ISSN 1062-3590, Biology Bulletin, 2013, Vol. 40, No. 1, pp. 107–110. © Pleiades Publishing, Inc., 2013.
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SHORT COMMUNICATION

Novel Spirochetes in the Crystalline Style of Fresh Water Gastropods

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Received April 7, 2011

Abstract—Freshwater gastropods of the families Amnicolidae, Bithyniidae, Baicaliidae, and Benedictiidae are shown for the first time to harbor spirochetes associated with the crystalline style in the intestinal tract. The examined gastropod species are omnivorous, phyto-detritophagous grazers or filter-feeders. Their habitats are various water bodies and biotopes, including deep water hydrothermal vents and gas hydrate zones in Lake Baikal.

DOI: 10.1134/S1062359012060131

INTRODUCTION

Despite the discovery of spirochetes in the digestive tract of mollusks more than 120 years ago (Paster et al., 1996), many issues relative to spirochete–host interactions remain unclear.

Spirochetes of the genus *Cristispira* Gross, 1910 (family Spirochaetaceae) are most studied from the digestive tract of bivalve mollusks that have the crystalline style in the stomach (Margulis and Hinkle, 2006). Members of the single species *C. pectinis* Gross, 1910 are large-sized (up to 180 μm in length and with a diameter range of 0.5–3 μm), Gram-negative, nonculturable, motile, and spiral-shaped (Tall and Nauman, 1981; Bergey and Holt, 1994; Margulis and Hinkle, 2006). Cristispiras swim freely near the functional end of the crystalline style, where amylolytic enzymes and an oxidase are released (Berkeley, 1962).

Spirochetes have thus far been identified in over 60 marine and several freshwater bivalves, as well as in gastropod mollusks with a gill: terrestrial Pupinidae (Morton, 1952), slipper limpets Calyptraeidae (Margulis, Hinkle, 2006), and two freshwater species *Semisulcospira bensoni* (Philippi, 1851) (Pleuroceridae) (Terasaki, 1960) and *Fluminicola coloradensis* Morrison, 1940 (Lithoglyphidae) (Sitnikova et al., 2012).

The objective of this work is to identify species of snails that harbor spirochetes, to carry out a preliminary study of spirochete morphology using scanning electron microscopy, and to characterize habitats and dietary preferences of spirochete-containing snails.

MATERIALS AND METHODS

Sixteen gastropod mollusk species collected in Lake Baikal (Eastern Siberia), hand-dredged by simple diving and the deep-sea manned submersible (GOA) *Mir* (Russia), and also snails *Kolhymamnicola ochotica* Zatrawkin et Bogatov, 1988, collected in the basin of the Kava river (northern Far East of Russia) by a sieve through water vegetation, were examined. In Lake Baikal, mollusks were caught in various seasons for three years in different lake locations at various depths and in different types of substratum. Baikal snails were dissected alive, and Far Eastern snails were prefixed in 80% ethanol (table).

The crystalline style (or protostyle in the case of Valvatidae) and food pellets from the stomach, as well as feces of live and fixed snails, were placed in a drop of water on a slide and examined by light microscopy for spirochetes. Spirochetes were also sought within enzymes liberated by the free end of the style (Fig. 1a). For *Lymnaea intercisa* Lindholm, 1909 and *Choanomphalus maacki* Gerstfeldt, 1859, which do not possess a style, only food boluses were examined.

Spirochetes from the crystalline style of *Benedictia baicalensis* (Gerstfeldt, 1859) were investigated in more detail using an Olympus light microscope (United States) with a conventional camera adapter and a Philips 525 scanning electron microscope (the Netherlands). After detecting spirochetes, crystalline styles were placed in sterile water and homogenized (1 : 9). After 24 h the suspension was filtered through Millipore polycarbonate filters (United States) with a pore size of 0.2 μm, followed by fixing with 2.5% glutaric dialdehyde in PBS, dehydrated in a graded series of ethanol, and critical point dried (Balzers CPD 030 Critical Point Dryer, BalTec AG, Liechtenstein). Filter pieces were mounted on stubs, sputter coated with gold (Balzers SCD 004, BalTec AG), and examined using scanning electron microscopy.

