Vol. 30: 165–169, 1997 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published September 30

Fluorescent labelling of actinospores for determining the portals of entry into fish

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ABSTRACT: A fluorescent dye, 5(6)-carboxyfluorescein diacetate, succinimidyl-ester (CFSE), was used for pre-labelling of actinospores to determine their portals of entry into fishes. Three types of actinospores, triactinomyxon (*Myxobolus arcticus*), raabeia (*Myxobolus cultus*) and aurantiactinomyxon (*Thelohanellus hovorkai*), were freshly isolated from the oligochaete hosts, and incubated with CFSE for 15 min. Myxosporean-free salmonid or cyprinid fishes were exposed to the CFSE-labelled actinospores for 30 min, and fixed with 2% paraformaldehyde. The fixed fins, skin and gills were examined for the presence of CFSE-labelled cells under a fluorescence microscope. Penetration of triactinomyxon and raabeia was mostly observed on the fins and skin. In contrast, aurantiactinomyxon penetrated mainly via the gills of common carp. Aurantiactinomyxon did not penetrate goldfish, which was non-susceptible to *T. hovorkai*. However, penetration of triactinomyxon was noted in the epithelium of sockeye salmon, which had a low susceptibility to *M. arcticus* in Japan. This is the first report of a CFSE-labelling technique used for tracking infectious organisms in transmission experiments.

KEY WORDS: Myxosporean · Actinosporean · Fluorochrome stain · CFSE · *Myxobolus arcticus* · *Myxobolus cultus* · *Thelohanellus hovorkai*

INTRODUCTION

Recent understanding of the complete life cycle of myxosporeans involving alternate oligochaete host and actinosporean stages (Wolf & Markiw 1984) has made it possible to clarify the mode of transmission of myxosporeans into fish. By exposing rainbow trout to triactinomyxon actinospores of *Myxobolus cerebralis*, the causative agent of salmonid whirling disease, portals of entry via the skin, fins, buccal cavity and digestive tract have been demonstrated (Markiw 1989). Furthermore, El-Matbouli et al. (1995) showed, from light and electron microscopic observations, that the penetrated parasites developed into the multiple-fission stages and finally reached the cartilage through the nervous system of rainbow trout. In these studies, however, an exposure of fish to a high concentration of actinospores (10 000 spores per fish) was required to detect the penetrated parasites and to investigate their routes into the cartilage (Markiw 1989, El-Matbouli et al. 1995). Although we also have succeeded in performing transmission experiments with several myxosporean species (Urawa & Awakura 1994, Yokoyama et al. 1995a, Yokoyama 1997), we so far could not detect the initial stages in the experimentally infected fish, most likely due to the difficulties in obtaining such a large amount of actinospores at a time.

The fluorescent dye 5(6)-carboxyfluorescein diacetate, succinimidyl-ester (CFSE) has been used as a marker in the analysis of cell fate during embryoid development (Paramore et al. 1992), and in following graft migration in the recipient during transplantation experiments (Fujioka et al. 1994). CFSE is a lipophilic molecule that is deacetylated by intracellular cytoplasmic esterases to yield flurescence, while succinimydil ester binds to amino groups of intracellular macromolecules (Fujioka et al. 1994). CFSE has been considered to be nontoxic for labelled cells and not to diffuse to adjacent tissues. Thus, CFSE labelling has

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been thought to be a useful technique for *in vitro* and *in vivo* cell localization.

In the present study, we used CFSE for pre-labelling of actinospores in transmission experiments in order to determine the portals of entry of actinospores into fish.

MATERIALS AND METHODS

Actinosporean collection. The aquatic oligochaetes *Lumbriculus variegatus* infected with the triactinomyxon stage of *Myxobolus arcticus* (Urawa & Awakura 1994), *Branchiura sowerbyi* with the raabeia stage of *Myxobolus cultus* (Yokoyama et al. 1995a) and *B. sowerbyi* with the aurantiactinomyxon stage of *Thelohanellus hovorkai* (Yokoyama 1997) were obtained in Japan from the Chitose River (western Hokkaido), a goldfish farm (Saitama Prefecture) and a carp pond (Niigata Prefecture), respectively. Waterborne actinospores were isolated by the multi-well plate method (Yokoyama et al. 1991); briefly, oligochaete worms were individually kept in 2 ml (for *B. sowerbyi*) or 1 ml (for *L. variegatus*) wells filled with dechlorinated tapwater, and released actinospores were collected within 24 h.

