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Hungactinomyxon, a new actinosporean type and collective group (Myxozoa) from *Branchiura sowerbyi* Beddard (Oligochaeta)

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Abstract

Actinosporeans are characterised by great morphological diversity. As it has been proved that the class Actinosporea is a synonym of the Myxosporea, actinosporean genera have only been regarded only as actinosporean collective groups. While most actinosporeans are released individually by their oligochaete hosts, members of the synactinomyxon, siedleckiella and antonactinomyxon collective groups are released as eight connected structural elements. These actinosporean types are differentiated by the type of unit and junction of the caudal processes. On the basis of these characteristics, a new actinosporean type, constructed from eight echinactinomyxon units, is described as hungactinomyxon. Adjacent units are joined by two of their three processes and form two interconnected cubes, each containing four echinactinomyxons. Molecular biological studies also suggest that this new actinosporean type differs from other actinosporean types built up from eight structural elements for which 18S rDNA sequences are available in GenBank.

Introduction

Almost 200 actinosporean types are known. On the basis of their morphology, they were earlier classified in the form of 12 genera of the class Actinosporea: *Hexactinomyxon* Štolc, 1899, *Synactinomyxon* Štolc, 1899, *Triactinomyxon* Štolc, 1899, *Sphaeractinomyxon* Caullery & Mesnil, 1904, *Tetractinomyxon* Ikeda, 1912, *Neoactinomyxum* Granata, 1922, *Guyenotia* Naville, 1930, *Aurantiactinomyxon* Janiszewska, 1957, *Raabeia* Janiszewska, 1955, *Siedleckiella* Janiszewska, 1955, *Antonactinomyxon* Janiszewska, 1957 and *Echinactinomyxon* Janiszewska, 1957 (based on Janiszewska, 1955, 1957). However, Wolf & Markiw (1984) discovered that actinosporeans were not independent taxa but the life cycle stages of myxozoans. Several research groups, among them Kent et al. (2001), regard actinosporean genera as morphological types of actinosporean stages of a given myxosporean species.

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Most actinosporeans develop in an annelid host (generally oligochaetes) in pansporocysts in the gut epithelium. In most of these pansporocysts eight spores are formed. Spores released from pansporocysts and shed into the intestinal lumen are usually separate individuals each having three polar capsules, a sporoplasm with secondary cells, three caudal processes (processes are absent in sphaeractinomyxon and tetractinomyxon type actinospores) and, for some types (triactinomyxon and hexactinomyxon), a style. In some cases, however, the eight actinospores develop in contact with one another, joined together by the tips of their caudal processes and are released in this arrangement. Actinosporean stages composed of eight spores have been designated, according to the shape of the individual spores and the mode of attachment, as synactinomyxon, siedleckiella and antonactinomyxon. Synactinomyxons have a globular form and unite with each other via their caudal processes. The characteristic body shape of synactinomyxon differs considerably from those of the other two collective groups. Seven different synactinomyxon types have been described (Štolc,

1899; Marques, 1984; McGeorge et al., 1997; Xiao & Desser, 1998; Özer et al., 2002). Representatives of the other two types usually are connected to each other by all three caudal processes, forming an octahedral (antonactinomyxon) or a cube-like shape (siedleckiella). In addition to the two type-species [*Siedleckiella silesica* Janiszewska, 1953 and *Antonactinomyxon antonii* (Janiszewska, 1954–55)] studied in detail by Janiszewska (1953, 1955 and 1957), four other actinospore types forming an octahedron are known. A new siedleckiella type was found by Uspenskaya (1995), who experimentally proved that it was the actinosporean stage of *Zschokkella nova* Klokačewa, 1914. Other siedleckiella types were found by Özer et al. (2002) in *Tubifex tubifex* (Müller) in Northern Scotland and Székely et al. (2003) in *Lumbriculus variegatus* (Müller) in Japan, respectively. Two antonactinomyxon types are known; in addition to the type-species *Antonactinomyxon antonii*, originally described by Janiszewska (1955) as *Siedleckiella antonii* from *Limnodrilus claparedeanus* Ratzel, another type was detected by Xiao & Desser (1998) in *T. tubifex* from Lake Sasajewun, Ontario, Canada. The present paper describes hungactinomyxon, an actinospore type containing eight structural elements, which notably differs in spore morphology from the actinospore types described previously. This novel actinosporean is characterised on the basis of both morphological and molecular studies.

