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# Inosine-arginine salt as a promising agent for *in vitro* activation of waterborne fish-pathogenic myxozoan actinospores

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ABSTRACT: Mucus-derived nucleosides serve as key host cues for myxozoan actinospore fish host recognition, but to date their use for experimental actinospore activation in the laboratory or application in disease prevention has not progressed very far. One obstacle has been the low solubility of pure inosine and guanosine. To overcome this, we used inosine-arginine salt (ino-arg), which incorporates both high activation properties and high solubility. We tested its efficacy both in microassays directly observing reactions of actinospores of 2 distantly related myxozoan species, *Myxobolus cerebralis* and *M. pseudodispar* in comparison to inosine, as well as its actinospore-inactivation properties by premature polar capsule discharge in an infection experiment. Ino-arg was considerably more effective in eliciting polar capsule discharge and sporoplasm emission at much lower concentrations than pure inosine and, in contrast to the latter, remained dissolved in aqueous solution. Ino-arg exposure of *M. pseudodispar* actinospores resulted in polar capsule discharge and sporoplasm emission before host contact and subsequently in a lower infection rate in roach *Rutilus rutilus*.

KEY WORDS: Myxozoa · Whirling disease · Actinospore activation · Polar filament discharge · Disease prevention · Aquaculture



## INTRODUCTION

Myxozoan parasite transmission to teleost fish hosts ultimately depends on their spread by actinospore stages. These planktonic stages are shed from their invertebrate host, mainly oligochaete worms, and encounter their fish hosts by passive floating and unique reactions in close proximity of a suitable host. A combination of chemical and mechanical signals initiates the invasion process (Yokoyama et al. 1995, Kallert et al. 2005, Eszterbauer et al. 2009). For actinospores of several species (e.g. *Myxobolus cerebralis, M. pseudodispar, M. parviformis, Henneguya nuesslini*), chemical recognition of nucleosides (i.e. inosine and guanosine) prime the actinospore to become mechanically sensitive (Kallert et al. 2010, 2011). To date, nucleosides have been the only natural host cues found in fish mucus to elicit distinct reactions by actinospores. When contact occurs, upon a mechanical stimulus they anchor themselves to the host surface by extrusion of their polar filaments, threads that are wound up in the myxozoan-specific polar capsules. These pull the endospore unit close to the epidermal surface and enable subsequent penetration of the amoeboid sporoplasm primary cell that thereafter enters the tissue and further migrates towards deeper tissue layers for secondary cell release (Kallert et al. 2009).

An easy-to-use method for *in vitro* activation of actinospores would not only enable researchers to test for actinospore reactivity and infectivity, but is an important step to analyse genetic factors involved in

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spore activation and host invasion as well as host specificity parameters (Eszterbauer et al. 2009). It is also a first step for *in vitro* culturing of myxozoans. The identification of the natural stimulants for actinospore activation initially opened the door for such study possibilities. However, inosine and guanosine, intermediate metabolites of ATP breakdown and the key components recognized by actinospore stages, have relatively low water solubility, and because of their hydrophobic character, they tend to disappear from the water column rapidly to form either molecular aggregates or bind to various substrates. While this may be a key characteristic for serving as a suitable host signal (accumulation in the mucus and virtual absence in the water column), use of these compounds in scientific studies and their application in aquatic environments remain rather difficult. Solubilisation requires heating and constant stirring; otherwise, these substances attach to debris or glass walls, or they build aforementioned aggregates. Substitution by other, more soluble nucleoside derivates was less or not at all effective compared to the pure substances in preliminary tests (our unpublished data). Moreover, these substitutes are usually very costly. An alternative might be the inosine-arginine salt (ino-arg) patented by Kurauchi et al. (2004), a substance which has a high water solubility even at low temperatures. It was developed for crop nutrition and growth promotion and is comparatively cheap in production. Therefore, ino-arg was considered suitable for application in actinospore activation.

