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EXPERIMENTAL ARTICLES

First Report on Bacteria of the Family *Spirochaetaceae* from Digestive Tract of Endemic Gastropods from Lake Baikal

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Abstract—Detection of bacteria of the family *Spirochaetaceae* in the crystalline style of 11 species of endemic gastropods from Lake Baikal is reported. Investigation by transmission and scanning electron microscopy showed that these spirochetes belonged to the genus *Cristispira*.

Keywords: Cristispira, gastropods, crystalline style, scanning electron microscopy, transmission electron microscopy

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Bacteria of the family *Spirochaetaceae* were first discovered in digestive tracts of oysters in 1882 by the French author A. Certes, who interpreted this finding as parasitic trypanosomes [1, 2]. Subsequently, Nelson [3] and Nogouchi [4] established that these organisms belonged to the genus *Cristispira*, family *Spirochaetaceae*, and were usual inhabitants of the digestive tract of marine bivalves which had crystalline style in their stomach. The crystalline style is an acellular gelatinous cylindrical formation with a multilayered concentric structure (Fig. 1a). It is known to contain digestive enzymes which decompose carbohydrates of the food and algal cell walls [5–7].

Bacteria of the genus *Cristispira* are saprophytic components of the normal mollusk microflora, large gram-negative, motile bacteria of spiral shape. *C. pectinis* is the type species of the genus *Cristispira* [8, 9].

In the stomach of marine bivalves, *Cristispira* are located close to the functional end of the crystalline style, where amylolytic enzymes and oxidases are released and mixed with the food [5]. However, it was shown that in oysters several hours after capture, most of the spirochetes were present within the bolus or at the surface of the crystalline style [8, 10, 11].

Cristispira have been found in over 60 species of marine bivalves and in several freshwater species: *Sphaerium corneum* (*Sphaeriidae*), *Lampsilis anodontoides* and *Strophitus* sp. (*Unionidae* [6], *Anodonta* sp. (*Unionidae*), and *Pisidium* sp. (*Pisidiidae*) [8]. *Cristispira* were also reported in branchiate gastropods—terrestrial *Cyclophoridae, Calyptraeidae* [6], and freshwater snails *Semisulcospira bensoni* (*Pleuroceridae*) [12]. Similar forms of small spirochetes with the number of axial fibrils less than that of *Cristispira* were found in freshwater gastropods [13]. The results of morphological investigation of these bacteria are, however, insufficient for their classification as *Cristispira* species.

Margulis and Hinkle [6] applied transmission electron microscopy to investigate the morphology of large symbiotic spirochetes of the family *Spirochaetaceae* (genera *Clevelandina, Hollandina, Pillotina, Diplocalyx,* and *Cristispira*) and determined the characteristics differentiating them from other genera of this family (*Treponema, Borrelia*, and *Spirochaeta*). *Cristispira* were found to differ from other genera of this family in their large size (30 to 180 μm), the presence of a crest (crista), resulting from the numerous (over 100) axial fibrils expanding the periplasmic space along one side of the cell, the absence of an invagination (groove) of the outer membrane towards the cytoplasmic membrane, and the presence of rosettes, the peripheral structures of the cytoplasmic cylinder. The rosettes described by Margulis and Hinkle were found only in *Cristispira*; their function is unknown [6, 10, 14–16].

The goal of the present work was to determine the taxa of Lake Baikal gastropods containing spirochetes, and to investigate their morphology and biodiversity.

MATERIALS AND METHODS

Sixteen species of gastropods from Lake Baikal were collected with a hand screen, by light divers, and from *Mir* deep submergence vehicles (DSV). The mollusks were collected for three years in different seasons, in remote sites, and from different biotopes: from silt, sandy, stony, or mixed grounds, from shallow (3–16 m) and deep zones of the lake (at 409 m in the zone of underwater hydrothermal vents of the Frolikha Bay [17] and at 1397 m in the zone of gas hydrate seepage from the St.-Petersburg mud volcano [18]).