Materials and methods

In order to compare actinospores with myxospores already identified genetically, a survey was initiated in a fish farm near Budapest between April and September 2003. In the Temperate Water Fish Farm (TEHAG), a large variety of cyprinid fishes and, to a lesser extent, carnivorous fish species including sheatfish *Silurus glanis* L., pike *Esox lucius* L. and pikeperch *Sander lucioperca* L. are cultured. Specimens of the three most common oligochaete species (*Limnodrilus hoffmeisteri* Claparède, *Tubifex tubifex* and *Branchiura sowerbyi* Beddard) were collected monthly from the draining fishbed of the ponds. Mud samples from the fishbed were carried to the laboratory; oligochaetes were washed and placed in small plastic dishes containing some mud covered by a layer of water. The water above the mud was filtered through a mesh of 21 μm every day. After filtration, the mesh was washed into a small amount of water and a few drops of this water were placed on a slide, covered with a coverslip and examined under a compound microscope under ×200 magnification. When actinospores were found in the water, oligochaetes were placed individually into cell-well plates (Yokoyama et al., 1991), which were covered with self-adhesive plastic foil to prevent the worms from crawling from one well to another. During the following days, the water above each oligochaete in each of the wells was regularly examined for the presence of released actinospores. The plates were refrigerated at 4 °C throughout the study, and the water in the wells was changed once or twice a week. The presence or absence of actinospores was checked by placing the cell-well plates under a stereomicroscope. When floating actinospores were found, a drop of water from the well was examined on a slide using light microscopy at higher magnification. Microphotographs were taken of the spores using an Olympus BH-2 microscope with a DP-10 digital camera. Subsequently, 30 newly released actinospores were measured and drawn according to the guidelines suggested by Lom et al. (1997).

Two samples were used for molecular biological examination. One of them contained 13 hungactinomyxon specimens collected from the water one by one, while the other sample contained *c.*45 hungactinomyxon specimens and a few guyenotia. Samples were centrifuged at 5000 *g* for 5 min, spore pellets suspended in 500 μl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS and 0.4 mg/ml proteinase K) and incubated at 55 °C for 3–4 hours. DNA was extracted using the Miniprep Express Matrix (BIO 101, USA), as described previously by Eszterbauer (2004). A nested PCR system was used for amplification. First, the DNA content was amplified with the 18e–18g′ universal primer pair (described by Hillis & Dixon, 1991 and modified by Andree et al., 1999). It was followed by a second PCR with primer pair MX5–MX3 (Andree et al., 1999). The total volume of the PCR reaction was 50 μl, which contained 10–50 ng extracted DNA, 1× Taq PCR reaction buffer (MBI Fermentas), 1.25 mM $MgCl_2$, 0.2 mM dNTP (Sigma), 50 pmol of each primer and 2 units of Taq DNA Polymerase (MBI Fermentas). A Biometra T1

thermocycler was used for amplification. Amplification conditions in the first round were: 95 °C for 50 sec, 56 °C for 50 sec and 72 °C for 80 sec, for 35 cycles, with a terminal extension at 72 °C for 7 min, followed in the second round with 95 °C for 30 sec, 50 °C for 30 sec and 72 °C for 60 sec, for 35 cycles, with a terminal extension at 72 °C for 7 min. PCR products were electrophoresed in a 1.0% agarose gel. The specific DNA fragment was isolated from the gel and purified with Geneclean III Kit (Bio 101).

The purified PCR fragments were cloned using the pGEM-T Vector System I (Promega) according to the manufacturer's manual. Positive clones were selected using the blue-white colour screening method and confirmed by digestion with restriction enzyme *Msp*I and then by sequencing with the universal pUC/M13 primer (Promega). Clones were then sequenced in both directions with primers listed in Table 2 (except universal primers) using the Applied Biosystems (ABI) BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems). For sequence assembling, the STADEN Sequence Analysis Package version 2001.0 (Staden, 1996) was used.

Results

During the fish farm survey, several actinosporean forms of triactinomyxon, aurantiactinomyxon, neoactinomyxum, raabeia and guyenotia were found (data to be presented elsewhere); among them, however, a double-cube form composed of eight echinactinomyxon-shaped units was recorded (Figures 1–3). This actinosporean was composed of two intertwined cubes each formed by the joining of the distal ends of the three processes of four echinactinomyxon-like units (Figure 4).

The description of this new formation, to be named hungactinomyxon, is as follows.

