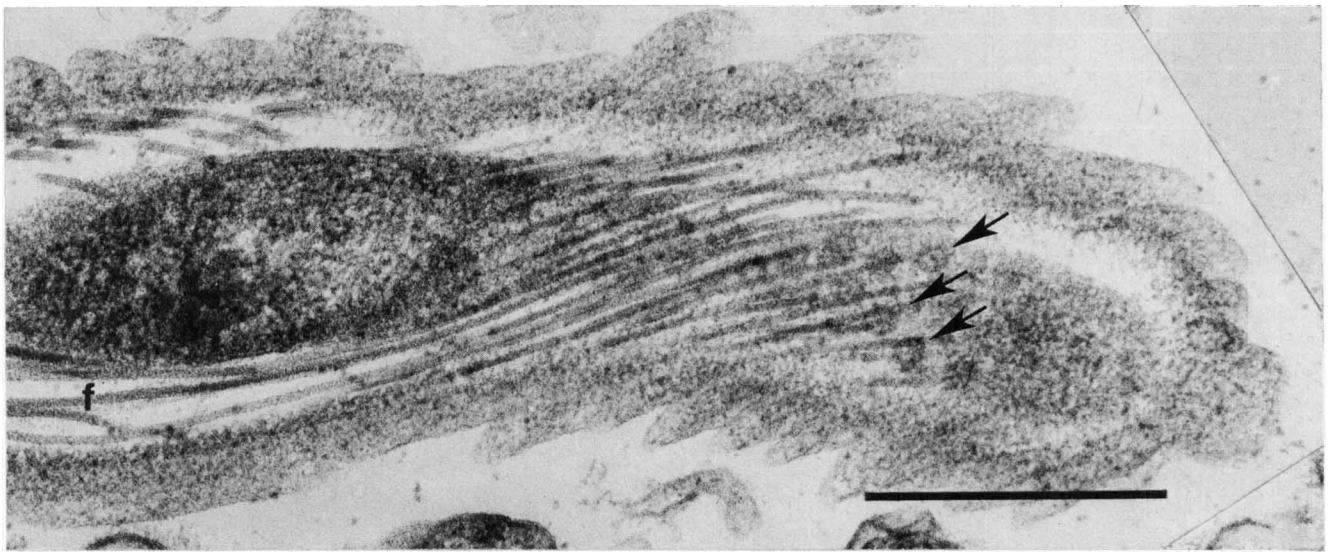


FIG. 2. Flagellar insertions (arrows) of *Pillotina* sp. from *Reticulitermes hesperus*. f,flagella. Bar = 0.5 μm. Magnification, ×79,800. Previously published by Margulis et al. (26). Published by permission of Springer-Verlag.

than their absence; even in spirochetes such as "*Pillotina*," in which tubules have been observed many times, never does every section of the organism have them (Fig. 3). Micrographs that are of high enough quality and show appropriate sections (anterior and apposed to the flagellar basal insertion) reveal the polar organelle (Fig. 4). Although not mentioned as such, the polar organelle is clearly visible in the large spirochetes described by Grimstone in 1963 (9) and in the *Cristispira* described by Ryter and Pillot (29).

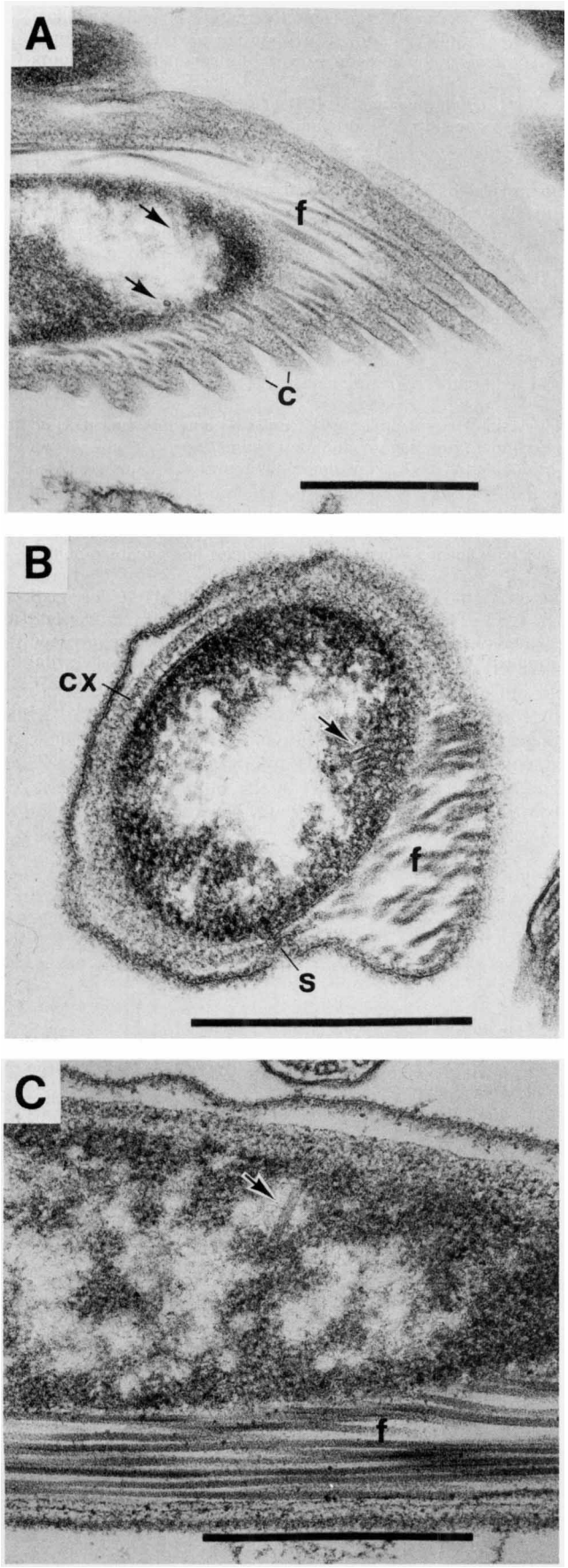
Spirochete morphotypes. The hypertrophied intestines of healthy members of any given termite species contain the same spirochete morphotypes. The great consistency of spirochete morphotypes (Fig. 5A and 6) in all healthy individuals of a given species, regardless of cast and age, demonstrates that these morphotypes are identifiable and distinct. Although in this paper we describe only large morphologically complex spirochetes, all of the termites examined also contained an abundance of smaller spirochetes (diameter, <0.4 μm) (Fig. 6).

