Cochliopodium minus, a scale-bearing amoeba isolated from organs of perch *Perca fluviatilis*

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ABSTRACT: A scale-bearing amoeba isolated from gills and other organs of perch *Perca fluviatilis* Linnaeus, 1758 was identified as *Cochliopodium minus* Page, 1976. This assignment was suggested by light microscopy and confirmed by ultrastructural study of both the strain isolated from perch and the environmental strain of *C. minus* (CCAP 1537/1A) serving as a control. This has been the first electron-microscopic definition of *C. minus* and, in addition, the first identification of an amoeba with a theca-like cover found to infect fish. The ability of the fish strain of *C. minus* to colonize gills and also internal body organs was proved experimentally.

KEY WORDS: Free living amoebae · *Cochliopodium minus* · Ultrastructure · Systemic infection of fish

INTRODUCTION

The most important outbreaks of amoebic gill diseases (AGD) resulting in mortalities have been recorded in salmonids. Among the agents, *Paramoeba* spp. evidently play the most important role in marine cultures, while amoebae of the families Cochliopodidae and Thecamoebidae have been recorded as agents of severe gill lesions in freshwater farmed *Oncorhynchus mykiss* (Sawyer et al. 1974, 1975, Daoust & Ferguson 1985, Noble et al. 1994, 1997). Unfortunately, none of the records of AGD attributed to amoebae of the family Cochliopodidae included an exact diagnosis of the agent. *Cochliopodium* spp. have been recorded from a broad range of habitats, both marine and freshwater (Bark 1973, Page 1988) but most of the species were described at the light-microscopical level. Three species of the genus *Cochliopodium* isolated either from activated sludge of a sewage treatment plant or from a small sphagnum swamp were described in detail by Bark (1973). In addition, 1 strain of *Cochliopodium* isolated by Nagatani et al. (1981) from an aquarium sediment was used by Yamaoka et al. (1984) for a scale formation study. Structural components of scales were

also examined in *C. bilimbosum* by Sadakane et al. (1996).

The goals of the present study were to report the isolation of a *Cochliopodium* sp. from the gills and other organs of perch *Perca fluviatilis* Linnaeus, 1758, and to present its ultrastructural features.

MATERIALS AND METHODS

The isolation, culture and cloning as well as maintenance of the cloned strain followed the general suggestions and systematic survey of culture methods of Kalinina & Page (1992) and were also based on our experience from previous research on free-living amoebae in freshwater fish (Dyková et al. 1996, 1997, 1998). The strain as well as the clone were cultured on non nutrient Bacto agar (Difco) seeded with *Bacillus subtilis*. In addition to light-microscopical methods of observation, transmission electron microscopy based on 2 fixation protocols, cacodylate buffered osmium tetroxide and glutaraldehyde with ruthenium red followed by postfixation with osmium tetroxide (Harris 1991), were applied to reveal surface architecture of the trophozoites. Amoebae cultured on agar plates were fixed *in situ*. Washed and pelleted material was embedded

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in Poly/Bed 812 - Araldite (Dykstra 1993). An identical procedure was applied to the strain of *Cochliopodium minus* Page, 1976 ordered from Culture Collection of Algae and Protozoa (CCAP 1537/1A) for comparison. The terminology used by Bark (1973), Nagatani et al. (1981) and Sadakane et al. (1996) was followed to allow comparison with previous descriptions of *Cochliopodium* strains. The ability of the isolated strain to colonize organs other than gills was tested experimentally in *Heteropneustes fossilis*, a bottom-dwelling fish species.

RESULTS

The *Cochliopodium* strain (accession No. 4694; a collection of strains is maintained at the Institute of Parasitology, České Budějovice) was isolated from the liver, kidney, brain, spleen and gills of perch *Perca fluviatilis* caught in the Vltava river, South Bohemia, Czech Republic, in July 1991. Passage No. 18 of this strain was stored in liquid nitrogen. The clone was derived from passage No. 21 of this strain in March 1997.

Light microscopy

Locomotive and resting forms of trophozoites observed in hanging drops (Fig. 1) were round to oval in shape. The prominent, centrally located endoplasm was rich in granules and surrounded by a flat zone of hyaloplasm at the periphery. Pseudopodia of variable shape and length (maximum 6.5 μm long) extended in all directions from the hyaloplasm.

