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Development and reproduction of *Saprolegnia* species in biofilms

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ABSTRACT

Saprolegnia spp. can cause mortality and economic losses in freshwater fish and eggs. Biofilm formation is generally regarded as a virulence factor, and biofilms can be an important cause of infection recurrence. Evidence of persistent sources of *Saprolegnia* infections on fish and eggs in fish farms support the assumption that *Saprolegnia* spp. might be able to form biofilms. In this study, we aimed to test the ability of *Saprolegnia* to form biofilms where it can survive, reproduce and resist different chemicals used for its control. Naturally formed biofilms were obtained from laboratory aquaria. *Saprolegnia* growth within these biofilms was demonstrated with light microscopy and confirmed by isolation. Isolates were identified morphologically and molecularly on the basis of ITS-sequences. Two isolates were identified as *Saprolegnia parasitica*, a species known to be highly pathogenic for fish, while the other belonged to *S. australis*. Selected *Saprolegnia* strains obtained from natural biofilms were then used to establish simple methods for *in vitro* induction of *Saprolegnia* biofilm. The ability of *Saprolegnia* isolates to form biofilms with subsequent production of infective motile zoospores within the biofilm was documented by light and confocal laser scanning microscopy. We demonstrate for the first time that isolates of *S. parasitica* and *S. australis* can form biofilm communities together with multiple microorganisms, wherein they grow and reproduce. It is therefore likely that natural biofilms constitute incessant *Saprolegnia* reservoirs in nature and aquaculture.

1. Introduction

Saprolegnia species belong to the class Oomycetes (Baldauf et al., 2000), some of which act as pathogens on fresh water fish, amphibians and crustaceans (Dieguez-Uribeondo et al., 2007; Krugner-Higby et al., 2010). Many salmonid fry raised in fish farms die as a result of oomycete infections, and total infection rates of 10% for salmonid eggs were estimated by Bruno et al. (2011). It is now widely accepted that microbes in nature rarely survive as solitary cells, but rather grow as biofilm (Harding et al., 2009). Biofilms are microbial communities that develop in association with a surface. They form a protected environment where microorganisms adopt a specific physiology. The discovery of biofilm formation by bacteria and yeasts has led to a better understanding of the mechanisms of virulence and persistence of pathogenic microorganisms (Harding et al., 2009). The secretion of extracellular enzymes and apical hyphal growth make fungi regarded as especially adapted for growth on surfaces (Jones, 1994). Fungi are therefore excellent candidates for biofilm formation (Harding et al., 2009). Oomycetes are not true fungi (Burki et al., 2007), but similar to fungi, *Saprolegnia* spores/cysts possess the ability to secrete adhesive materials that have affinity to bind to lectins (Lehnen and Powell, 1989; Burr and Beakes, 1994; Beakes et al., 1994). Sources of *Saprolegnia* infection cannot always be identified. Thoen (2011) reported that the *Saprolegnia* spore counts in effluent water of most Norwegian salmon hatcheries were higher than in inlet

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water. This suggests that there are unknown origins of *Saprolegnia* and biofilm could be one of the sources for saprolegniosis in fish farms and hatcheries. Based on this and due to recurrence of *Saprolegnia* infection within limited time intervals we hypothesize that *Saprolegnia* spp. are able to form biofilms. To explore the hypothesis, both naturally developed and artificially produced *Saprolegnia* biofilms were studied. Developmental and structural studies were performed by means of conventional light microscopy and confocal laser scanning microscopy which allows visualization of three dimensional structures of fully hydrated biofilm samples, while survival within biofilm was studied by exposure to known *Saprolegnia* control agents.

2. Materials and methods

2.1. Sampling of natural biofilms

Five biofilm samples were collected from the laboratory aquaria of the Norwegian School of Veterinary Science/Norwegian Veterinary Institute. The aquarium is equipped with tanks and water systems similar to what is used in commercial salmon hatcheries. The sampling site is therefore considered relevant and comparable to field conditions. Samples were taken by direct removal of biofilms attached to the bottom of aquarium tanks that were part of a water re-circulation system, using sterile forceps. Water parameters were recorded. Temperature was 11.5–13.0 °C during the period of biofilm formation. Water supply to the aquarium was from the lake Maridalsvannet, distributed by the municipal waterworks of Oslo, de-chlorinated by carbon filtering. The chlorination and de-chlorination processes are meant to decimate the microbial load in the water, but *Saprolegnia* spores are not greatly affected. Further microbes introduced with fish in the recycling systems contribute to a natural water environment. The flow rate was 5.0 l/min, with current created by horizontally directed distribution of inlet water. Recycling rate was 90–95% (variation depending on number of other tanks at use (total water consumption)). The 600 l fiberglass tanks were enamel painted and open. Natural biofilm samples were kept under constant humidity and were examined by light microscopy to confirm the presence of *Saprolegnia* hyphae.