Hungactinomyxon, new type

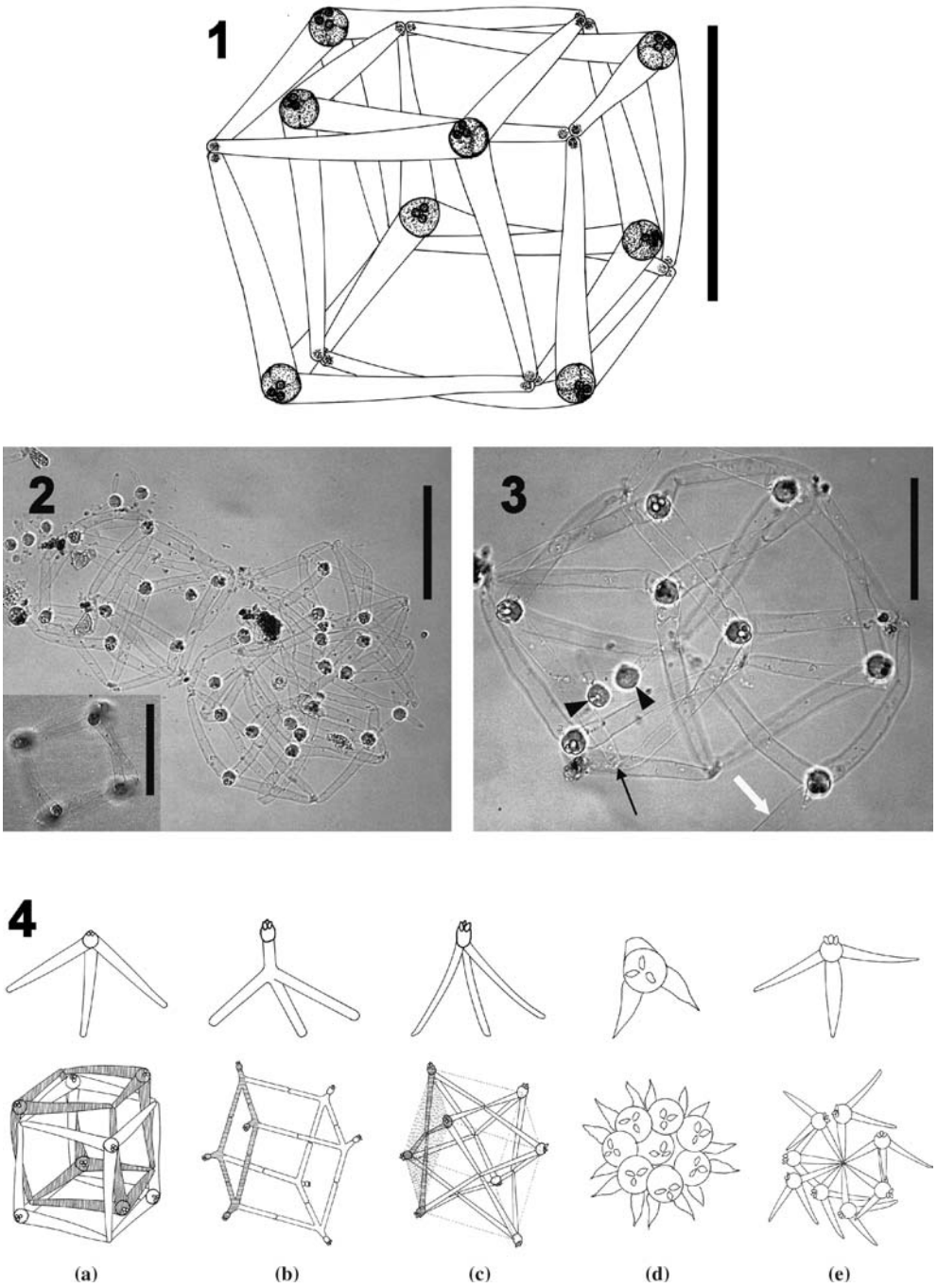
Mature spores are composed of a styleless spore body with three equal-sized, finger-like, lateroposterior caudal processes. The processes gradually taper toward the point of junction with the processes of two other spores. The length of the caudal processes is 71 (60–84) μm, the width of the process at the base of the spore body 9 (7–11) μm and at the junction 3.2 (3–3.5) μm; the spore body is spherical and 11.5 (10–13) μm in diameter. The three equal-sized pyriform polar capsules are situated very close to each other at the spore apex, and measure 4 (3–5) μm in length and 2.8 (2.5–3.5) μm in width. Each process of each spore joins one process of two different spores. Inside the overall cube reticulation, four spores are joined to each other by their ends, and this unit of four spores is interlaced with a second unit, forming a stable configuration of eight spores. Less frequently, masses of unseparated double-cubes (Figure 2) and discerptible units built up only from four spores were observed floating in the water.