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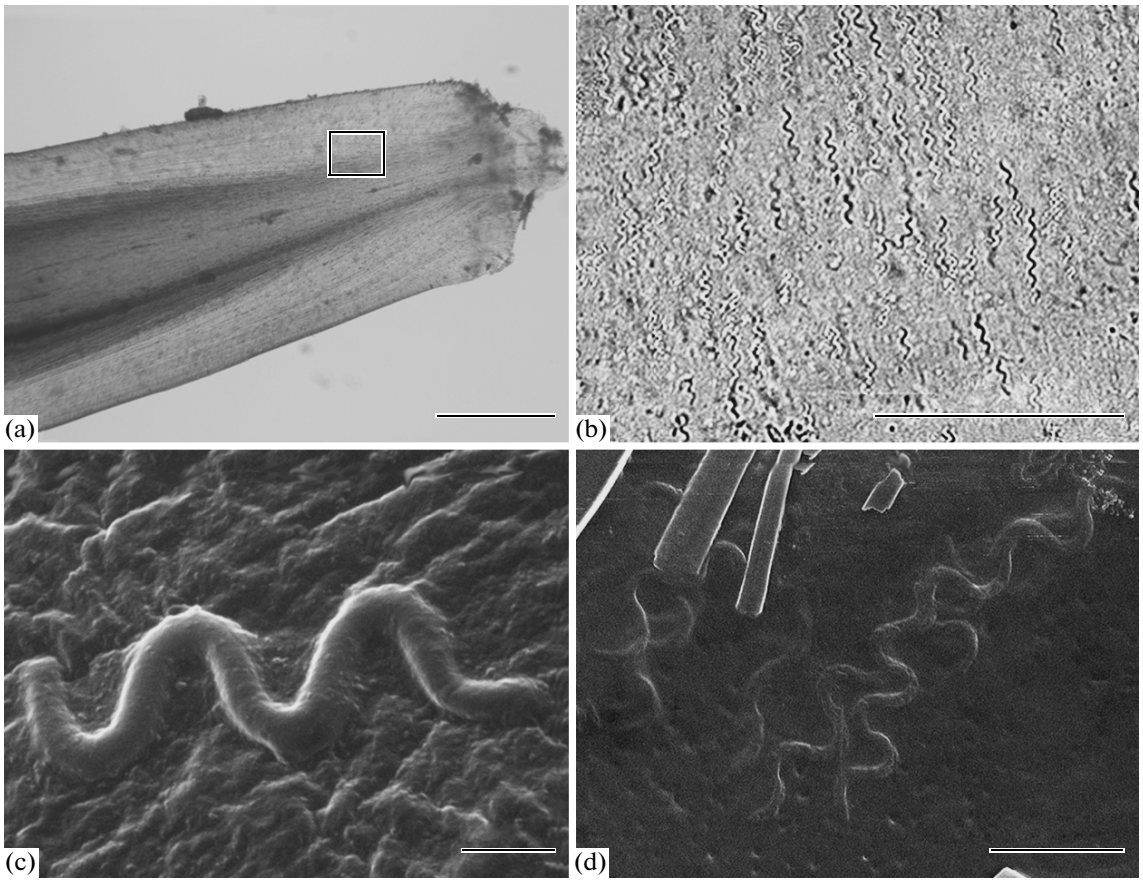

Fig. 1. Light microscopy of the *B. baicalensis* crystalline style: crystalline style with spirochetes (scale bar, 200 μm) (a); enlarged fragment marked by a frame of Fig. 1a (scale bar, 50 μm) (b). SEM of the *B. baicalensis* crystalline style and stomach content: spirochete on the surface of the crystalline style (scale bar, 2 μm) (c); and spirochetes in the stomach content (scale bar, 10 μm) (d).

The mollusks were dissected in vivo. The material investigated is listed in Table 1.

In order to detect the presence of spirochetes, the crystalline style (Fig. 1a) or the protostyle in the case of *Valvatidae* from the stomach of live gastropods, the bolus, and the feces were investigated in a drop of water under an Olympus light microscope with attached camera. For *Lymnaea intercisa* and *Choanomphalus maacki*, the species devoid of the crystalline style, the bolus was investigated in the same way.

The spirochetes from the crystalline style of *Benedictia baicalensis* were investigated by light microscopy and under a Philips 525 scanning electron microscope. For light microscopy, the preparations were Gram-stained. For scanning electron microscopy (SEM), after 24-h incubation the suspension was filtered through polycarbonate membranes (Millipore, 0.2 μm pore diameter). The filters were fixed with 2.5% glutaraldehyde in phosphate buffer, postfixed with 1% OsO_4, dehydrated in ascending concentrations of ethanol, and dried in a Balzers CPD 030 Critical Point Drier (BalTec AG, Liechtenstein). Sections of the filters were then placed on special tables and coated with gold (Balzers SCD 004).