Here we evaluated the efficacy of ino-arg in laboratory usage for polar capsule discharge triggering and sporoplasm emission tested with 2 myxosporean actinospores. For the study, we chose *Myxobolus cerebralis*, the parasite causing whirling disease in salmonids, and *M. pseudodispar*, a widely distributed muscular parasite of roach and other cyprinids throughout Europe. Furthermore, we asked whether this chemical may be a potent preliminary activator of actinospores in the absence of fish to reduce numbers of infective stages in water and subsequently to quantitatively interfere with transmission to host fish, reducing disease severity.

## MATERIALS AND METHODS

### Parasites

Actinospores (<48 h old) of *Myxobolus cerebralis* and *M. pseudodispar* were obtained from long-term laboratory cycles as described previously (Kallert et al. 2005, 2007). They were filtered through 20 µm nylon gauze from the supernatant water of mud-bottom tanks containing oligochaete worms infected with the parasites, and they were further concentrated by gauze filtration for experimental use and kept in plain filtrate residue water at 12°C at all times. The concentration of actinospores used for microassays varied between 2500 and 20 000 $ml^{-1}$. The same actinospore isolate was used for each microassay replicate.

### Fish and mucus preparation

Specific pathogen-free (SPF) roach *Rutilus rutilus* were laboratory-reared from eggs collected from the leaves of aquatic plants in a small stream near Budapest, Hungary. Fingerlings were used for infection at 1.2 cm (5 mo of age). They were kept in aerated glass tanks at 23°C and fed on commercial ornamental fish food. Rainbow trout for mucus homogenate preparation (positive controls) were obtained live from a commercial fish farm in Germany. Mucus homogenate was prepared as described previously by Kallert et al. (2005). The concentration of mucus was calculated based on its dry weight after complete lyophilisation of 200 µl aliquots. A homogenate of 1 mg $ml^{-1}$ final concentration was used for actinospore activation.

### Ino-arg preparation

Ino-arg was prepared according to the patent instructions (US patent no. 2004/0192553 A1; Kurauchi et al. 2004). Briefly, 10 g of inosine (Sigma, pure grade) and 6.5 g of arginine (Sigma, HPLC grade) (equimolar amounts) were solubilised by stirring and heating (70°C) to obtain a concentrated solution in 33.5 ml water. This was slowly added to 1 l of anhydrous ethanol under constant vigorous stirring. The resulting white precipitate was paper filtered (round paper filters, Scholl) and dried at 45°C overnight. The white powder of ino-arg was then diluted with 5 mM sodium phosphate buffer pH 7.5 to concentrations of 0.1, 0.05 and 0.01 mg $ml^{-1}$, respectively.

### Experiments

#### Activation microassay

To measure polar filament discharge and sporoplasm emission, we applied the vibration microassay

including trout mucus homogenate as described previously (Kallert et al. 2005). The actinospore solution of *Myxobolus cerebralis* and *M. pseudodispar* was mixed with test substrate at a ratio of 2.33:1 (total volume 30 µl) on a slide and vibrated (50 Hz, 3 mm amplitude) immediately after covering with a 22 × 22 mm cover slip. Activated actinospores were then counted using phase contrast microscopy for approximately 5 min immediately after activation. Reaction rates were calculated from all viable actinospores per slide with clear reactions or their absence. Discharge and emission were always counted as separate reactions; a spore is either just discharged or has emitted its sporoplasm. We did not count actinospores with emitted sporoplasms that were not close or attached to the apical region or the ones without sporoplasms. Only sporoplasms inside the actinospore shell which reacted to stimuli or the emitted sporoplasms in close contact were counted (e.g. see Fig. 1 of Eszterbauer et al. 2009). All test substrates were buffered to pH 7.5 by sodium phosphate buffer. As negative control substrate, aqueous solutions of the non-stimulating nucleoside derivate 3′methyl-guanosine were used with the final concentration of 0.1 and 1 mg $ml^{-1}$. For the inosine substrate, inosine powder (Sigma) was diluted with 5 mM sodium phosphate buffer (pH 7.5) to concentrations 0.5 and 0.1 mg $ml^{-1}$, respectively. Preparation of substrates was conducted with respect to the degree of inosine solubilisation comparable to its use in a field-like situation by leaving samples standing after preparation and buffering at 12°C for 20 min with only minimum vortexing prior to addition to the actinospore suspension. These conditions were chosen to more closely resemble the practical application of inosine and ino-arg in our laboratory setup.