The large spirochetes, which are characterized by their size, lack of hooked ends, and habitat (the anaerobic or microaerophilic digestive tracts of animal hosts), belong to the family *Spirochaetaceae*. Cockroach and termite gut spirochetes differ from previously described genera. They are enteric in these insect hindguts and are intolerant of atmospheric oxygen. Thus, their habitat distinguishes them from *Spirochaeta*, spp., which are free-living, and from *Cristispira* spp., which are aerotolerant inhabitants of the crystalline styles of mollusks. Unlike *Borrelia*, the termite and roach spirochetes are not pathogenic (26). *Treponema* spp. cells (Table 3) tend to be small, usually 0.1 to 0.4 μm in diameter, and have relatively few flagella (<10 flagella), whereas the cells of the spirochetes which we examined tend to have diameters in excess of 0.4 μm and 15 to 70 flagella. Furthermore, the cockroach and termite hindgut spirochetes exhibit the following morphological features which are virtually absent from the other spirochete genera: crenulations, sillons, and elaborate coatings on their outer membranes. Their morphological complexity, coupled with their large size and number of flagella, definitively distinguishes the cockroach and termite hindgut spirochetes from other spirochetes.

Applying the criteria described above to the large spirochetes studied by electron microscopy from the guts of wood-eating cockroaches and termites yields at least four major morphotypes (Table 4). Examples of these morphotypes are shown in Fig. 2 through 7. For these morphotypes we revive the three genera and species described below and formally propose the new genus *Clevelandina* and the new species *Clevelandina reticulitermitidis*.

Description of *Pillotina* (ex Hollande and Gharagozlou 1968) gen. nov., nom. rev. *Pillotina* (Pil.lo.ti'na. M.L. gen. *Pillotina*, in honor of J. Pillot, a French microbiologist). Symbiotic in the guts of wood-eating cockroaches and termites; probably anaerobic or microaerophilic. Helical cells are 0.6 to 1.5 μm in diameter. Stellate profile with approximately 30 to 70 flagella distributed throughout the periplasmic space as viewed in transverse section. Outer membrane crenulated (i.e., pleated or folded) (Fig. 1B, 2, 3A, and 5A and B). Outer coat of outer membrane either present or absent. One of the grooves between the ridges is deeper and narrower, forming the sillon. The type species is *Pillotina calotermitidis* from the termite *Calotermes praecox*.

Description of *Pillotina calotermitidis* (ex Gharagozlou 1968) sp. nov., nom. rev. *Pillotina calotermitidis* (cal.o.ter. mit.'id.is. M.L. gen. *calotermitidis*, named after the termite host *Calotermes* [synonyms, *Kalotermes, Postelectrotermes*] *praecox*). Helical cells are 1.0 to 1.5 μm in diameter, with 20 parallel ridges extending in a loosen spiral around the long axis nearly the full length, seen as crenulations in cross section. Alternate pairs of lower and higher ridges in the outer membrane are symmetrically placed on either side of the sillon. There are approximately 70 periplasmic flagella which are 15 to 18 nm in diameter. Angle subtended by the flagella, 350°; concavity absent; no flagellar bundle. The distribution of the flagella is delimited by a lamina between the inner and outer membranes. An outer coat of the outer membrane is absent. The thickness of the inner coat of the outer membrane ranges from 22 to 77 nm (measured from the bottom of the crenulations toward the center of the cell) (average, 49.5 nm). The ratio of the thickness of the inner coat of the outer membrane to the outer membrane thickness ranges from 3.3 to 11.9 (average, 6.7). An outer coat of the inner membrane is absent. The protoplasmic cylinder is



circular to oblong in cross section and 0.5 to 0.7 μm in diameter. The ratio of the protoplasmic cylinder diameter to the cell diameter is 0.47 to 0.50 (average, 0.49). Microtubules, ca. 25 nm in diameter, are occasionally found within the protoplasmic cylinder. The type specimen (10) is from *Calotermes praecox* from the Island of Madeira, Portugal.