Gastropods that harbored and did not harbor* spirochetes in the intestinal tract

Collection site, date	Species and numbers of individuals examined
Lake Baikal, Maloe More strait, depth 3–10 m, sand, sandy–stony bottom, 29.03.2008	*Benedictia baicalensis* (Gerstfeldt, 1859) ($n = 5$)
Slyudyanka, depth 16 m, sand, 11.11.2008	*B. baicalensis* ($n = 1$), *B. limnaeoides* Schrenck, 1867 ($n = 4$), *Kobeltocochlea martensiana* (Dybowski, 1875) ($n = 2$), *Baicalia carinata* (Dybowski, 1875) ($n = 4$), *B. carinatocostata* (Dybowski, 1875) ($n = 2$)*, *Parabaikalia oviformis* (Dybowski, 1875) (n =3)*, *P. florii* (Dybowski, 1875) ($n = 3$); *Megalovalvata demersa* Lindholm, 1909 ($n = 3$)*; *Lymnaea intercisa* Lindholm, 1909 ($n = 2$)*, *Choanomphalus maacki* Gerstfeldt, 1959 (n =3)*
Listvennichnyi Bay, depth 3–14 m, stony substratum, 06.05.2009	*Benedictia baicalensis* ($n = 20$), *Maackia herderiana* ($n = 4$), *M. costata* (Dybowski, 1875) ($n = 3$); *Baicalia turriformis* Lindholm, 1909 ($n = 2$)
The same site, 05.06.2010	*Benedictia baicalensis* ($n = 5$)
Middle hollow, Methane Gas Seep, St. Peterburg, depth 1397 m, silted sand, 5.07.2010	*Kobeltocochlea falsipumyla* Sitnikova, 2001 ($n = 3$)
Chivirkuyskii Bay, Zmeinaya Creek, depth 1.5 m, *Elodea canadiens* thickets, meshwork, 20.07.2010	*Boreoelona contortrix* (Lindholm, 1909) ($n = 3$)
Frolikha, hydrothermal vents, depth 409 m, silt, gravel, 24.07.2010	*Benedictia pumyla* Lindholm, 1924 ($n = 2$)
Magadan oblast, coastal area of the Sea of Okhotsk, Kava River, IBSS-no. 4325, 30.07.2001	*Kolhymamnicola ochotica* Zatrawkin et Bogatov, 1988 ($n = 1$)

RESULTS

The digestive tracts of 12 out of 17 freshwater mollusks studied (table) harbored large spiral-shaped bacterial cells (Fig. 1b), which were identified, correspondingly, as spirochetes. All live mollusks carried spirochetes inside the crystalline style immotile; however, immediately after leaving it, the spirochetes exhibited vigorous locomotion.

Spirochetes were found in mollusks from Lake Baikal, collected in various seasons for three years in spatially separated localities of the lake in different biotopes in shallow waters (depths of 3 to 16 m), as well as in deep waters (down to 1397 m), including areas with gas hydrate deposits (Granin et al., 2010) and hydrothermal vent (Golubev, 1993).

Alongside endemic mollusks from Lake Baikal, host-associated spirochetes were also seen in the northern Asian snail *Boreoelona contortrix* (Lindholm, 1909) (Bithyniidae), common to northern lake bays and adjacent water reservoirs, as well as Northern Far East species *K. ochotica* (Amnicolidae).

Food boluses and feces of the above-mentioned mollusks also contained spirochetes, but in small numbers. The food of all individuals was dominated by plant food consisting of diatoms, cyanobacteria, chrysophyte algae, organic detritus, bacteria, and occasionally sponge spicules and fine sand grains.

No spirochetes were observed in two baicaliids *Baicalia carinatocostata* (Dybowski, 1875) and *Parabaikalia oviformis* (Dybowski, 1875), as well as in 8 of 27 dissected *B. baicalensis* individuals, collected at different times and in different lake regions. All examined snails of the families Valvatidae, Lymnaeidae, and Planorbidae that did not possess a style did not have spirochetes either.

Spirochetes associated with the crystalline style of *B. baicalensis* are spiral-shaped bacteria, 13 to 25 μm in lenght and 0.8 to 1.1 μm in width. The spiral has between 2 to 4 turns. The helix pitch ranges from 4.8 to 5.2 μm. The cell has blunt-tipped ends (Fig. 1c).