In the first activation microassay, substrate concentrations were chosen on the basis of preliminary tests and experience gained from previous experiments and in accordance with the inosine content of trout mucus as published by Kallert et al. (2011). In a second microassay experiment, we intended to confirm the results from the first experiment with another ino-arg preparation and a different day's actinospore isolate and include an even lower (0.01 mg $ml^{-1}$) ino-arg concentration to highlight the differential efficiency properties.

### Decoy assay

In this part of the study, we tested the possibility of reducing infective units (actinospores) prior to individual fish exposure after the incubation of *Myxobolus pseudodispar* actinospores in water with ino-arg in comparison to pure inosine and a water control without additives. To assess the potential for reduced numbers of infective actinospores by ino-arg in larger water volumes and to lower subsequent fish infection, we treated water (300 ml) containing *M. pseudodispar* actinospores (1000 per glass and single fish) with inosine (0.01 mg $l^{-1}$), ino-arg (0.018 mg $l^{-1}$) and tap water (gently stirred twice with a glass rod) as a negative control for 20 min prior to addition of SPF fish (10 specimens per group). Individually exposed fish were left in the aerated solution for 2.5 h before transfer to aerated aquaria. Fish of the same group were kept together. After 3 mo, fish were anaesthetized in 200 mg $l^{-1}$ MS222 solution (Sigma) and killed by cranial cut. After dissection, the musculature from 1 side fillet of each specimen was homogenized with Ultra Turrax, and the homogenate was diluted to 7 ml with cooled tap water. The numbers of myxospores were counted in 4 replicates per homogenate using a Bürker chamber with phase contrast microscopy.

### Statistics

To test for normal distribution of data, we used the Kolmogorov-Smirnov test, while homogeneity of variances was confirmed by the Levene test. Data from myxospore enumeration in the infection experiment and the activation microassays were compared using ANOVA. Probability levels for reaction rates between all groups were analysed by a multiple *t*-test (Tukey LSD). For all statistics, SPSS 11.5 and StatistiXL were used.

## RESULTS

### Activation microassay

In both microassay experiments, dissolved ino-arg showed a better activation effect than pure inosine. In all cases (except sporoplasm release of *Myxobolus cerebralis* in microassay no. 2), negative controls were significantly different from mucus homogenate reaction rates ($p < 0.05$; Table 1). In the first experiment, we observed rather low reaction rates of polar filament discharge (Table 1). Sporoplasm emission rates showed species-specific differences. Higher sporoplasm emission rates were detected for *M. pseudodispar* than for *M. cerebralis*. The sporoplasm

Table 1. Activation microassay no. 1 using *Myxobolus cerebralis* and *M. pseudodispar* actinospores (6 replicates), and microassay no. 2 with a new inosine-arginine salt production lot including lower concentrations and different actinospore isolates of both species (12 replicates). Mean rate (%) of actinospores showing polar filament discharge and sporoplasm emission upon chemical stimulation followed by instant mechanical activation. Ino: inosine (final concentration thereafter); IA: inosine-arginine salt; mucus: trout mucus homogenate (final concentration 1 mg $ml^{-1}$); negative control: methyl-guanosine 1 mg $ml^{-1}$; N: number of actinospores counted. Asterisk indicates significant difference from negative control ($p < 0.05$)