Labelling with CFSE. A stock solution of 5(6)-carboxyfluorescein diacetate, succinimidyl-ester (CFSE; Molecular Probes, Inc.) was prepared at 10 mM in 100% dimethylsulfoxide (DMSO), and kept at 5°C until use. For labelling, CFSE stock solution was added to an actinosporean suspension (2000 to 3000 spores ml^{-1}) at 10 µl per ml spore suspension, mixed with a quick vortex, and then left for 15 min at a room temperature. After labelling, actinospores were recovered by filtration with a 50 µm (for triactinomyxon and raabeia) or 20 µm (for aurantiactinomyxon) nylon mesh, and resuspended in distilled water. In order to check fluorescence intensity, small volumes of spore suspension were fixed in 1% formalin.

Transmission experiments to fishes. Myxosporean-free fish fry [masu salmon *Oncorhynchus masou* (average body weight; 0.21 g, average fork length 2.76 cm), sockeye salmon *O. nerka* (average body weight 0.15 g, average fork length 2.60 cm), goldfish *Carassius auratus* (average body weight 0.04 g, average standard body length 1.1 cm) and common carp *Cyprinus carpio* (average body weight 0.04 g, average standard body length 1.2 cm)] were exposed to the labelled actinospores in a glass beaker (100 to 400 ml) at a dose of 1000 spores per fish. After 30 min exposure, 5 fish were anaesthetized with di-phenoxyethanol, rinsed with distilled water, and then fixed in 2% paraformaldehyde. Remaining fish were transferred to a myxosporean-free tank and subsequently kept at a constant temperature (ca 20°C for common carp and goldfish, ca 10°C for masu and sockeye salmon). After removal of exposed fish from the beaker, actinospores were recovered by filtration with a nylon mesh in order to examine release of infective cells from spore valves. Several months post-exposure, surviving fish were dissected, and examined for infection with myxosporeans.

Fluorescence microscopy. The fixed fins, skin and gills were mounted on a glass slide and examined for CFSE-labelled cells using a fluorescence microscope (Nikon, Labophoto-2) equipped with a 100 W mercury lamp and a B2 band excitation filter (450 to 490 nm).

RESULTS

Labelled actinospores exhibited intensely bright green fluorescence, particularly in the infective cells (Figs. 1 & 4). Fluorescence intensity appeared to be unchanged by fixation. During fish exposure, most of the triactinomyxon actinospores (80 to 90%) released infective cells, suggesting that CFSE labelling did not affect the infectivity of actinospores.

Following exposure, triactinomyxon infective cells were clearly seen in the fins and skin of masu and sockeye salmon (Fig. 2). Anchoring polar capsule cells (Fig. 3) were also recognized at the site of entry of infective cells. As penetrated parasites divided and invaded the deeper strata of the epithelium, fluorescence intensity gradually faded. However, fluorescing polar capsules remained at the probable point of penetration. Infective cells were rarely detected in the basal part of the gill filament of masu and sockeye salmon (Table 1). There was no marked difference in frequency of actinosporean penetration between masu and sockeye salmon. Six months post-exposure, *Myxobolus arcticus* spore formation was observed in the central nervous system of masu salmon (5 infected fish out of 5 examined fish). However, no spores were found in any of the sockeye salmon (n = 22) (Table 1).

Penetration of raabeia infective cells was frequently observed in the fins and skin of goldfish and common carp (Table 2). Penetration into the gills was not seen. *Myxobolus cultus* spores were not detected from either fish species even 3 and 4 mo post-exposure.

In contrast to the above 2 species, aurantiactinomyxon penetrated via the gills. Opened spore valves and penetrating infective cells were occasionally detected between the gill lamellae (Fig. 5). Aggregates of infective cells appeared round or rod-shaped, and were observed in the distal and/or basal part of the gill lamellae (Fig. 6). Penetration of infective cells was observed in common carp gills, but not in goldfish gills (Table 2). Three to four months post-exposure, *Thelohanellus hovorkai* spores were found in common carp (8 out of 9 fish), whereas no myxosporeans were detected in goldfish (0 out of 17 fish).

Table 1. Penetration of triactinomyxon actinospores of *Myxobolus arcticus* into salmonid fishes and myxosporean spore formation in the central nerve tissues

	Masu salmon			Sockeye salmon		
	Fins and skin	Gills	Myxosporean spore formation	Fins and skin	Gills	Myxosporean spore formation
Triactinomyxon	++	+	5/5*	++	+	0/22

++: frequently observed; +: rarely observed; *no. of infected fish / no. examined fish

Table 2. Penetration of aurantiactinomyxon actinospores of *Thelohanellus hovorkai* and raabeia actinospores of *Myxobolus cultus* into cyprinid fishes