Description of *Diplocalyx* (ex Gharagozlou 1968) gen. nov., nom. rev. *Diplocalyx* (Dip.lo.ca.'lyx. Gr. *diploos*, twofold; L. n. *calyx*, cup, cover; M.L. n. *Diplocalyx*, twofold cover). Symbiotic in termites; probably anaerobic or microaerophilic. Helical cells are 0.7 to 0.9 μm in diameter. Approximately 40 to 60 periplasmic flagella are confined to a tight bundle between the inner and outer membranes, and there is a sillon which varies in position in different morphotypes (26). A thick outer coat of the inner membrane, forming a calyx, is present except at the flagellar bundle and sillon. Crenulations are lacking in the outer membrane. Microtubules, ca. 21 nm in diameter (Fig. 3B), and polar organelles are occasionally seen. The type species is *Diplocalyx calotermitidis* from the termite *Incisitermes flavicollis*.

Description of *Diplocalyx calotermitidis* (ex Gharagozlou 1968) sp. nov., nom. rev. *Diplocalyx calotermitidis* (cal.o.ter.mi.'ti.dis. M.L. gen. *calotermitidis*, named for the host termite *Calotermes* [synonyms *Kalotermes*, *Incisitermes*] *flavicollis*). Helical cells, lobate in cross section, are 0.8 μm in diameter, with approximately 40 flagella (diameter, 20 nm) in a tight bundle. The angle subtended by the flagella ranges from 50 to 70°. The angle of concavity ranges from 150 to 160°. The thickness of the outer coat of the outer membrane varies from 3.8 to 13.5 nm (average, 12 nm). The ratio of the thickness of the outer coat of the outer membrane to the outer membrane thickness is between 0.6 and 2.0 (average, 1.7). The thickness of the inner coat of the outer membrane ranges from 6 to 14 nm (average, 10 nm). The ratio of the thickness of the inner coat of the outer membrane to the inner membrane thickness varies from 0.9 to 2.1 (average, 1.5). The thickness of the outer coat of the inner membrane (the calyx) ranges from 42 to 51 nm (average, 48 nm). The ratio of the thickness of the outer coat of the inner membrane to the inner membrane thickness is between 6.4 and 7.7 (average, 7.3). The protoplasmic cylinder is slightly oblong and 0.38 μm (narrowest point) to 0.43 μm (widest point) in diameter. The ratio of the protoplasmic cylinder diameter to the cell diameter is between 0.48 and 0.53 (average, 0.51). The outer coat of the inner membrane is bisected by the sillon. The position of the sillon is opposite the flagellar bundle. The type specimen (8) is from *Calotermes flavicollis* found in southern France.

Description of *Hollandina* (ex To et al. 1978) Gen. nov., sp. nov., nom. rev. *Hollandina* (Hol.lan.din.'a. M.L. gen. *Hollandina*, in honor of Andre Hollande, Jr., a French protistologist). Symbiotic in the hindguts of wood-eating cockroaches and termites; probably anaerobic or microaerophilic. Helical cells rounded to oblong in cross section and 0.4 to 1.0 μm in diameter. A thick coat on the outer surface of the outer membrane is usually present, and

FIG. 3. Cytoplasmic tubules (arrows). Distribution and orientation vary. f, flagella. (A) *Pillotina* sp. from *Reticulitermes hesperus*. Tubules are 18 nm in both cross section and longitudinal section. c, crenulations. Bar = 0.5 μm. Magnification, ×52,200. (B) *Diplocalyx* sp. from *Incisitermes minor*. Two tubules are 21 nm in diameter. s, sillon. cx, calyx. Bar = 0.5 μm. Magnification, ×83,100. (C) *Hollandina* sp. from *Incisitermes minor*. The cytoplasmic tubule is 20 nm in diameter. Bar = 0.5 μm. Magnification, ×79,800.

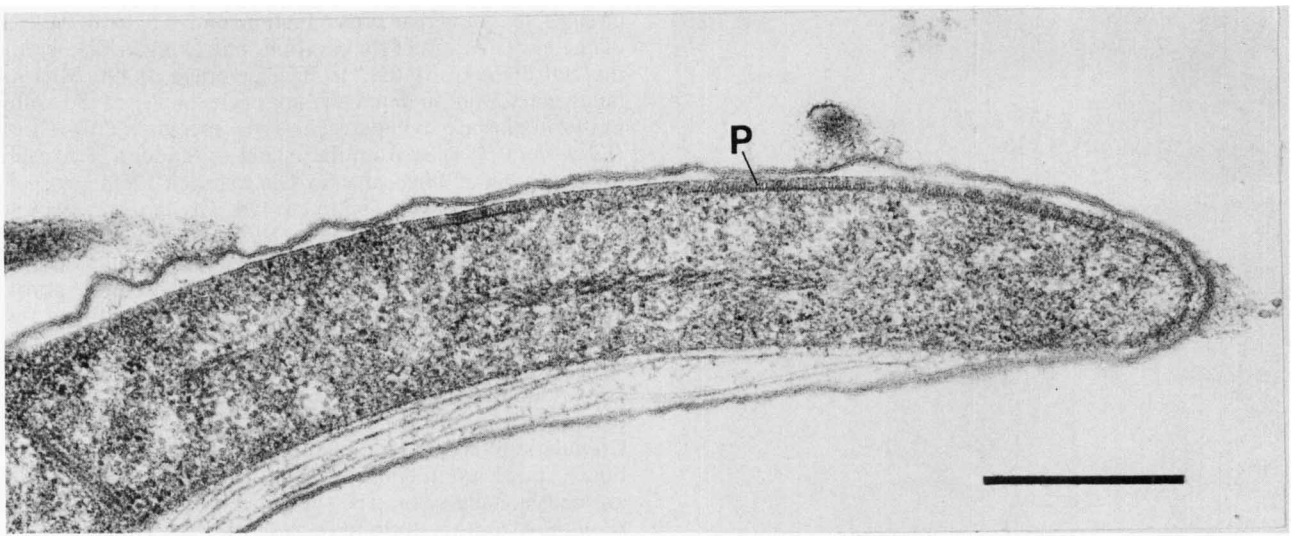


FIG. 4. Polar organelle (P) of a *Hollandina* sp. from *Incisitermes minor*. Bar = 0.5 μm. Magnification, ×52,600. Previously published by Margulis et al. (26). Published by permission of Springer-Verlag.