When measured in a hanging drop of tap water, the average length (L) of the most active trophozoites was 25.5 (16–38) μm , the breadth (B) was 26.5 (12–52) μm and the L/B ratio was 1:1.02. The rate of locomotion averaged 54 $\mu\text{m min}^{-1}$. The trophozoites were less active under the coverslip in distilled water, the rate of their locomotion being 17.4 $\mu\text{m min}^{-1}$. Their L was 21.4 (16–28) μm , B was 16.4 (10–24) μm and L/B ratio was 1.3:1. The diameter of the nucleus averaged 3.4 μm and of the nucleolus 2.3 μm . Trophozoites attached to coverslips of hanging drop preparations kept in a wet chamber for 12 h were observed to be inactive. They were almost discoid, having an L of 26.0 μm , B of 25.5 μm and L/B ratio 1.02:1.

Figs. 1 to 3. **Fig. 1.** Trophozoites of *Cochliopodium* sp. (clone 4694/l isolated from organs of *Perca fluviatilis*) as observed in hanging drops. Nomarski DIC. Scale bar = 10 μm . **Fig. 2.** *Cochliopodium* sp. (clone 4694/l), scanning electron micrograph showing scale-bearing surface of the trophozoite. Scale bar = 10 μm . **Fig. 3.** Trophozoites of *Cochliopodium minus* (clone CCAP 1537/1A; original designation '47' in Type Culture). Nomarski DIC. Scale bar = 10 μm

Light microscopy revealed the specific shape of the trophozoites. Concentric separation of the cytoplasm into the raised zone of granuloplasm and the flat rim of hyaloplasm was recorded. The presence of scales on the sur-

face of trophozoites, which is of principal value for generic diagnosis, was at first only observed in transmission electron microscopy (see Figs. 4–8) and later also documented by scanning electron microscopy (Fig. 2).

Figs. 4 to 8. Fig. 4. Trophozoite of *Cochliopodium* sp. (clone 4694/I) fixed on agar plate and sectioned in dorsoventral plane. Tectum (T) consisting of scales, nucleus (N), nucleolus (n), mitochondria (m) with tubular cristae. Scale bar = 2 μ m. Figs. 5 & 6. Scale-bearing surface of trophozoites. Scale bar = 1 μ m. Fig. 7. Microscales of *Cochliopodium* sp. (clone 4694/I) sectioned in various planes. Funnel-like part of scales in dorsal view (D). Rectangular transverse sections through the scale column (C). Fragments of scale base plates indicated by arrows. Scale bar = 500 nm. Inset: Cross-section of the column. Fig. 8. Funnel-like part of scales in dorsal view showing web-like pattern with rays, concentric interconnections and striped rim. Scale bar = 200 nm

Transmission electron microscopy

Cultured trophozoites fixed *in situ* were mostly elongated in cross section (Fig. 4). Their dorsal section was covered with a layer of slightly overlapping scales not extending to the peripheral hyaloplasm, while the ventral scale-free surface was attached to the agar. The layer of scales extended up to 330 nm above the plasma membrane (Figs. 5 & 6). Nuclei were oval in shape and in some sections made the impression of having lobes. In the cytoplasm, symbiotic and phagocytized bacteria and granules of unknown origin were observed. Mitochondria contained branched tubular cristae and a prominent Golgi apparatus was observed several times close to the scale cover of the plasma membrane.

Each scale consisted of a flat lattice base plate attached to the plasma membrane of the trophozoite and a hollow column which at its capital flared open wide in the shape of a funnel (Figs. 5 & 6). In the dorsal view (Fig. 8), the top, i.e. distal part, of the scale had the appearance of a spider web. The web-like structure consisted of 17 to 19 rays slightly widening

towards the periphery which were concentrically interconnected (Figs. 7 & 8). The diameter of the funnel top was 560 to 620 nm and its perimeter was marked with a narrow striped rim (Fig. 8). The column of each scale was reinforced with 4 stalks which diverged toward the base plate (Figs. 5 & 6) and were shaped like birds' feet in the distal part. The transverse section through the middle part of the scale column was rectangular (Fig. 7), with sides equal in length to 180 nm. When grown on agar plates, trophozoites formed double-walled cysts with diameters of 10 to 15 μm . The outer layer, i.e. the ectocyst, consisted of scales; the thin inner layer was closely apposed to the outer layer. In the outer scale layer of the cysts a scaleless aperture has always been observed.

Taxonomic assignment

The general morphology of the scale-bearing amoeba isolated from organs of *Perca fluviatilis* corresponded in all respects to the genus *Cochliopodium* (Hertwig & Lesser, 1874) as redefined by Bark (1973). The assign-

Figs. 9 to 12. Fig. 9. *Cochliopodium minus* (clone CAAP 1537/1A). Part of the tectum showing 3 scales sectioned in dorsoventral plane. Scale bar = 200 nm. Fig. 10. Section through microscale of *C. minus* (CAAP 1537/1A) showing the top part of its column, which resembles the flared bell of a trumpet. Scale bar = 200 nm. Fig. 11. Base plates of microscale with lattice structure and points of attachment of 4 stalks, which resemble bird's feet (arrows). Scale bar = 200 nm. Inset: Detail of attachment. Fig. 12. Scales of *C. minus* (clone CCAP 1537/1A) in dorsal view. Scale bar = 200 nm

ment to the species *C. minus* Page, 1976 resulted from comparison of light microscopic and ultrastructural characteristics of the 2 clones: the newly isolated clone from perch (4694/I) and that from Culture Collection of Algae and Protozoa (CCAP 1537/1A) isolated by Page from a freshwater pond in 1965. Because data on the ultrastructure of *C. minus* (CCAP 1537/1A) were not available, we had to study this strain prior to comparison.