The spirochete suspension for transmission electron microscopy (TEM) was fixed with 4% glutaraldehyde in phosphate or cacodylate buffer (pH 7.4) for 30 min and then with 1% OsO_4 in the same buffer for 2 h. Dehydration was carried out in ascending ethanol concentrations and anhydrous acetone. The samples were embedded according to the recommendation for the Araldite 502 Kit and polymerized for 48 h at 60°C. Ultrathin sections were made using an Ultracut R microtome (Leica, Austria) and contrasted with 2% ethanol solution of uranyl acetate and with lead citrate by Reynolds [19]. The sections were examined under a Leo 906E transmission electron microscope (Carl Zeiss, Germany). The photographs were obtained with a MegaView II digital camera (LEO Elektronenmikroskopie GmbH, Germany).

Morphology of the spirochetes was investigated according to the protocol of Margulis and Hinkle, who determined the characteristics required for differentiation between the genera of the family *Spirochaetaceae* (Figs. 2a, 2b) [6]. Eleven features were used for com-

Table 1. Investigated gastropod species and occurrence of spirochetes

no.	Sampling site, date, and procedure	Analyzed species	Presence of spirochetes
1	Maloe More strait, depth 3–10 m, stony–sandy ground, collection by divers, March 29, 2008	*Benedictiidae*: *Benedictia baicalensis*	+
2	Slyudyanka littoral, depth 16 m, sandy ground, collection by divers, November 11, 2008	*Benedictiidae*:	
		Benedictia baicalensis	+
		B. limnaeoides	+
		Kobeltocochlea martensiana;	+
		Baicaliidae:	
		Baicalia carinata	+
		B. carinatocostata	–
		Parabaikalia oviformis	–
		P. florii	+
		Valvatidae:	
		Megalovalvata demersa	–
		Lymnaeidae:	
		Lymnaea intercisa	–
		Planorbidae:	
		Choanomphalus maacki	–
3	Listvennichnyi Bay, depth 3–14 m, stony ground, collection by divers, May 6, 2009	*Benedictiidae*:	
		Benedictia baicalensis	+
		Baicaliidae:	
		Maackia herderiana	+
		M. costata	+
		Baicalia turriformis	+
	At the same site, June 5, 2010	*Benedictiidae*: *Benedictia baicalensis*	+
4	St.-Petersburg mud volcano, middle lobe, depth 1397 m, silted sandy ground, DSV *Mir*, July 5, 2010	*Benedictiidae*: *Kobeltocochlea falsipumyla*	+
5	Chivyrkui Gulf, Zmeinaya Bay, depth 1.5 m, *Elodea canadiens* growth, hand screen collection (N.M. Pronin), July 20, 2010	*Bithyniidae*: *Boreoelona contortrix*	+
6	Frolikha Bay, hydrothermal vent, depth 409 m, silt and gravel ground, DSV *Mir*, July 24, 2010	*Benedictiidae*: *Benedictia pumyla*	+

Note: "+" and "–" indicate the presence and absence, respectively.

parative characterization. The first four were determined on microphotographs obtained by SEM. Cell diameter (feature 1) was measured for the broadest and narrowest sites of a cell. Features 2, 3, and 4 are cell length and the length and amplitude of the spiral wave, respectively (Fig. 2a). The features from the fifth to the eighth were determined by TEM. Feature 5 is the angle formed by the bundle of axial fibrils, characterizing the fibril distribution in the periplasmic space. Features 6 and 7 are the number of axial fibrils and the presence of their bundle. Feature 8 is the ratio of the diameter of the cytoplasmic cylinder to the cell diameter. Feature 9 is the absence or presence of a groove (invagination of the outer membrane towards the cytoplasmic membrane). Feature 10 is the presence or absence of rosettes. The polar membrane (or polar organelles, feature 11) is a region of low electron den-