crenulations and lacking. Cells have from 15 to 60 flagella distributed in the periplasmic space. A sillon is absent or present (Fig. 6). Polar organelles (Fig. 4) and microtubules (Fig. 3C) have been observed in some types. The type species is *Hollandina pterotermitidis* from the termite *Pterotermes occidentis*.

Description of *Hollandina pterotermitidis* (ex To et al. 1978) sp. nov., nom. rev. *Hollandina pterotermitidis* (pter.o.ter.mi.ti.'dis. M.L. gen. *pterotermitidis*, named for the host termite *Pterotermes occidentis*). Helical cells oblong in cross section and 0.40 to 0.42 μm (narrowest point) to 0.53 to 0.65 μm (widest point) in diameter. The outer membrane is in close proximity to the inner membrane over a wide area for most of the cell length. A narrow groove (the sillon) is present toward the center. Cells have 15 flagella that are 15 to 16 nm in diameter. The angle subtended by the flagella ranges from 110 to 130° (average, 122°). The angle of concavity ranges from 170 to 180° (average, 175°). The thickness of the outer coat of the outer membrane ranges from 17 to 20 nm (average, 19 nm). The ratio of the thickness

TABLE 3. Comparative morphology of anaerobic symbiotic spirochetes: treponemes, brachyspiras, and borrelias

Taxon	Length (μm)	Width (μm)	Wavelength (μm)	Amplitude (μm)	No. of flagella	Shape of ends[a]	Presence of cytoplasmic tubules	Presence of polar organelle	Presence of sheathed flagella	Presence of flagellar bundles	Reference(s)
Treponemes and brachyspiras (no vectors)											
Treponema calligyrum	8–12	0.19–0.26	1.15	0.30	2–3	ta	+[b]	NDT[c]	+	+	16
Treponema genitalis	4–11	0.18	1.1	0.15	2–4	ta	+	NDT	+	+	15
Treponema hyodysenteriae	8–10	0.23–0.45	3.5[d]	0.5[d]	8–9	ta	−[d]	NDT	+[d]	+[d]	22
Treponema innocens	8–14	0.31–0.40	3.5[d]	0.5[d]	7–10	ta	−[d]	NDT	+[d]	+[d]	22
Treponema microdentium	4–10	0.15	0.9	0.15	1–3	bl	+	NDT	+	+	13
Treponema minutum	9–18	0.18	1.36	0.20	2–3	ta	+	NDT	+	+	13
Treponema pallidum	6–20	0.15	1.1	0.2–0.3	3	pt	+	NDT	+	+	12, 16
Treponema refringens	4–12	0.20–0.25	1.8	0.2–0.3	4–6	ta	+	NDT	+	+	17
Brachyspira aalborgi	1.7–6.0	0.2	2	ND[e]	4	ta	−	NDT	+	+	20
Rumen spirochetes[f]											
Strain PB (*Treponema saccharophilum*)	12–20	0.6–0.7	2.4	0.54	≥32	bl	ND	NDT	NDT	+	27, 28
Strain CA	10–15	0.4–0.5	ND	ND	16–20	ND	ND	ND	NDT	ND	27
Strain DA	12–15	0.6	ND	ND	≥32	ND	ND	ND	NDT	ND	27
Borellias (vector transferred)											
Borrelia baltazardi	14–20	0.35	2.0	0.35	25	pt	−	NDT	−	+	21
Borrelia burgdorferi	12–17	0.30	2.8	ND	7–11	pt	−	NDT	+	+	18
Borrelia merionesi	12–17	0.40	1.7	0.30	15–20	pt	−	NDT	−	+	14
Borrelia persica	16–23	0.45	2.0	0.35	25–30	pt	−	NDT	−	+	21
Borrelia recurrentis	12–16	0.50	1.7	0.30	15–20	pt	−	NDT	−	+	14

[a] ta, Tapered; bl, blunt; pt, pointed.
[b] +, Present; −, absent.
[c] NDT, Not detected.
[d] Unpublished data of K. Hovind-Hougen.
[e] ND, No data available.
[f] These anaerobic, host-associated spirochetes may require a new taxonomic treatment (28).