DISCUSSION

It is a matter of fact that *Cristispira* spirochetes in bivalve and gastropod mollusks are always associated with the crystalline style (Bergey and Holt, 1994; Margulis and Hinkle, 2006; et al.). These bacteria are located within the style inner matrix, occupying all areas of the crystalline style (Tall and Nauman, 1981). However, not all individuals of one species harbor spirochetes under similar habitat conditions (Margulis and Hinkle, 2006). In the course of our studies, we noted an as-yet unexplainable absence of spirochetes in several *B. baicalensis* individuals and two baicaliids having a style in the stomach.

Marine bivalve mollusks, which host spirochetes, are sestonivorous, utilizing planktonic plant organisms and organic particulate matter as food sources (Lucas and Newell, 1984). There is evidence that the crystalline style produces amylolytic enzymes used for digesting plant food (Yonge, 1932; Berkeley, 1962). It was also shown that cellulose degradation occurs regardless of spirochetes in the style (Margulis and

Hinkle, 2006) that releases the same group of enzymes: amylase and chitobiase (Mayasich and Smucker, 1987). Consequently, spirochetes are not obligatory for digestion of plant food in mollusks.

This finding is corroborated in our study. Among the spirochete-harboring gastropods studied, *B. baicalensis* and *B. limnaeoides* endemic to Lake Baikal are omnivorous since, alongside plant food in the form of plankton and benthic diatomic algae, they also utilize plant and invertebrate detritus; they are capable of passing sediments through the intestine and scavenge dead fish particles. The species *Kobeltocochlea martensiana*, as well as the above-mentioned benedictines, is grazer. It largely scrapes plant detritus and possibly animal detritus off green sponges of the genus *Lubomirskia* Dybowski, 1880 (Roepstorf et al., 2003; Sitnikova and Roepstorf, 2004). Snails of the families Bithyniidae and Baicaliidae are filter-feeders. Their stomachs are full of seston components such as planktonic diatoms, plant detritus, and bacteria (Lilly, 1953; Roepstorf et al., 2003). In our study the food boluses of the Far Eastern snail *K. ochotica* consisted of diatomic algae and undigested plant matter at the moment of examination. Spirochete-containing terrestrial gastropod Pupinidae feed on leaves and plant matter (Morton, 1952). Limpets are filter-feeders of seston (Newell and Kofoed, 1977). *Semisulcospira* freshwater snail scrapes microscops dominated by diatoms (Nagai et al., 1979). *Fluminicola* snails feed on periphyton (Hershler and Frest, 1996), including various microscopic algae.

Freshwater snails from Baikal (*B. carinatocostata* (Dybowski, 1875) and *P. oviformis* (Dybowski, 1875)), which were found free of spirochetes, have similar dietary preferences as baicaliids. The valvatid snail, *Megalovalvata demersa*, the protostyle of which is spirochete free, a filter-feeder of benthic diatoms, plant, and animal debris (Roepstorf et al., 2003; Sitnikova and Roepstorf, 2004). Grazing phyto- and detritophagous snails of the families Lymnaeidae and Planorbidae (Tsikhon-Lukanina, 1987) lacking the style were found free of spirochetes in food pellets as well. This suggests that the presence of spirochete in a food boluses cannot be attributed to their dietary preferences.

The freshwater gastropods and marine bivalves studied in this work showed that spirochetes are associated with the crystalline style and are not obligate bacteria.

Species identification of the spirochetes remains poorly understood, since thorough investigations are still ongoing. However, there is evidence that spirochetes from the crystalline style of *B. baicalensis* belong to the genus *Cristispira*, but considerably differ from the single known species *C. pectinis* associated with the crystalline style of marine bivalves. The examined bacterial cells are much shorter than that of *C. pectinis* (13–25 μm versus 30–180 μm), with a smaller diameter range (0.8–1.1 μm versus 0.5–3) and with a smaller numbers of turns (4 versus 6) (Lawry et al., 1981; Tulupova et al., 2012).

