The Variation in Pathogenicity Between Saprolegnia Parasitica and Saprolegnia Ferax Depends on Structural Differences

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ABSTRACT

The relation between pathogenicity and the structural characteristics of a parasitic fungus (Saprolegnia parasitica) and a saprophytic fungus (Saprolegnia ferax) was examined. Factors examined included zoospore numbers, the amount of adhesive materials, and the surface structure of zoospores, cysts and hyphae. Studies were carried out by germination of zoospores on salmon (Oncorhyncus mykiss Walbus) skin. The number of zoospores that attached to (encysted and for germinated) on salmon skin were higher in S. parasitica than in S. ferax. Also, S. parasitica secreted more adhesive materials than S. ferax during zoospore attachment, encystment and germination. In addition, S. parasitica zoospores and cysts have many hair bundles of different sizes and lengths, while hair bundle structures was not detected on zoospores and cysts of S. ferax. The hyphal surface of S. parasitica has a coarse and amorphous structure (amorphous verrucose) “first recorded” which was completely absent on the hyphae of S. ferax. These structural differences between S. parasitica and S. ferax may cause the pathogenicity of S. parasitica as highly fish pathogenic strain.

Keywords: Structure, Pathogenicity, S. parasitica, S. ferax.

INTRODUCCION

Saprolegnia is probably the most important fungal pathogen of aquatic animals causing saprolegniasis disease [24]. Infection caused by Saprolegnia has been responsible for massive death in wild and cultured fish specially salmon [25,29,30]. Once the infection is established, it is often fatal and difficult to treat. This suggests that the strains of Saprolegnia will continue to be problematic pathogens especially under stressful conditions prevailing in aquaculture.

Fungal adhesion is considered as an important event in animal diseases and an essential first step in the colonization process and parasitism, leading to the penetration and colonization of the host cell [27]. In the aquatic environment where turbulence and high flow rates are usually present [15], the dispersed spores should attach firmly to a suitable substratum for successful germination and colonization. Fungal adhesion may be serving as a base around which the infection is established.

Zoospores follow a homing sequence which include, zoospore taxis; encystment; cyst adhesion and germination [8]. Deacon and Saxena [7] deduced that most of these stages in the sequence are pre-programmed and depend only on environmental cues. It is usually mediated by recognition of chemical diffusates and surface components of the host substrate.

Induction of zoospore encystment can occur as a result of recognition of a host surface component. The adhesion and germination of cysts are closely linked and rapid germination of encysted zoospores would lead to rapid establishment on a host or other nutrient resources [8].

The present study was conducted to evaluate the differences in attachment, encystment and germination between a parasitic strain of fungus (S. parasitica) and a saprophytic strain (S. ferax) on salmon (Oncorhyncus mykiss Walbus) skin, in order to clarify the relation between pathogenicity and structural characteristics, such as zoospore numbers, the amount of adhesive materials and the surface structure of zoospores, cyst and hyphae.

Materials and Methods

1- Production of zoospores:

Zoospores of Saprolegnia parasitica H2 (NJM 8604)and Saprolegnia ferax (ATTC 36146) were produced according to the method of Kitancharoen and Hatai [18]. The concentration of zoospores was adjusted to 4X10^7 spores/ml.
2- Fish:

Two healthy rainbow trout (Oncorhynchus mykiss Walbaum), with an average weight of 37.0 ± 2.0 gm and an average length 15.0 ± 1.0 cm, were collected from Oshino Trout Hatchery Experimental Station, Yamanashi Prefectural Fisheries Technology Centre, Oshino, Yamanashi, JAPAN. The fish were anaesthetized with AF-100 (Tanabe Co. Ltd, Tokyo) and samples of skin were aseptically collected.

3- Zoospore attachment:

The skin samples were immersed in the spore’s suspension for 5, 15, 30, 60, 90, 120 and 180 min. to allow zoospores attachment.

4- Scanning electron microscopy:

a- Fixation:

Skin samples with attached and germinating zoospores were fixed in Trump’s fixative (McDowell and Trump, 1976) for 20 min., washed in phosphate buffer saline (pH. 7.3) three times for 15 min., re-fixed in 2% osmium tetroxide for 12 hours, washed 4-5 times in distilled water for 15 min. and dehydrated in ethanol series. The specimens were immersed in t-Butyl alcohol (2-methyl-2-propanol) three times for 10-20 min. at 4°C.

b- Critical point drying:

The dehydrated samples were critical point dried using liquid CO₂ as a drying agent and a JEOL JFD-300 dryer.

c- Ion sputtering:

Dried specimens were mounted on aluminum stubs and coated with gold/palladium using a JOEL JFC-1100E ion sputter.