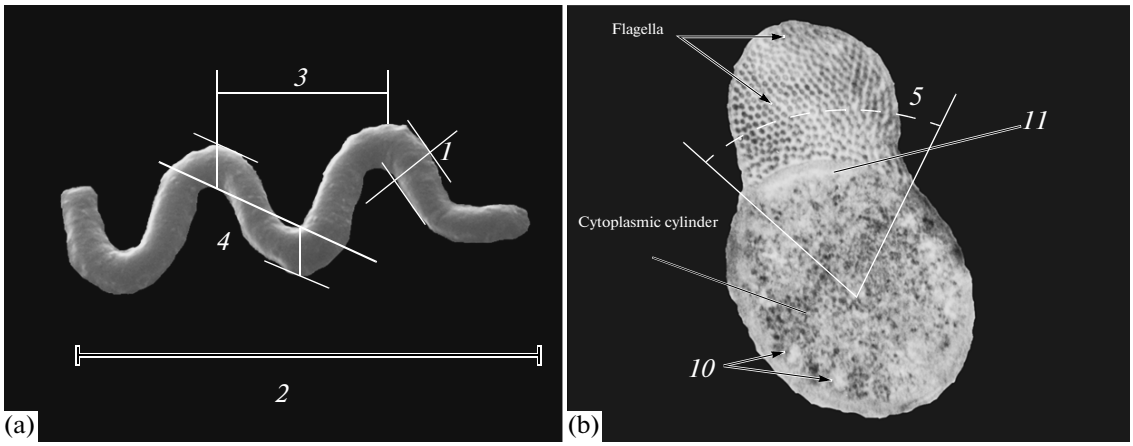

Fig. 2. Features used for the morphometric analysis of spirochetes. Morphological features determined by scanning electron microscopy: diameter (*1*), length (*2*), wavelength (*3*), and amplitude (*4*) (a). Morphological features determined by transmission electron microscopy: angle of a bundle of axial fibrils (*5*), rosettes (*10*), and polar membrane (*11*) (b).

sity within the cytoplasmic cylinder adjacent to the cytoplasmic membrane (Fig. 2b).

Morphology of 48 spirochete cells from the mollusk *Benedictia baicalensis* was determined.

RESULTS

Out of 16 gastropod species investigated, spirochetes were found in 11, including 10 Lake Baikal endemics (Table 1). Apart from the endemics, spirochetes were found in a North Asian snail *Boreoelona contortrix* (*Bithyniidae*), which is common in the northern bays of the lake and adjacent water bodies. In all individuals, spirochetes were observed within the crystalline style. Although they were often nonmotile and oriented perpendicular to the axis of the style, they began active movement immediately after emerging from it. Motion of three types was observed: movement along spiral or wavy trajectories, bending, and rotation around the axis of the spiral. Similar to other members of the phylum *Spirochaetes*, they exhibited gliding movement.

Spirochetes were also present in the bolus and feces, although their number was insignificant (up to 5 cells per microscope field at ×300 magnification). In all snails, vegetable food prevailed in the bolus and feces; detritus, bacteria, and soil sand particles were also present.

In two species of the family *Baicaliidae*, *Baicalia carinatocostata* and *Parabaikalia oviformis*, as well as in 8 out of 27 examined specimens of *B. baicalensis* collected at different time from different Baikal sites, spirochetes were not found. None of the investigated members of the families *Valvatidae, Lymnaeidae*, and *Planorbidae*, which do not possess crystalline styles, contained spirochetes.

The number of spirochetes within the crystalline style of *B. baicalensis* varied from 6 to 50 cells per 100 μm^2 (Fig. 1b). Single cells of spirochetes were found in the bolus and feces.

In a crystalline style submerged in a small amount of water at 7°C, the spirochetes remained motile for 24 h.

Light and scanning electron microscopy of the cells revealed that they were spiral-shaped gram-negative bacteria 17.6 ± 4.8 (10.5–30.1) μm long and 0.9 ± 0.2 (0.6–1.2) μm thick. The spiral of the bacterial cell had 2 to 4 coils, with rounded or blunt cell edges. The length of the spiral wave was 5.9 ± 1.3 (3.6–7.4) μm and its amplitude was 3.3 ± 0.8 (1.9–4.9) μm (Table 2, Figs. 1c, 1d). TEM revealed over 100 periplasmic flagella (axial fibrils). Diameter of the cells and the cytoplasmic cylinder determined by TEM were 1.0 ± 0.2 (0.5–1.3) and 0.7 ± 0.1 (0.4–0.9) μm, respectively. The ratio of the diameters of the cytoplasmic cylinder and the cell was 0.8 ± 0.1 (0.6–0.9). The angle of flagella distribution in the periplasm was 132 ±28.1 (78.3–184.1). The three-layered cell wall structure of the spirochetes was typical of gram-negative bacteria. The axial fibrils were located between the outer membrane and the peptidoglycan layer. The fibrils were 27.5 ± 3.5 (23.8–33.5) nm in diameter. The thickness of the outer membrane was 10.5 ± 3.2 (5.2–16.7) nm, the thickness of the peptidoglycan layer was 4.9 ± 2.0 (1.7–7.6) nm, and the thickness of the cytoplasmic membrane was 9.6 ± 3.1 (4.9–13.8) nm (Figs. 3c, 3d). Spherical zones of low electron density were observed at the periphery of the cytoplasmic cylinder. They were not found at the sites of attachment of the axial fibrils. Their average size was 37.7 ± 3.8 (34.2–43.8) nm. Areas of low electron density adjacent to the cytoplasmic membrane from the side of the cytoplasmic cylinder (polar membranes) were observed (Figs. 3a, 3b). Electron-dense spherical structures (40–70 nm) were present mostly at the periphery of the cytoplasmic cylinder (Figs. 4a, 4b).