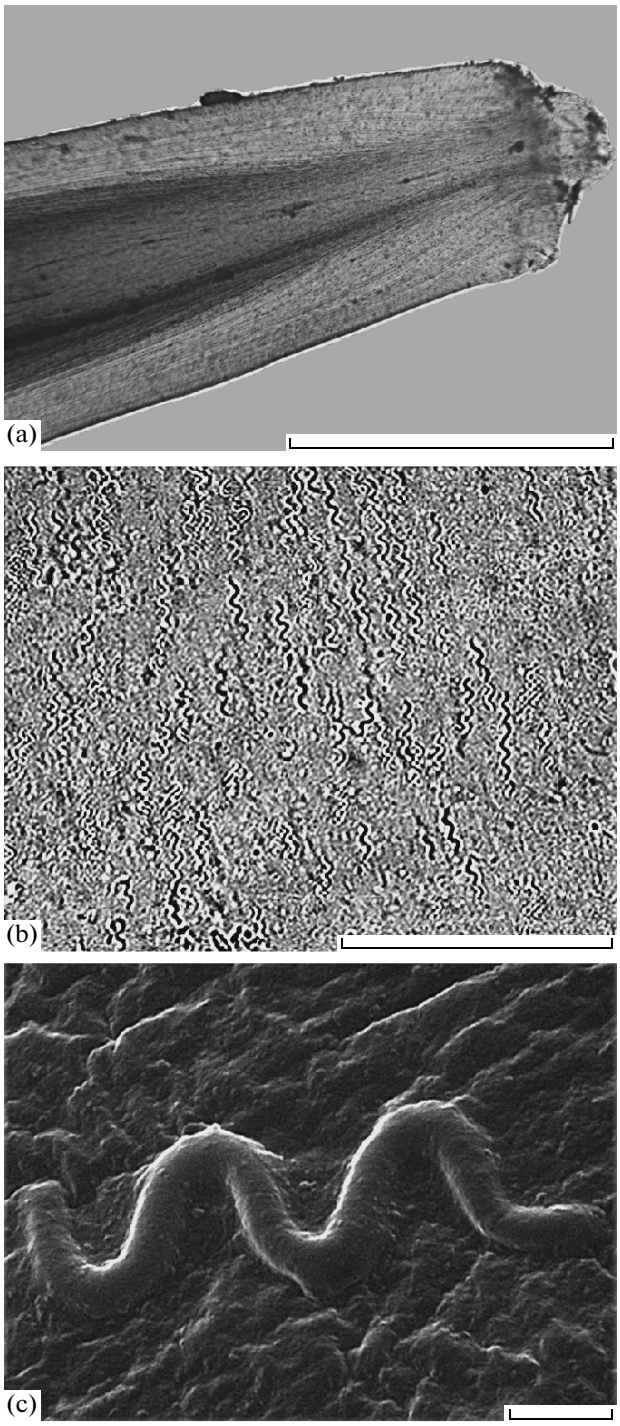

Fig. 1. The crystalline style and spirochetes in the freshwater mollusk *Benedictia baicalensis*: (a) the free end of the style; (b) spirochetes within the style (light microscopy); (c) the spirochete morphology (electron scanning microscopy). Scale bar: a, 0.5 mm; b, 0.1 mm; c, 10 μm.

It is likely that the mollusk–spirochete interactions should be referred to not as symbiosis, but as commen-

salism, wherein the crystalline style content is favorable for spirochete colonization. However, in order to understand clearly the role of spirochetes in the mollusk, further studies are required.

CONCLUSIONS

The 12 species of freshwater gastropods from 4 families were shown to harbor spirochetes differing in morphology from *C. pectinis*, which colonizes marine bivalve mollusks. As in bivalves, in freshwater gastropods spirochetes are associated with the crystalline style. Snails found to host spirochetes exhibited different trophic strategies (filter-feeders and grazers), are omnivorous or phyto-, detrito-, and bacteriophagous snails, and inhabit different water bodies and biotopes of the same water pool, including deep water hydrothermal vent and gas hydrate seeps in Lake Baikal. To define species of the spirochetes, further genetic studies are required.

ACKNOWLEDGMENTS

The authors thank A.B. Kupchinskii (Limnological Institute, Siberian Branch, Russian Academy of Sciences), N.M. Pronin (IOEB, Siberian Branch, Russian Academy of Sciences) and V. Nischeta (GOA *Mir*) for assistance in mollusk collection.

This work was supported by a grant from the Presidium of the Siberian Branch, Russian Academy of Sciences (project no. 23.8); DVO, Russian Academy of Sciences (project no. 12-1-P30-01); and the Russian Foundation for Basic Research (project nos. 10-05-01078 and 12-05-98011).

Gastropod collection aided by GOA *Mir* was organized and financially supported by the Baikal preservation foundation and the Metropol group of companies.

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