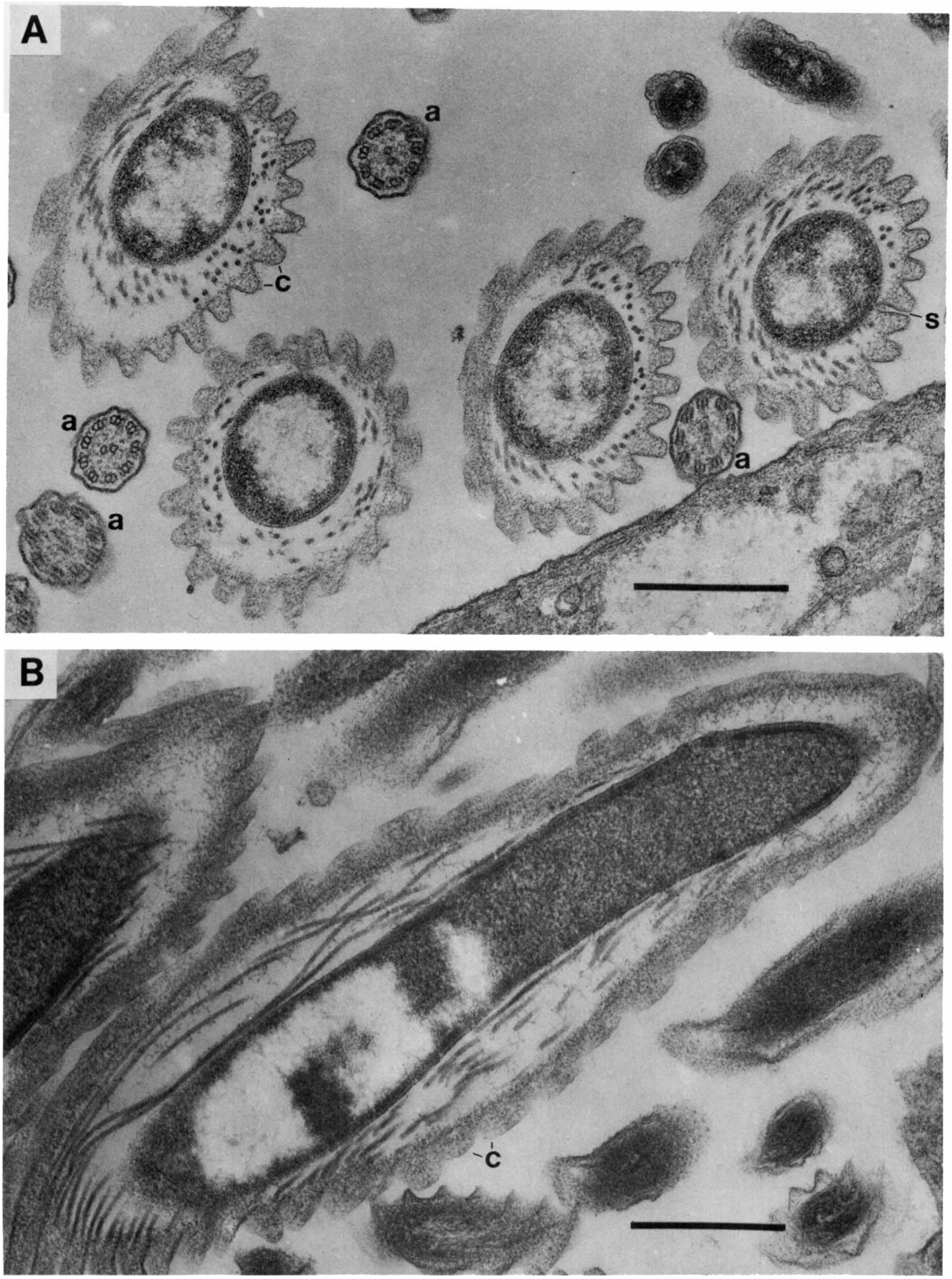


FIG. 5. *Pillotina* sp. from *Reticulitermes hesperus*. (A) Transverse sections showing crenulations (c), sillon (s), and consistency of ultrastructure. Bar = 0.5 μm. Magnification, ×52,600. (B) Longitudinal section. Bar = 0.5 μm. Magnification, ×53,000.

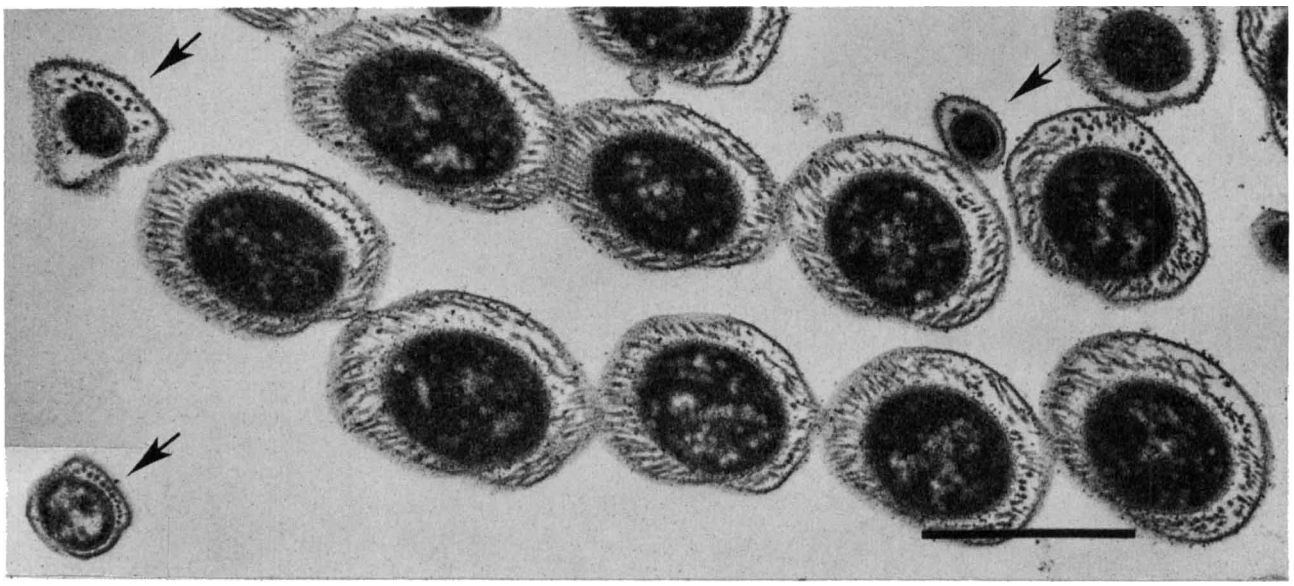


FIG. 6. *Hollandina* sp. from *Reticulitermes hesperus* showing consistency of ultrastructure and other small spirochetes (arrows). Bar = 0.5 μm. Magnification, ×55,600.