| Activation microassay | Polar filament discharge Mean | SEM | Sporoplasm emission Mean | SEM | N |
|---|---|---|---|---|---|
| **Microassay 1** | | | | | |
| *M. cerebralis* | | | | | |
| Negative control | 12.12 | 2.24 | 0 | – | 165 |
| Ino 0.5 mg $ml^{-1}$ | 27.75 | 3.65 | 9.56* | 3.63 | 251 |
| IA 0.05 mg $ml^{-1}$ | 31.06 | 4.49 | 12.31* | 4.52 | 268 |
| Mucus | 32.56* | 2.13 | 14.34* | 6.21 | 251 |
| *M. pseudodispar* | | | | | |
| Negative control | 6.92 | 2.82 | 0 | – | 159 |
| Ino 0.5 mg $ml^{-1}$ | 11.29* | 3.15 | 28.74* | 8.19 | 174 |
| IA 0.05 mg $ml^{-1}$ | 11.63* | 1.87 | 35.82* | 4.80 | 201 |
| Mucus | 18.03* | 9.03 | 51.20* | 7.0 | 125 |
| **Microassay 2** | | | | | |
| *M. cerebralis* | | | | | |
| Negative control | 22.50 | 6.76 | 0 | – | 195 |
| Ino 0.1 mg $ml^{-1}$ | 36.36* | 4.38 | 2.76 | 1.52 | 181 |
| IA 0.01 mg $ml^{-1}$ | 27.16 | 4.40 | 1.62 | 0.87 | 243 |
| IA 0.1 mg $ml^{-1}$ | 58.27* | 2.36 | 3.62 | 1.33 | 219 |
| Mucus | 38.67* | 5.08 | 6.22 | 1.30 | 193 |
| *M. pseudodispar* | | | | | |
| Negative control | 7.32 | 2.68 | 2.38 | 1.20 | 252 |
| Ino 0.1 mg $ml^{-1}$ | 11.95 | 2.50 | 22.53* | 3.75 | 342 |
| IA 0.01 mg $ml^{-1}$ | 15.73 | 3.12 | 17.08* | 3.68 | 267 |
| IA 0.1 mg $ml^{-1}$ | 38.64* | 7.91 | 30.34* | 3.86 | 379 |
| Mucus | 34.62* | 3.84 | 43.65* | 5.31 | 323 |

emission rate was highest in the mucus homogenate positive control for both species. When directly compared in the microassay, ino-arg reached higher activation levels than solubilised inosine, especially in the sporoplasm emission response (Table 1), although the difference between ino-arg and inosine was not significant.

In the second experiment, the activation of both reactions in both species' actinospores by ino-arg was significantly greater ($p < 0.05$) than that elicited by inosine solution of the same concentration. In the case of *Myxobolus pseudodispar* polar capsule discharge, the same was observed for the 10-fold lower ino-arg concentration ($p < 0.05$). Ino-arg in a 0.1 mg $ml^{-1}$ concentration outperformed the highly effective substrate prepared from natural trout mucus regarding polar filament discharge by 20% (Table 1, $p < 0.05$ for *M. cerebralis*). Sporoplasm emission was always significantly different ($p < 0.001$) after ino-arg (0.1 mg $ml^{-1}$) addition when compared to control. In *M. pseudodispar*, polar filament discharge induced by 0.1 mg $ml^{-1}$ ino-arg was also higher, but not significantly different from that by mucus.

### Decoy experiment

Roach were heavily infected in the control group (without prior addition of a substrate), and only 4 out of 10 specimens in this group survived until the end of the experiment, while 7 survived in each of the 2 other groups. The relative mean numbers of myxospores in single homogenized fillets (1 side) of infected roach were 181 250 ± 38 500 (negative control), 200 000 ± 88 250 (inosine) and 90 000 ± 43 750 (ino-arg) (myxospore number per ml ± standard error of the mean). Although not significantly different ($p = 0.28$, *t*-test), ino-arg decreased parasite load to a mean of 45% of that obtained by pure inosine addition.