Table 2. Comparative morphological characterization of the spirochetes studied and other genera of the family *Spirochaetaceae*

Feature	Studied spirochetes	*Cristispira*	*Pillotina*	*Hollandina*	*Diplocalyx*	*Clevelandina*	*Spirochaeta*	*Borrelia*	*Treponema*
Cell diameter, μm	0.6–1.2	0.5–3.0	0.6–1.5	0.4–1.0	0.7–0.9	0.4–0.8	0.2–0.8	0.2–0.5	0.1–0.7
Ratio of the diameters of the cytoplasmic cylinder and the cell	0.70–0.90	0.90	0.56–0.67	0.63–0.90	0.47–0.81	0.60–0.81	ND	ND	ND
Number of periplasmic flagella	≥100	≥100	30–70	30–60	40–60	30–45	2	15–20	1–16
Angle formed by the fibrillar bundle, °	78.3–184.1	90–160	190–350	105–330	50–100	140–330	ND	ND	ND
Bundle of periplasmic flagella	+	+	–	+/–	+	+/–	ND	ND	ND
Cell length, μm	10.5–30.1	30–180	ND	ND	ND	ND	5–520	3–30	1–20
Wave amplitude, μm	1.9–4.9	4–6	ND	ND	ND	ND	ND	ND	ND
Wavelength, μm	3.6–7.4	10–20	ND	ND	ND	ND	ND	ND	ND
Groove	–	–	+	+/–	+	+	ND	ND	ND
Rosettes	+	+	–	–	–	–	ND	ND	ND
Polar organelles	+	+	+	+	+	ND	ND	ND	ND

Note: "+" and "–" indicate the presence and absence, respectively. "ND" stands for no data. The data of Margulis and Hinkle [6] were used in the table.

DISCUSSION

Spirochetes of the genus *Cristispira* are known to be associated with the crystalline style of bivalves and gastropods [6, 8, 10]. Most of bacterial cells are submerged into the internal matrix of the style throughout its length [11]. The reasons for association of *Cristispira* with this organ and the essence of their ecological relationship with the host organism are presently not clearly known. Importantly, under similar environmental conditions, not all specimens of a mollusk species carry these spirochetes [6]. In the present work, some of the individuals of *B. baicalensis* and two other baikaliid species possessing crystalline styles were also not found to contain spirochetes.

Marine bivalves, hosts of the spirochetes, feed on planktonic algae and detritus [20, 21]. Digestion of cellulose was found to occur independently on the presence of spirochetes in the style [6]. The latter in both cases contains the same enzymes—amylase, chitinase, and chitobiase [22]. Spirochetes are therefore not required for assimilation of vegetable food by the mollusks.

This conclusion is confirmed by the results of the present work. Among the gastropod species containing spirochetes, *B. baicalensis* and *B. limnaeiodes* are omnivorous, since, apart from vegetable food (planktonic algae and benthic diatoms), they also feed on the detritus of plant and animal origin, are able to run the sediments through their intestines and to capture tissues of dead fish. The species *Kobeltocochlea martensiana*, similar to *Benedictia*, is "grazing on the substrate", scraping the plant detritus from the surface of the *Lubomirskia* green sponge [23, 24]. Members of the families *Bithyniidae* and *Baicaliidae* are filter-feeders, with components of seston (planktonic diatoms, plant detritus, and bacteria) being the content of their stomachs [9, 24].