of the outer coat of the outer membrane to the outer membrane thickness is between 2.6 and 3.0 (average, 2.8). The thickness of the inner coat of the outer membrane ranges from 2 to 6 nm (average, 4.3 nm). The ratio of the thickness of the inner coat of the outer membrane to the outer membrane thickness is between 0.3 and 0.9 (average, 0.7). The thickness of the outer coat of the inner membrane ranges from 7 to 13 nm (average, 9.5 nm). The ratio of the thickness of the outer coat of the outer membrane to the inner membrane thickness varies from 0.8 to 2.0 (average, 1.4). The oblong protoplasmic cylinder is 0.33 to 0.38 μm (narrowest point) to 0.41 to 0.46 μm (widest point) in diameter. The ratio of the protoplasmic cylinder diameter to the cell diameter ranges from 0.78 to 0.93 (average, 0.86).

TABLE 4. Distinctions among genera of large symbiotic spirochetes based on morphological criteria

Criterion[a]	Genera						
	Cristispira[b]	*Pillotina*	*Hollandina*	*Diplocalyx*	*Clevelandina*	*Borrelia*[c]	Rumen spirochetes (*Treponema*)[d]
Primary							
Diam (μm)	0.5–3.0	0.6–1.5	0.4–1.0	0.7–0.9	0.4–0.8	0.5	0.4–0.7
Approximate no. of flagella	≥100	30–70	30–60	40–60	30–45	15–20	8–16
Presence of sillon	−[e]	+	+,−	+	+	−	−
Presence of crenulations	−	+	−	−	−	−	−
OCOM/OM[f]	ND[g]	0	2.4–8.0	0.6–2.0	0–2.8	1–3	ND
ICOM/OM[h]	ND	3.3–11.9	0–1.3	0.9–2.1	2.8–5.0 (chambered)	0	ND
OCIM/IM[i]	ND	0	1.0–2.5	6.4–7.7	0–3.6	1–2	ND
PC/diameter[j]	0.90	0.56–0.67	0.63–0.90	0.47–0.81	0.60–0.81	0.90–0.95	0.65–0.75
Angle subtended by flagella (°)	90–160	190–350	105–330	50–100	140–330	30–50	70–80
Presence of flagellar bundles	+	−	+,−	+	+,−	−	+
Auxiliary							
Length (μm)	30–180	ND	ND	ND	ND	12–17	10–20
Amplitude (μm)	4–6	ND	ND	ND	ND	0.3	ND
Wavelength (μm)	10–20	ND	ND	ND	ND	1.7	ND
Presence of cytoplasmic tubules	NDT[k]	+	+	+	NDT	−	NDT
Presence of polar organelle	+	+	+	+	NDT	−	NDT

[a] See Fig. 1.
[b] See references 5, 23, and 29.
[c] *Borrelia recurrentis* (16). Most other borrelias are smaller than 0.5 μm in diameter.
[d] Data from references 27 and 28. These anaerobic, host-associated spirochetes may require a new taxonomic treatment (28).
[e] +, Present; −, absent.
[f] Ratio of thickness of outer coat of outer membrane to outer membrane thickness.
[g] ND, No data available.
[h] Ratio of thickness of inner coat of outer membrane to outer membrane thickness.
[i] Ratio of thickness of outer coat of inner membrane to inner membrane thickness.
[j] Ratio of diameter of protoplasmic cylinder to cell diameter.
[k] NDT, Not detected.