## DISCUSSION

In the present study, the actinospore stages of 2 myxozoan parasites, *Myxobolus cerebralis* and *M. pseudodispar*, were used in activation microassays *in vitro*. *M. pseudodispar* actinospores are more delicate regarding their apical architecture and thus always react much quicker and to a greater extent than the more rigidly built *M. cerebralis* stages (Kallert & El-Matbouli 2008). Therefore, we examined the first 5 min after the actinospore activation to be able to compare the discharge rates of polar capsules, which are not concealed to such a high extent by emerging sporoplasms that accumulate over the course of incubation. In the first activation microassay, we used a relatively high inosine concentration (i.e. 0.5 mg $ml^{-1}$) compared to the inosine content in a highly effective mucus preparation, so the difference was not as elevated (i.e. not significant). Ino-arg could exceed the reaction level of inosine and reach that of the mucus homogenate (especially for *M. cerebralis*) due to the sufficiently high proportion of dissolved inosine. The sporoplasm emission rate was highest in the mucus homogenate positive control for both species, as sporoplasm emission took place more rapidly, most likely due to slightly higher mucus homogenate viscosity and better mechanical

stimulation. Compared to *M. pseudodispar*, *M. cerebralis* showed lower sporoplasm emission rates during the relatively short counting time, which might be explained by their more rigid actinospore architecture.

Inosine solubilisation requires heating, and it has a water solubility of only 2.1 g $l^{-1}$ at 20°C. In cold water, it rapidly forms aggregates due to its slightly hydrophobic nature (log partition factor p = 1.4, Novotny et al. 2000), which can be readily observed under the microscope. Because of this, its triggering activity as a host cue decreases rapidly at ambient temperatures. The chemical formula of ino-arg is $C_{16}H_{26}N_8O_7$, and when complexed with arginine, it dissociates readily in polar solutions. This makes all functional groups of the nucleoside available, since a major conformation change is not expected, which is reflected by the excellent activation efficacy for actinospores that we observed in this study. Since not only polar filament discharge was triggered, but a high proportion of sporoplasm emission was achieved such as takes place in full mucus preparations, artificial effects (e.g. by discharge due to osmotic or pH-effects) can be excluded. The stimulation thus can be regarded as the natural reaction taking place in the vicinity of a fish host prior to invasion. The fact that ino-arg is effective even at much lower concentrations than pure inosine underlines its homogenous availability to the actinospore receptor(s) in water. Furthermore, it remains solubilised (retains its activating properties) even when left in solution for 4 d, without losing its triggering effect (data not shown).

Parasite load is an indirect measure of infectivity ratio among waterborne actinospores (Ryce et al. 2004). The more units there are in the water column, the more will be able to enter a fish host within a given time and theoretically more mature myxospores will develop. It is not known whether some of the fish used in the experiment died from overexposure or for other reasons such as secondary infections. However, unfortunately, the differences in the results did not reach sufficient levels of significance due to this incidental mortality. When actinospores react by polar filament discharge without anchoring to a host, this affects mainly the capsulogenic cell itself. After polar capsule discharge, these cells disintegrate and the underlying sporoplasm may be hampered by osmotic problems. This also occurs instantly, when, in addition to discharge, the sporoplasm is activated and actively emerges from the shell valves. After a short period, the sporoplasm stops moving, and disintegration due to osmotic imbalance likely takes place. Due to the rapid emergence and subsequent sporoplasm cell death, these specimens will not be infective for fish. Although not statistically significant due to the few fish used for this challenge and the high variation between individual parasite loads, our results indicate the potential of ino-arg to act as a potent preliminary polar capsule discharge/sporoplasm emission trigger, leaving parts of the parasite load noninfective in the water.

Diseases caused by myxozoans are still a major cause for economic losses in the wild and in aquaculture. Means to remove infective actinospores from water usually involve UV-irradiation or mechanical filter methods (e.g. sand filtration), or they require the use of water disinfectants, which can be toxic for fish or the environment or are even illegal. An overview of protective measures both in aquaculture and field systems was provided by Steinbach et al. (2009). According to our results, after adequate toxicological and water and food safety tests, addition of low concentrations of ino-arg could be used for at least partial, but effective, water clearance of infective actinospores to support disease prevention even at low water temperatures. Furthermore, it can serve as an easy-to-use laboratory standard for actinospore activation for various studies. The latter application could be important for researchers aiming to study myxozoan genetics and the basics of the invasion processes (receptor studies, genetic regulation and cell recognition) and may well be a valuable tool for future high-yield *in vitro* culturing or cell culture of myxozoans without the contamination associated with mucus use.

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