The specimens were examined at 20KV using a JOEL-6100 scanning electron microscope.

Results and Discussion

Tip growth is a major form of cell growth, characteristic of fungal hyphae and plant cells such as root hairs and pollen tubes. It involves localized apical extension, which produces highly polarized tubular cells typical of species and life cycle stages. It requires coordination of apical cell wall deposition, tip extension, apex-directed cytoplasmic migration and organelle movements [28].

Hyphae of Saprolegnia maintain a tip-high gradient of cytoplasmic Ca²⁺ which is essential to tip growth. The gradient extends further along the periphery than the centre of the hyphae, consistent with an extended region of Ca²⁺ influx. Both cytoplasmic Ca²⁺ and the gradient’s slope are higher in faster growing hyphae, indicating greater exocytosis or apical extensibility in response to elevated Ca²⁺ [15].

Fungal spore attachment is especially critical in aquatic habitats and is essential for colonization, since the fungal spores in aquatic habitats may be washed ashore, attached to floating substrata or sink to the bottom. Therefore, the unattached zoospore could be easily carried from a suitable substrate by floating water force, thus preventing colonization [9]. Other factors considered to affect attachment of fungal spore include; surface recognition [21]; ionic charges (surface potential) of both spores and substratum [2]; hydrophobicity [6] and host surface topography [22]. Zoospore attachment studies were reported for S. parasitica [29] and S. ferox [9].

Among Oomycetes, the rapid appearance of the adhesive material that bound the zoospores to the host surface suggests the presence of stored preformed adhesive materials within the zoospore in the peripheral vesicles which are found around the zoospore ventral groove containing glycoprotein and glucan contents [8]. Cytochemical investigations demonstrated the partial carbohydrate nature of the adhesive material, indicating the presence of N-acetyl-D-glucosamine residues and large amounts of sulphhydril-containing polysaccharides, therefore, the most germinating rates of Saprolegnia species are from isolates germinated on salmonid mucus obtained from wild brown trout, Salmo trutta L. [11], since the mucus is consists of a mucopolysaccharides; containing sialic acid (n-acetyleneuraminic acid) [25,10] which may be the fundamental and the basic constituent for the formation of the adhesive materials.

Adhesive materials can also be a reservoir for digestive enzymes needed to penetrate the host cell, such as phosphatase, estrase and protease, prior to zoospore germination [16].

Chitin synthasae are active essentially at the hyphal tips of Saprolegnia species are vital for hyphal growth. Chitin is most likely synthesized transiently at the apex of the cells before cellulose, the major cell wall component in oomycetes [12]. The apical region of growing hyphae in S. ferox is cytoplasm-rich, and, adjacent to the cellular membrane, contains a high concentration of filamentous actin that is organized into a network termed an apical cap. This cap is considered to be important in tip morphogenesis and may have other roles in tip growth. Hyphae tips, also, contain a population of F-actin, which permeates their cytoplasm and may be important in cytoplasmic and organelle movements. Many actin-containing networks, are well known to be regulated by diverse ions, especially Ca²⁺ which may be of fundamental importance to the tip growth process by stretching of the plasma membrane via interactions between the integral membrane ion channel proteins and the actin cytoskeleton [20].
Fig. 1: (A): Secondary cyst of *S. parasitica* with hairs (arrowed). (B): Secondary cyst of *S. ferax* without hairs. (C): *S. parasitica* germinating secondary zoospore with long hairs (arrowed). (D): *S. ferax* germinating secondary zoospore naked from hairs.

Fig. 2: (A): Newly germinating secondary zoospore of *S. Parasitica* with hairs (thin arrow) and adhesive material around the zoospore forming the adhesive pad of globular nature (thick arrow). (B): Newly germinating secondary zoospore of *S. Ferax* with adhesive material around the zoospore forming the adhesive pad. (C): Extracellular materials at the tip of the germinal tube (thick arrow) of *S. parasitica* which extend as finger like structure (thin arrow) to insure the firm implantation on fish skin. (D): Little secretion of extracellular materials at the tip of the germinal tube of *S. ferax*. 
Durso et al. [8] showed that the adhesive material secreted by S. ferax is released by the cystospore which extends a short distance along the germ tube during attachment and was released at the tip of the germ tube during elongation and colonization to facilitate spreading on the substrate (Fig. 3B). Consistent with the hypothesis, a high incidence of adhesive material was observed with the early initiation of a bud of S. parasitica that would elongate into a germ tube (Fig. 3A).