The species of freshwater snails which were not found to contain spirochetes have similar rations (in the case of *Baicalia carinatocostata* and *Parabaikalia oviformis*). *Megalovalvata demersa*, which does not contain spirochetes in its protostyle, is a filter-feeder consuming benthic diatoms and plant and animal detritus [23, 24]. Members of the families *Lymnaeidae* and *Planorbidae*, which have no crystalline styles in the stomach, also were not found to contain spirochetes. These mollusks are "substrate-grazing" phyto- and detritophages [25]. Thus, the presence of spirochetes in the digestive tract of the mollusks can not be explained by the spectrum of their feeding.

Similar to marine bivalves, in the studied freshwater gastropods the spirochetes were localized in the crystalline style and were not obligatory [6, 10, 22].

The results obtained by TEM and SEM made it possible to compare the Lake Baikal spirochetes to other members of the family *Spirochaetaceae*. Cell size

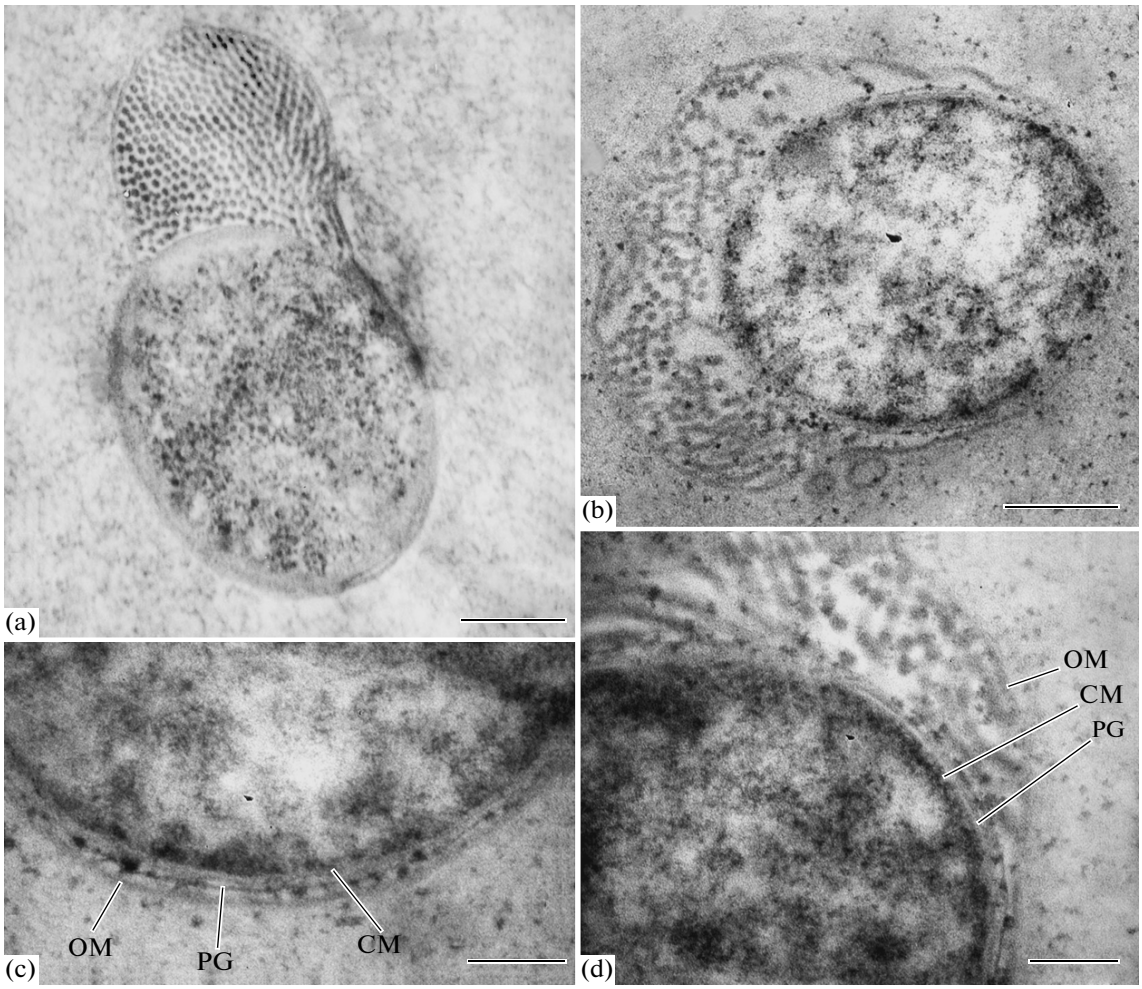

Fig. 3. Transmission electron microscopy of the Lake Baikal spirochetes. Scale bars 200 nm (a), (b) and 100 nm (c), (d). OM, outer membrane, PG, peptidoglycan layer, and CM, cytoplasmic membrane.