Taxonomic summary

Type-host: *Branchiura sowerbyi* Beddard.
Type-locality: Temperate water fish farm, Százhalombatta, Hungary.
Prevalence of infection: 2 of the 109 worms (1.8%) during the whole period, (2 of the 20 worms (10%) found in May).
Phototypes: In the collection of the authors.
Etymology: Hungactinomyxon is a composed name referring to Hungary

Remarks

This form resembles siedleckiella and antonactinomyxon types in its cubic shape and closely connected eight actinospores, but differs from them by the structure of the cube which is composed of two units each containing four spores. Both antonactinomyxon and hungactinomyxon are built up from echinactinomyxon units but differ from each other in the way in which their caudal processes join together. In hungactinomyxon, a process of one spore forms a junction with only a single process of two other actinospores. In the antonactinomyxon type, however, four processes of different spores form the junction. Hungactinomyxon and siedleckiella differ from each other in the type of junction, with respect to both the type of the eight units and the number of caudal processes joining together. The siedleckiella type is composed of eight triactinomyxon-type spores, but hungactinomyxon is built from



Figures 1–4. 1. Diagrammatic illustration of the hungactinomyxon spore complex. 2. Mass of unseparated double-cubes of hungactinomyxons. Free spores of guyenotia are also apparent. Inset: a regular double-cube of a hungactinomyxon, apical view. 3. A freshly released hungactinomyxon floating in water and composed of echinactinomyxon units with united caudal processes. One process (white arrow) of an echinactinomyxon unit torn from the junction (arrow). Two guyenotia actinospores are found inside the grid (arrowheads). 4. Diagrammatic illustration of reticulated actinosporean collective groups: hungactinomyxon (a), siedleckiella (b), antonactinomyxon (c) and two synactinomyxon (d, e) types (lower row) and their actinospore unit (upper row); drawings not to scale. *Scale-bars*: 100 μm.

Table 1. Main characteristics of the actinosporean spores released by oligochaetes as clusters of eight spores.

Actinosporean type (species)	Type of the spore units	Number of processes in one unit at junctions	Number of processes forming a junction	Shape of the reticulation	Oligochaete host
Siedleckiella silesica Janiszewska, 1953	triactinomyxon	3	2	cube	*Tubifex* sp.
Siedleckiella stage of *Zschokkella nova* Uspenskaya, 1995	"	3	2	"	*T. tubifex*
Siedleckiella Özer et al., 2002	"	3	2	"	*T. tubifex*
Siedleckiella Székely et al., 2003	echinactinomyxon	3	2	"	*Lumbriculus variegatus*
Antonactinomyxon antonii (Janiszewska, 1955)	"	3	4	"	*Limnodrilus claparedeanus*
Antonactinomyxon Xiao & Desser, 1998	"	3	4	"	*T. tubifex*
Hungactinomyxon, present sudy	"	3	3	double-cube	*Branchiura sowerbyi*
Synactinomyxon tubificis Štolc, 1899	spore resembles aurantiactinomyxon with one shorter and two longer processes	1	8	star-like structure, circle	*T. tubifex*
Synactinomyxon type A McGeorge et al., 1997	"	1	8	"	*T. tubifex*
Synactinomyxon type 1 Özer et al., 2002	"	1	8	"	*T. tubifex, L. variegatus*
Synactinomyxon type 2 Özer et al., 2002	"	1	8	"	*T. tubifex*
Synactinomyxon longicauda Marques & Ormieres, 1982	echinactinomyxon	1	8	"	*T. tubifex*
Synactinomyxon type B* McGeorge et al., 1997	"	1	8	"	*T. tubifex, L. variegatus*
Synactinomyxon Xiao & Desser, 1998	"	1	8	"	*T. tubifex*
Synactinomyxon type 3 Özer et al., 2002	"	1	8	"	*T. tubifex, L. variegatus*

*Appears to be identical with *Synactinomyxon longicauda*.

echinactinomyxons. The synactinomyxon type is also composed of eight associated actinospores, but they join each other with a single process and form a globe rather than a cube (Figure 4; Table 1).

Among hungactinomyxons released by *Branchiura* specimens, actinospores of the guyenotia type were also found in mixed infection (Figures 2–3).