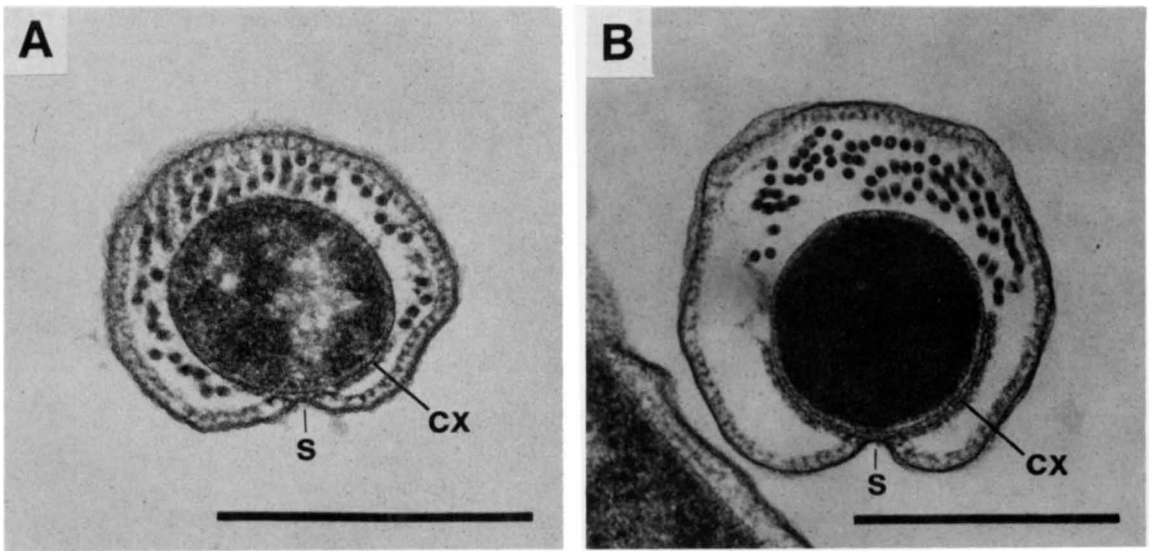


FIG. 7. *Clevelandina* ultrastructure. s,sillon. cx,calyx. (A) *Clevelandina reticulitermitidis* from *Reticulitermes tibialis*, cross section. Bar = 0.5 μm. Magnification, ×83,500. (B) *Clevelandina* sp. from *Reticulitermes flavipes*, cross section. Bar = 0.5 μm. Magnification, ×69,000. Courtesy of J. Breznak. Previously published by Breznak (6) and Margulis et al. (26). Published by permission of The Williams & Wilkins Co.

The type specimen is from the hindgut of the termite *Pterotermes occidentis* from the Sonoran Desert in Arizona.

Description of *Clevelandina* gen. nov. *Clevelandina* (Cleve.land.in'a. M.L. gen. *Clevelandina*, in honor of L. R. Cleveland [1892-1969], an American biologist). Symbiotic in the hindguts of termites; probably anaerobic or microaerophilic. Helical cells are oblong in cross section and 0.4 to 0.8 μm in diameter. Cells have 30 to 45 perpiplasmic flagella. The angle subtended by the flagella tends to be greater than 180°, and the inner coat (calyx) is correspondingly reduced. The cells are distinguished by a thick inner coat (layer) of the outer membrane with a chambered appearance and an outer coat of the inner membrane which is present except where the circumference is covered by flagella. Sillon present; no crenulations (Fig. 7A and B). The type species is *Clevelandina reticulitermitidis* from the termite *Reticulitermes tibialis*.

Description of *Clevelandina reticulitermitidis* sp. nov. *Clevelandina reticulitermitidis* (re.ti.cu.li.ter.mi.'ti.dis. M.L. gen. *reticulitermitidis*, named for the host termite genus *Reticulotermes*, in which it is found). Helical cells are slightly oblong and 0.52 to 0.61 μm (narrowest point) to 0.67 to 0.73 μm (widest point) in diameter. Approximately 45 flagella 17 to 20 nm in diameter are distributed throughout the periplasm. The angle subtended by the flagella is 260 to 270°; concavity absent; no flagellar bundle. The thickness of the outer coat of the outer membrane ranges from 0.0 to 18.6 nm (average, 11.5 nm). The ratio of the thickness of the outer coat of the outer membrane to the outer membrane thickness varies between 0.0 and 2.8 (average, 1.7). The inner coat of the outer membrane, which ranges from 25 to 27 nm thick (average, 25.3 nm), forms a chambered appearance in cross section. Chambers vary from 20 to 22 nm wide (average, 21 nm). The ratio of the thickness of the inner coat of the outer membrane to the outer membrane thickness varies from 3.8 to 4.1 (average, 3.9). The thickness of the outer coat of the inner membrane (calyx) varies between 12 and 20 nm (average, 14.6 nm). The ratio of the thickness of the outer coat of the inner membrane to the inner membrane thickness is between 1.8 and 3.8 (average, 2.8). The oblong protoplasmic

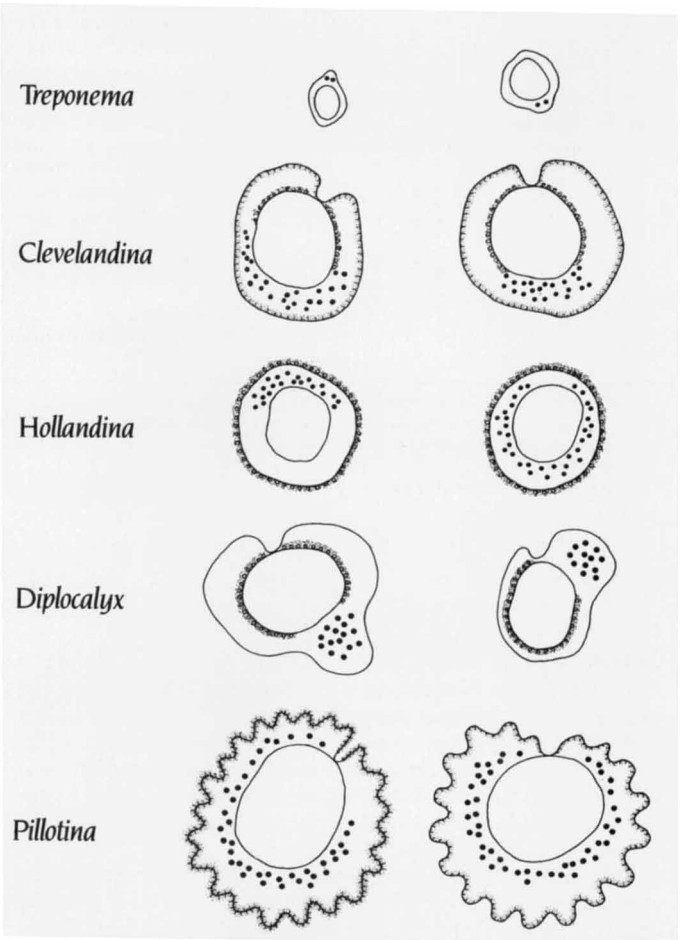


FIG. 8. Idealized drawings of *Treponema* and spirochete genera that are symbiotic in wood-eating cockroaches and termites (drawing by Laszlo Meszoly).