and association with hosts differentiate the genus *Cristispira* and the spirochetes of Baikal mollusks from the genera *Borrelia, Treponema*, and *Spirochaeta*. The genera *Pillotina, Diplocalyx*, and *Clevelandina* are characterized by the presence of a groove. Such grooves were not found in the studied spirochetes and in the genus *Cristispira*. Among members of the genus *Hollandina*, the groove is not a permanent feature. *Cristispira*, however, has the highest number of axial fibrils (over 100, compared to 30–45 in *Clevelandina* and 30–70 in *Pillotina* and *Hollandina*). The spherical structures of low electron density were found to be rosettes (Fig. 2b). The presence of rosettes in the Lake Baikal spirochetes is another indication of their affiliation to *Cristispira*. Other symbiotic genera are not known to form rosettes [6].

The electron-dense formations located mostly in the peripheral part of the cytoplasmic cylinder are probably inclusions (Figs. 4a, 4b). Their diameter varied from 40 to 70 μm. Most of these inclusions were homogeneous, although some of them had granular structure.

Thus, although the cell morphology supports classification of the studied bacteria as members of the genus *Cristispira*, they differ from *C. pectinis*, the only described species of this genus, in smaller size (the Lake Baikal spirochetes are almost 5 times shorter, with 3 times lower wave length, and 2.3 times smaller amplitude), habitat, and host species (*C. pectinis* occurs in the crystalline style of marine bivalves).

These differences indicate that the spirochetes from the crystalline style of endemic gastropods of Lake Baikal belong to a new species of the genus *Cristispira*. Further investigation with molecular biological techniques will elucidate the phylogenetic position of the Baikal spirochetes and contribute to the description of the new species.

Thus, investigation of the morphological characteristics of the spirochetes from the gastropods of Lake Baikal by TEM and SEM suggests their classification as members of the genus *Cristispira*. Molecular genetic

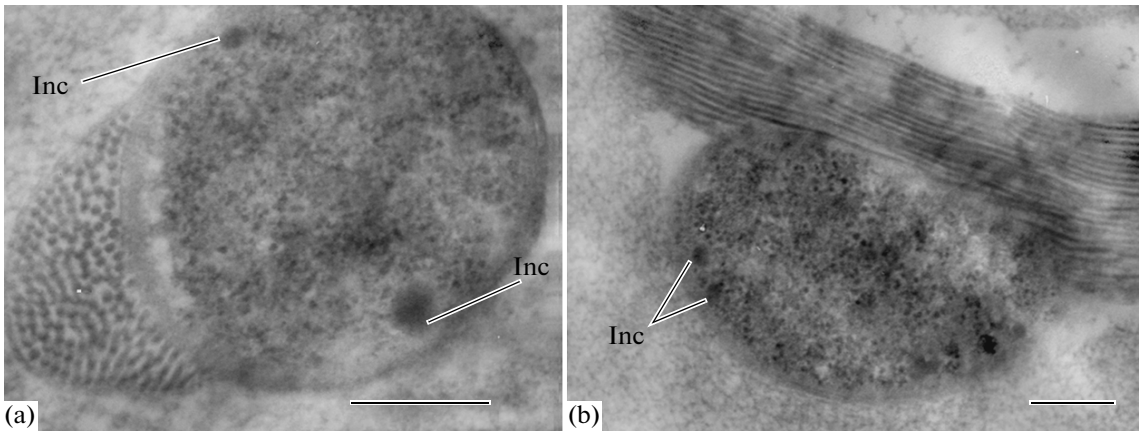

Fig. 4. Transmission electron microscopy of the Lake Baikal spirochetes. Scale bars 200 nm. Inc, inclusions.

analysis will probably confirm their status as a new species. Spirochetes were found in 11 species of Baikal mollusks, which feed mostly on food of plant origin and inhabit various biotopes of the lake. They are associated with the crystalline style and are not obligatory symbiotic microorganisms.

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