Molecular biological examination

The universal 18e–18g' primers (Table 2) and the more specific primer pair MX5–MX3 successfully amplified *c*.1,900 and *c*.1,600 bp fragments of the 18S rDNA, respectively, from both samples examined. PCR with the universal primers resulted in aspecific fragments as well; therefore, the DNA fragment of the correct size was isolated from the agarose gel and purified with Geneclean III Kit (Bio101) before the second PCR step.

Assembled sequences of both samples examined were 100% identical. A 1,648 bp long consensus DNA sequence was deposited in the GenBank under the accession number AY779062. Comparing this sequence to myxosporean sequences available in GenBank, *Myxobolus lentisuturalis* Dyková, Fiala & Nie, 2002 (AY119688) was the most similar (93.9%), followed by *M. cultus* Yokoyama, Ogawa & Wakabayashi, 1995 (AB121146) (93.5%). In GenBank, the partial 18S rDNA sequences of two collective groups of actinosporeans containing eight units are available. Hungactinomyxon was only 74.2% similar to the antonactinomyxon (AF378355) from *Lumbriculus hoffmeisteri* and 74.0% similar to the DNA sequence of synactinomyxon (AF378354) collected also from *L. hoffmeisteri* in Ontario, Canada. *Synactinomyxon* 'type 1' (AJ582002) and *S. longicauda* Marques & Ormières, 1982 (AJ582003) were found to be less than 70% similar to the hungactinomyxon.

Three of twelve clones had different sequences, which were identical to a partial 18S rDNA sequence of a guyenotia (AY779063) studied previously in our laboratory (Eszterbauer et al., unpublished).

Discussion

Since a review paper on the recent knowledge of Myxozoa (Kent et al. (2001), actinosporean stages of myxozoans described earlier as species are unanimously regarded as actinosporean types of known or undescribed species belonging to the Myxozoa. The authors of this paper agree with the latter approach, and actinosporean spores varying in shape and size are designated in the paper as actinosporean types.

Based on Janiszewska's (1955, 1957) classification, a number of actinosporean types have been described on the basis of spore body shape, the presence or absence of a style, the shape and branching of the caudal processes if present and the arrangement of individual spores. Actinosporeans containing eight spore units developing in a pansporocyst and connected by their processes are named siedleckiella, antonactinomyxon and synactinomyxon. The synactinomyxon collective group possesses a globular-like body shape, which differs considerably from that of hungactinomyxon.

New results (Kent et al., 2001), however, show that the number of actinosporean forms might be much higher than known at present, and new collective groups (endocapsa, tetraspora) have also

Table 2. Primers used for PCR and/or sequencing.

Name	Sequence	Reference
18e	5′-CTG GTT GAT TCT GCC AGT-3′	Hillis & Dixon, 1991
18g′	5′-CGG TAC TAG CGA CGG GCG GTG TG-3′	Hillis & Dixon, 1991
MX5	5′-CTG CGG ACG GCT CAG TAA ATC AGT-3′	Andree et al., 1999
MX3	5′-CCA GGA CAT CTT AGG GCA TCA CAG A-3′	Andree et al., 1999
MB5r	5′-ACC GCT CCT GTT AAT CAT CAC C-3′	Eszterbauer, 2004
MB3f	5′-GAT GAT TAA CAG GAG CGG TTG G-3′	Eszterbauer, 2004
MC5	5′-CCT GAG AAA CGG CTA CCA CAT CCA-3′	Molnár et al., 2002
MC3	5′-GAT TAG CCT GAC AGA TCA CTC CAC GA-3′	Molnár et al., 2002

been described (Hallett & Lester, 1999; Hallett et al., 1999). The siedleckiella type actinospore described by Székely et al. (2003) from *Lumbriculus variegatus* in Japan has been found to have an extraordinary shape, as it is built from echinactinomyxons instead of triactinomyxons, which are the usual units of siedleckiella (Table 1). Although its cube-like shape and the way of attachment greatly resemble siedleckiella, this actinosporean form should have been described as a novel type and collective group.

The present investigations contribute to the large number of variations that exist in the structure of actinosporean types built from eight units. Differences might appear in the shape of actinosporean elements (spores with or without styles) as well as in the number of uniting caudal processes and the type of their junction. The hungactinomyxon spore type described herein differs from all known actinosporean formations containing eight spore units in having two interlaced subunits each of four spores. Furthermore, its molecular sequence data is novel, and it is presented thus as a both a novel type and collective group.

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