TABLE 5. Summary of distribution of spirochetes that are symbiotic in wood-eating cockroaches and termites[a]

Host family	Host genus	Location of host	Spirochete genus	Reference(s)
Order Blattaria				
Cryptocercidae (Protoblattidae; wood-eating cockroaches)	*Cryptocercus punctulatus*	United States	*Hollandina*	10
Order Isoptera				
Hodotermitidae (damp-wood termites)	*Porotermes adamsoni*	Australia	—[b]	
Kalotermitidae (dry-wood termites)	*Bifiditermes condonensis*	Australia	—	
	Ceratokalotermes apoliator	Australia	—	
	Cryptotermes brevis	United States	—	
	Cryptotermes cavifrons	United States	—	
	Cryptotermes gearyi	Australia	—	
	Glyptotermes iridipennis (*Kalotermes iridipennis*)	Australia	—	
	Glyptotermes neotuberculatus	Australia	—	
	Kalotermes approximatus[c]	United States	—	
	Kalotermes banksiae	Australia	—	
	Calotermes flavicollis[c]	France, Spain	*Diplocalyx*	8
	Kalotermes jouteli (*Neotermes jouteli*)	United States	—	
	Kalotermes minor (*Incisitermes minor*)	United States	*Hollandina*	26
	Kalotermes schwarzi (*Incisitermes schwarzi*)	United States	*Hollandina*	26, 31
			Pillotina	26, 31
	Kalotermes snyderi[d]	United States	*Hollandina*[e]	
	Incisitermes milleri	United States	—	
	Marginitermes hubbardi (*Kalotermes hubbardi*)	United States	*Diplocalyx*	32
	Neotermes insularis	Australia	—	
	Paraneotermes simplicicornis	United States	—	
	Postelectrotermes praecox (*Calotermes praecox*)[c]	Madeira, Portugal	*Pillotina*	10
	Pterotermes occidentis	Mexico, United States	*Hollandina*	31
Mastotermitidae	*Mastotermes darwiniensis*	Australia	*Hollandina*	7
Rhinotermitidae (subterranean termites)	*Coptotermes aginaciformis*	Australia	—	
	Coptotermes formosanus	Hawaii	*Hollandina*	32
	Heterotermes aureus	United States	—	
	Reticulitermes flavipes	United States	*Clevelandina*	6
			Pillotina	6
	Reticulitermes hesperus	United States	*Clevelandina*[f]	
			Hollandina	26
			Pillotina	26
	Reticulitermes tibialis	United States	*Clevelandina*	26
			Hollandina	26

[a] See reference 26 for sources of termites unless otherwise noted.
[b] —, Absence of ultrastructural information on which identification could be based.
[c] Originally published nomenclature used (*Calotermes* is equivalent to *Kalotermes*).
[d] See Table 1.
[e] Unpublished data of B. Dorritie.
[f] Data not shown.

cylinder ranges from 0.34 to 0.42 μm (narrowest point) to 0.42 to 0.53 μm (widest point) in diameter. The ratio of the protoplasmic cylinder diameter to the cell diameter varies from 0.66 to 0.73 (average, 0.68). The type specimen (Fig. 7A) of *Clevelandina reticulitermitidis* is from *Reticulitermes tibialis* from Joshua Tree National Monument, Nev. (this termite was incorrectly identified as *Reticulitermes hesperus* by Margulis et al. [26]).

Distinctions among the genera. *Pillotina* is the only spirochete genus from termite or cockroach guts with crenulations. *Diplocalyx* has a flagellar bundle, sillon, and calyx. *Clevelandina* has a distinct chambered inner coat of the outer membrane. *Hollandina* tends to have thick coatings on the outer surface of the outer membrane and to lack crenulations, a calyx, and a chambered coat. Idealized drawings of the genera are shown in Fig. 8.

Distribution of large symbiotic spirochetes. Large spirochetes are found associated with the digestive systems of wood-eating cockroaches and termites. The spirochetes from *Reticulitermes flavipes* (6) are clearly *Pillotina* sp. and *Clevelandina* sp. according to the criteria set forth in this paper. Other spirochetes described previously are also identifiably *Hollandina* sp. (e.g., the large surface spirochetes attached to the protist *Mixotricha paradoxa* from the Australian termite *Mastotermes darwiniensis* [7] and the unidentified spirochetes pictured by Grimstone [9]; also see reference 11). The geographical and host distributions of these large spirochetes are summarized in Table 5.

Microtubulelike structures. Eucaryotic microtubules are nearly invariant at 24 nm in diameter, except in some protists, where they approach 36 nm. However, cytoplasmic tubules in spirochetes (e.g., Fig. 3) and other procaryotes vary over a factor of at least four (Fig. 3). The diameter of the treponeme tubules may be as small as 6 nm (17, 19), whereas the diameter of *Pillotina* tubules may be as large as 25 nm (10). These differences in distribution, size, and morphology imply the existence of different types of cytoplasmic tubules in procaryotes. Some may be components of viral particles (25). The possible evolutionary importance of these tubules has recently been discussed (2).

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