After the adhesive material discharge, the texture of adhesive material was homogenous, but where it spreads along the substrate, it formed a branching network with globular structure (Fig. 2A). After, the adhesive material could extended beyond the diameter of the spore. This material was more abundant in the case of S. parasitica than with S. ferax on germinating zoospores (Figs. 2A&B).

With germings, there is an extracellular sheet of material near the site of origin of the germ tube. It is sometimes extended along the contact surface between the emerging germ tube and substrate and spreads for a distance away from the sides of the germ tube. This material appeared to be continuous with the adhesive pad material between the secondary cystospore and substrate (Figs. 2C&D).

Extracellular material between the tip of the germ tube and substrate was visible on germings with well-developed germ tubes. This extra-cellular material spread slightly in advance of the tip of the germ tube which is lectin in nature. This lectin also bound to portions of the adhesion pad between the secondary cystospore and its substrate; and the material at the germ tube tip was not continuous with this previously released adhesive material [9]. This material was most visible at the tip of the germ tube because the rounded contour of the tip separated it from the surface of the substrate. The pad of adhesion material extending toward the sides of the cystospore and slightly along the germ tube was not continuous with the material at the tip of the germ tube. The adhesive pad consists of three zones (a fibrous, a finely granular band and a coarsely granular) adjacent to the cyst coat [19].

Changes in the structure of the adhesion pad material indicated that it had fluidity when first released, but later its fluidity became constrained, possibly by progressive polymerization. Regions in which globular material was visible at the early stages and prominent at the later stages, might be the sites where polymerization of adhesive material occurs [9]. Lehnen and Powell [19] found that the material is initially released as a mound and the concavity of the exocytosing K2-body membrane physically constrains it from massive expansion. The material forming the leading edge of the conical-shaped adhesion pad expands along the zoospore membrane at the edge of the discharge site. The conical mounds of the material transform into disc-shaped adhesion pads, sometimes flowing along the germ tube for a short distance. The appearance of the adhesion pads depends upon the substrate and the pattern in form which depends on the capillary forces involved in the spread of the adhesion material. Adhesion material from secondary cystospore does not extend much beyond the edge of the spore. Thus, there may be constrains, either with time or space, which limit the fluidity of adhesive material. Within 5 min. of K2-body discharge, secondary cystospore were resistant to physical attempts at detachment, and the spores that came in contact with the surface became firmly attached. The further increase in the strength of attachment to a given substrate could be due to hyphal appressorial formation. Appressorial formation begins when the germ tube or hyphal apex ceases forward growth and serves as an adaptation for fungal attachment [27]. Appressorial formation in Saprolegnia helps the fungus to extract nutrients from the substrate which is influenced by topographical signals, substrate hydrophobicity and available nutrients [22]. Saprolegnia secondary zoospores began to swim sluggishly; settled onto the surface of substrates and encysted and within 15-20 min. germ tubes emerged from secondary cystospores.

Amorphous verrucose was identified on the hyphal wall of Saprolegnia parasitica (Figs. 3C&4C), but not all on Saprolegnia ferax (Fig. 3D). Amorphous verrucose has a coarse granulated structure which may act as a special hyphal structure that result in the firm attachment of fungal hyphae to the substrate which ensures fast germination and thus increases the pathogenicity of S. parasitica to fish.

Contact between the fungal mycelium and the food source is established by three mechanisms, the first is the modification of hyphae, e.g. appressoria; the second is the location of extracellular secretory materials and the third is the amorphous mucilaginous hyphal sheaths [17]. A fast germination rate in combination with mucilage production along the germ tube was shown to result in a firm attachment to substrate [1].

Most isolates of Saprolegnia showed some degree of poly plantism, that is, a repeated release of secondary zoospore. Polypl antism was revealed by the presence of empty secondary cyst cases with dehiscence pores in S. parasitica (Fig. 4A) and in S. ferax (Fig. 4B). The dehiscence pores (Figs. 2A&B) represented the place of the discharged secondary encystment vesicles (SEV) secreting the electron-dense outer wall where the cyst coat spines are embedded [5]. The cyst spines can be seen in cyst "ghosts" (Fig. 4C) which are left after the liberation of secondary zoospores. These spines appeared only on the parasitic strain (S. parasitica) but not the saprophytic strain (S. ferax) (Fig. 4D). The discharge of both adhesive and encystment vesicle is required to increase the cyst attachment to a substrate. This discharge might afford an explanation of the selective advantage of repeated zoosporere-


emergence. Polyplantism would, in effect, increase the number of chances to a single spore for employing its active attachment mechanisms [3]. The spores of the pathogen S. parasitica attached more rapidly and in greater number than those of the saprophytic S. ferax.