2.2. Isolation and purification

Pieces of naturally formed biofilm were incubated in Glucose-Yeast (GY) broth at 15 ± 1 °C for 2–3 days (Stueland et al., 2005). To inhibit the bacterial growth, chloramphenicol (200 μg ml^{-1}) was added to the medium (Fregeneda-Grandes et al., 2007). The growing hyphae was cut into small pieces and transferred to sterile aquarium water (SAW) for zoospore production. Single spore isolation was performed on GY agar with antibiotics and incubated at 21 ± 1 °C for 2–5 days (Onions et al., 1981). These procedures were repeated until pure cultures were obtained. Pure cultures were stored on autoclaved hemp seeds at 4 ± 1 °C (Stueland et al., 2005).

2.3. Identification

2.3.1. Morphological identification

All strains were identified morphologically according to Seymour (1970) and Willoughby (1985). After producing single spore cultures on GY agar, a small plug of the growing mycelium was placed in GY broth and incubated for 2–3 days at 15 ± 1 °C. Bundles of hyphae were washed with SAW and incubated with autoclaved hemp seeds in SAW at 5, 15 and 20 ± 1 °C. The hemp seed cultures were examined for production of oogonia and antheridia twice a week using a stereomicroscope over a 12-week period (Stueland et al., 2005).

2.3.2. Molecular identification

The purified *Saprolegnia* isolates from biofilm were subjected to molecular identification. Genomic DNA was extracted from 20 mg mycelia from each isolate using CTAB miniprep extraction protocol (Gardes and Bruns, 1993). The ITS region was amplified using the universal fungal primers ITS1-ITS4 (White et al., 1990). The 25 μl reaction mixture consisted of 1.7 μM of each primer, 2 μl of genomic DNA, puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, UK) and milliQ water. PCR was performed on a DNA Engine Tetrad® Peltier Thermal Cycler. The PCR amplicons were visualized by gel electrophoresis on 1.5% agarose gel stained with Gelred (Huang et al., 2010). PCR products were purified with ExoSAP-IT (Amersham Bioscience, Buckinghamshire, UK) according to the protocol. The products were then sequenced in both directions with their respective primers, using the BigDye® Terminator v3.1 Ready Reaction mix (Applied Biosystems, Life Technologies, USA). The sequenced PCR products were purified with BigDye® XTerminator Purification kit (Applied Biosystems, Life Technologies, USA) according to manufacturer's instructions, and subsequently analyzed on an ABI PRISM® 3100 – Avant Genetic Analyzer (Applied Biosystems). Sequence contigs were assembled and quality controlled in BioEdit (Hall, 1999). The sequences were compared to publically available sequences using the NCBI nucleotide BLAST (Basic Local Alignment Search Tool; Altschul et al., 1997), and identified on the basis 100% identity to well annotated *Saprolegnia* reference strains.

2.4. Zoospore production, encystment and adjustments

In order to produce spores to be used for formation of biofilm, *Saprolegnia* hyphae were excised from colonized GY agar plates and incubated in GY broth at 15 ± 1 °C for 2 days to obtain further hyphal growth. Subsequently, bundles of these young hyphae were washed twice in SAW, transferred to two glass bottles containing one liter of SAW and incubated at 21 ± 1 °C for 24 h to allow extensive zoospore production. The zoospore suspensions were filtered through a sterilized tea filter (0.5 mm pores) into another glass bottle, a procedure that is expected to result in zoospore encystment (Stueland et al., 2005). The cysts were counted using a haemocytometer (Bürker türk). The cyst suspensions were adjusted by dilution to obtain the required density 1.0×10^4 spores l^{-1} (Thoen et al., 2011) and used in the