	Common carp			Goldfish		
	Fins and skin	Gills	Myxosporean spore formation	Fins and skin	Gills	Myxosporean spore formation
Aurantiactinomyxon	–	++	8/9*	–	–	0/17
Raabeia	++	–	0/7	++	–	0/17

++: frequently observed; –: not observed; *no. of infected fish / no. examined fish

DISCUSSION

This is the first report of the CFSE labelling technique used for tracking infectious agents in transmission experiments. Even at a relatively low dose of actinospores (1000 spores/fish), penetration of infective cells into fish was easily detectable. The advantages of this labelling technique include: (1) it is simple, stable and easily applicable to many kinds of infectious organisms; (2) *in vivo* and *in vitro* visual detection of labelled cells is rapid and easy under a fluorescence microscope, even if the labelled cells are only few in quantity and hard to distinguish by morphological characteristics; (3) CFSE labelling does not affect viability of infectious organisms. On the other hand, the technique has the following possible limitations: (1) it can be applied only in a short period post-infection, because fluorescence intensity will diminish as labelled cells undergo cell-division and further development processes; (2) histological tracking may be difficult, because common solvents for conventional paraffin processing will cause the fluoresence to be lost. Frozen section or immunohistochemical methods (Garton & Schoenwolf 1996) might solve this problem. The failure of the infection experiment with raabeia labelled with CFSE suggests that the labelling might have affected the developmental process, but this is not clear at this time, because a control exposure of goldfish to unlabelled raabeia was not conducted at the same time.

The chemoresponse of actinospores to fish skin mucus suggested that the routes of entry might be the body surface of fish, possibly the fins, skin and gills (Yokoyama et al. 1993, 1995b). In the present study, there was a remarkable difference in the routes of entry among the 3 types of actinospores tested. Penetration via the body surface by the triactinomyxon of *Myxobolus arcticus* seems to be consistent with the results described for the triactinomyxon of *M. cerebralis* (Markiw 1989, El-Matbouli et al. 1995). However, the aurantiactinomyxon case appeared to be quite different from the triactinomyxon case. Styer et al. (1991) demonstrated that the exposure of channel catfish to aurantiactinomyxon spores from the oligochaete *Dero digitata* led to a granulomatous inflammation of the gills, namely, PGD (proliferative gill disease). Pote & Waterstrat (1993) found the motile stage of aurantiactinomyxon spores following exposure of spores to channel catfish gills. It is possible that aurantiactinomyxon-type actinosporeans may prefer the gills as the initial route of infection. The mechanism of the gill-preference of aurantiactinomyxons needs further study. In this study, only the skin, fins, and gills were examined as portals of entry, but other possible routes such as buccal cavity and intestine should be investigated by histological sections. Also, the morphological characteristics of the early stages of the myxosporean should be more closely described by light and electron microscopy.

The host-selection mechanism of actinosporeans remaines to be studied. The triactinomyxon of *Myxobolus arcticus* penetrated into both masu and sockeye salmon, but no spore formation occurred in sockeye salmon, suggesting that the parasites could not have