Trophozoites of *Cochliopodium minus* (CAAP 1537/1A) (Fig. 3) observed in hanging drops were identical and their measurements fitted in the size range of *Cochliopodium* sp. (4694/I). In addition, the type of scales, their shape and size (Figs. 9 to 12) corresponded to those of the *Cochliopodium* sp. under study. The heights of scales above the plasma membrane, the diameters of basal plates and the diameters of the distal parts of scales differed only insignificantly (on the scale of nanometers). The same applied to the number of rays in distal part of scales (16 or 17 in *C. minus* from CCAP, and often more, (17 to 19, in *C. minus* from *Perca fluviatilis*).

Experimental infection

Cochliopodium minus was isolated from gills and other organs (kidney, liver and brain) of 3 out of 6 specimens of *Heteropneustes fossilis*, a bottom-dwelling fish species, kept for 5 wk in water enriched with trophozoites and cysts from culture of *C. minus* (4964/I). *C. minus* was not isolated from any of 5 control fishes of the same origin.

DISCUSSION

Comparison of the size of trophozoites and scale ultrastructure of the clone isolated from perch with the relevant data available from 4 species of the genus (1 named and 3 unnamed species) excluded 2 *Cochliopodium* species: the type species *C. bilimbosum* Auerbach, 1856 and the species designated as *Cochliopodium* sp. 3 by Bark (1973). Features similar to the present *Cochliopodium* sp. were described in *Cochliopodium* sp. 2 by Bark (1973) and by Nagatani et al. (1981) in strain NYS, which Yamaoka et al. (1984) used (under the name *Cochliopodium* sp.) for scale formation study. While the sizes (mean *L* and *B*) of trophozoites, diameters of nuclei and nucleoli and the size of scales fitted in the same range for the *Cochliopodium* sp. under study and *Cochliopodium* sp. 2 described by Bark (1973), the scale morphology differed. In *Cochliopodium* sp. 2 the 4-ribbed column was topped with a flat wheel-shaped structure, which in the dorsal view

had 2 concentric rings and 20 spokes. The ultrastructural comparison of scales in the present *Cochliopodium* sp. and *Cochliopodium* sp. strain NYS revealed similarity in the shape but divergence in size. All scale metric data (the height above the plasma membrane, the diameter of the top of the funnel and the number of rays in the web-like structure) were larger in the NYS strain. The same applied to the diameter of trophozoites measured by light microscopy.

In addition to the type species *Cochliopodium bilimbosum* Auerbach, 1856, 13 species of the genus *Cochliopodium* named from 1856 to 1926 were listed by Bark (1973). Since only light-microscopical features were available in the species described before, Bark (1973) faced an extremely difficult task trying to compare his own findings with the previously described species. Thanks to his study, the light-microscopical description of the type species, *C. bilimbosum*, was supplemented by ultrastructural data and the specific scale structure was recognized. Thus, only 1 of the 3 microscale patterns discovered by Bark (1973) could be attributed to the previously described *Cochliopodium* species. The other 2 types of scales characterize the 2 still unnamed different *Cochliopodium* spp. In addition to the type species, Page (1988) included in the genus *Cochliopodium* 2 other species, *C. actinophorum* (Auerbach, 1856) and *C. minus* Page, 1976. While *C. actinophorum* and *C. bilimbosum*, the species with large trophozoites and light-microscopically perceptible microscales, differ essentially, *Cochliopodium* sp. isolated from organs of perch could be identified with *C. minus* (CAAP 1537/1A) isolated by Page from a freshwater pond (Madison, Wisconsin, USA).

In older classifications (Levine et al. 1980) the genus *Cochliopodium* was assigned to the order Arcellinida Kent, 1880, i.e. among testate amoebae. Page (1987), however, established a special order, Himatismenida Page, 1987, for this genus, which he regarded as a transitional category between Gymnamoebia Haeckel, 1862 and Testacealobosia de Saedeleer, 1934. Irrespective of their actual taxonomic position, this is the first amoeba with a test-like cover (tectum) of unattached cell surface recovered as a systemic parasite of fish.

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