**Fig. 3:** (A): Highly amount of adhesive materials around the tip of the germinal tube of *S. parasitica*. Note, the branching network nature of the adhesive materials with globular structure (arrowed). (B): Little amount of adhesive materials around the tip of the germinal tube of *S. ferax*. (C): *S. parasitica* secondary zoospore with hairs (thick arrows). Note the coarse granulated structure of the amorphous verrucose (thin arrow). (D): *S. ferax* secondary zoospore with smooth surface.

The cyst coat of *S. parasitica* has a range of hair bundle types (Figs. 1A, 1C,2A,2C, 3A&3C), while the cyst coat of *S. ferax* does not have these hair bundles (Figs. 1B,1D,2B,3B&3D). The mean bundle length was found to be linearly related to the mean number of hairs per bundle. The explanation for these relationships may be that the vesicles containing the pre-formed cyst hairs, within the motile secondary zoospores, are of quite different sizes and volumes, as illustrated by Beakes [3]. Large vesicles contain many long hairs and small vesicles contain a few short cyst hairs, or even only one [13]. Similarly, there was a significant correlation between the mean length of the hooked-hairs and the mean distance between bundles of hooked-hairs, or single hooked-hairs if present. Significant differences in the mean hooked-hair length, number of hooked-hairs per bundle and inter-bundle distance were found between different *Saprolegnia* isolates [13]. Thus, isolates of *S. parasitica* had bundles of long hooked-hairs, with many hairs in each bundle and a relatively large inter-bundle distance on the secondary cysts.

The cyst wall is made up of two distinct layers. An outer 15-20 nm thick electron-dense layer in which the spines are embedded which are derived from the peripheral matrix of the bar body or primary encystment vesicles (PEVs). The second layer is an electron-transparent inner wall layer (250-400 nm) and is believed to be composed of amorphous and microfibrillar glucans [14]. The outer most layer of the cyst wall, in which the spines were embedded, appeared uniformly smooth, and there was no sign of underlying wall microfibrils that were often observed in secondary cysts [14]. The spines are linked with the outer electron-dense layer by glycol bonds. The outer electron-dense layer and spines survived after the treatment with cellulase, glucuronidase, pectinase and trypsin. In contrast, the inner electron transparent layer of the cyst wall is partially removed by cellulase and completely extracted by glucuronidase [3].

The most noticeable differences between saprophytic and fish-lesion isolates of *Saprolegnia* are shown by the secondary cyst coat ornamentation. Saprophytic isolates (*S. ferax*) have short spines (<
1.0 μm) which are born singly or in tufts of two or three. *S. parasitica* is distinguished by their bundles of (15-20 μm long) and (5-10 μm long) boathook spines [13].

The boathooks of *S. parasitica* isolates are clearly attached separately (not branched) to the amorphous outer cyst coat. The basal plaque of each spine is firmly embedded in the thin (8-10 nm) outer layer of the cyst wall and detached spines were only observed in association with spheres of peripheral matrix material. In cross section, the bundles of boathook spines are associated with fibrillar material, which may maintain bundle integrity [5]. The tips of the boathooks are characteristically bifurcated and reflexed. The obvious role of the bifurcated spines is to act as attached organelles. Pickering *et al.* [25] concluded that the bifurcated hooks on the secondary cysts of *Saprolegnia* are somehow involved with attachment of the cyst to a substratum. However, it has also been suggested these spines could act as buoyancy aids or possibly be primarily involved in the recognition response of fungus and pathogen [26]. The boathook clusters would increase chances to secondary cyst to entangle in the fish epidermis in an analogous fashion to an anchor. The explosive discharge and uncoiling of the cluster of boathook spines might enhance the chances of attachment. The secondary zoospores of *S. parasitica* are characterized by bundles of long hairs [29] which may facilitate infection by enabling the zoospores to attach to the fish more efficiently. The apparent trend towards grouping of the hooked-hairs and towards an increase in the length of these hairs has been interpreted as an adaptation to a parasitic existence [4].

Fig. 4: (A): *S. parasitica* empty secondary cyst cases with boathook spines which still attached (arrowed). (B): *S. ferax* empty secondary cyst cases. (C): *S. parasitica* empty germinal tube and secondary cyst with hairs which still attached (thick arrow). Note the amorphous verrucose (thin arrow). (D): *S. ferax* empty secondary cyst and germinal tube which still attached.

REFERENCES