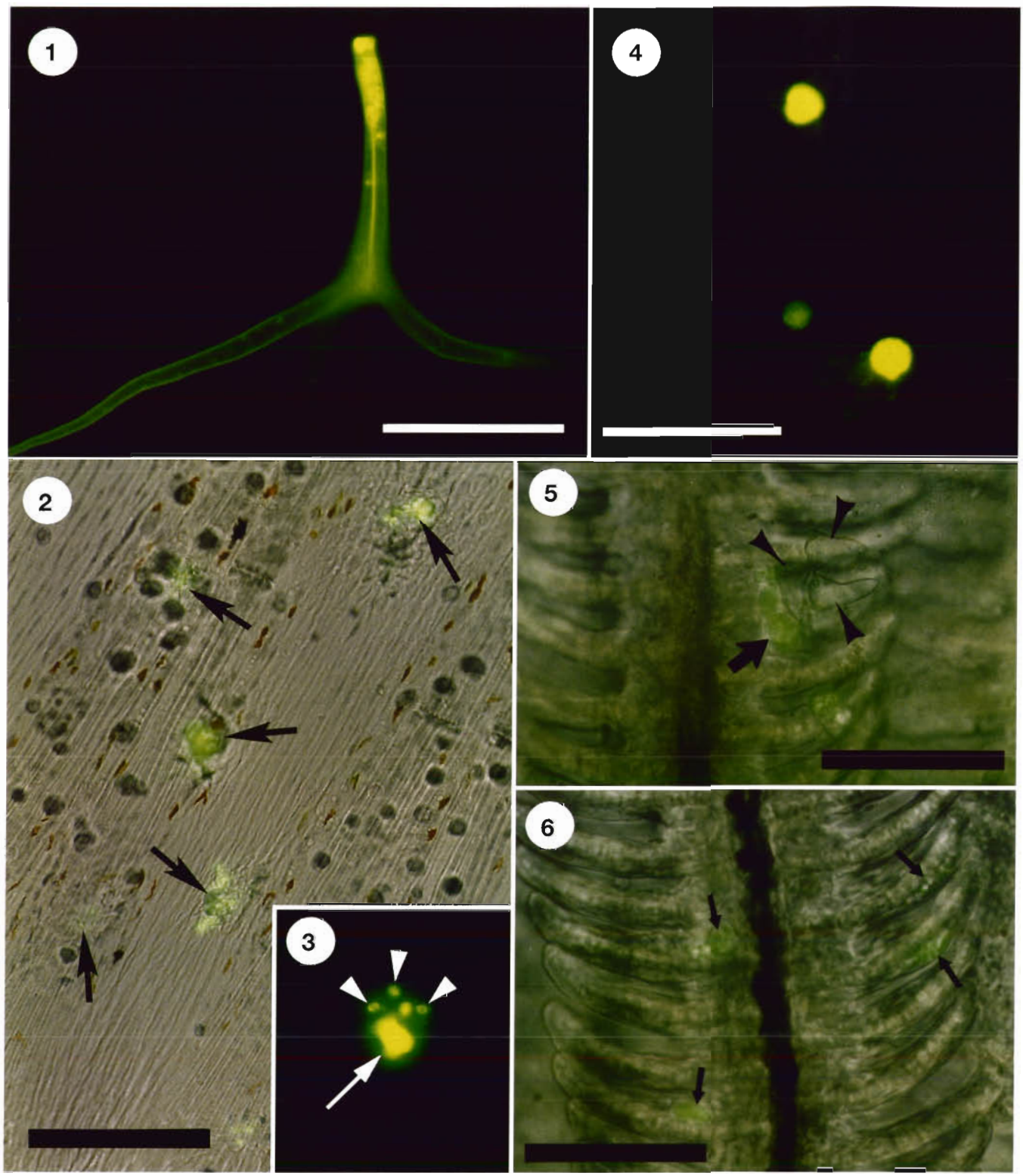

Fig. 1. *Myxobolus arcticus*. CFSE-labelled triactinomyxon actinospore. Scale bar = 100 µm

Figs. 2 & 3. Caudal fin of sockeye salmon exposed to CFSE-labelled triactinomyxon. Fig. 2. Penetration of triactinomyxon infective cells (arrows) into the fin. Double exposure with normal light and fluorescence. Scale bar = 100 µm. Fig. 3. Anchoring polar capsules (arrowheads) and penetrated infective cells (arrow)

Fig. 4. *Thelohanellus hovorkai*. CFSE-labelled aurantiactinomyxon actinospores. Scale bar = 100 µm

Figs. 5 & 6. Gills of common carp exposed to CFSE-labelled aurantiactinomyxon. Double exposure with normal light and fluorescence. Scale bar = 100 µm. Fig. 5. Opened spore valves (arrowhead) and infective cell (arrow) between the gill lamellae. Fig. 6. Invasion of aurantiactinomyxon infective cells (arrows) into the gill filaments

developed normally in sockeye salmon. The observation that even unlabelled triactinomyxon spores could not infect sockeye salmon (Urawa et al. 1995) supports this idea. However, Canadian *M. arcticus* has been reported to easily infect sockeye salmon (Kent et al. 1993). It is possible that there is a geographical difference in the susceptibility of fish species between Japanese and Canadian strains of *M. arcticus* (S. Urawa unpubl. data). In contrast to *M. arcticus*, it appeared that the aurantiactinomyxon of *Thelohanellus hovorkai* could recognize the specific host (common carp) at the time of entry, since no penetration of aurantiactinomyxons was observed in goldfish. Yokoyama et al. (1995b) indicated that a non-specific mucin component must be a trigger of infective cell release of raabeia spores of *M. cultus*, suggesting that the invasion of raabeia of *M. cultus* might be non-specific for fish species. It is unknown how aurantiactinomyxons could distinguish the natural host (common carp) from an unnatural host (goldfish). In order to elucidate the mechanism on host-specificity of myxosporean infection, further studies will be required.

In conclusion, portals of entry of actinospores into fish were successfully determined using the CFSE labelling technique, suggesting that CFSE may have significant potential as a marker in transmission experiments with other infectious agents as well.

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Editorial responsibility: Wolfgang Körting, Hannover, Germany

Manuscript received: January 24, 1997
Revised version accepted: June 5, 1997