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Construction and Screening of an Expression cDNA Library from the Triactinomyxon Spores of *Myxobolus cerebralis*, the causative agent of Salmonid Whirling Diseases*

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This thesis is dedicated to

My Parents, my wife and my kids; Ahmed, Mohamed and Reem

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1. Introduction

Whirling disease and its associated myxosporean agent, *Myxobolus cerebralis,* were first described in Europe in 1898 among farmed rainbow trout (Hofer 1903). The disease spread throughout Europe and eventually to the USA through the international fish trade (Hoffman 1970, El-Matbouli *et al.* 1992). Whirling disease is considered not only a problem in the fish culture industry but is also a major threat to the survival of wild rainbow trout in North America (Hedrick *et al.* 1998). The disease has been recognised as a central cause of the catastrophic decline of wild rainbow trout populations in the states of Idaho, Montana, Colorado and Utah, USA (Nehring & Walker 1996, Hedrick *et al.* 1998). Brown trout are considered the natural host of *Myxobolus cerebralis*, for even though they become infected, they remain asymptomatic (Hoffman *et al.* 1962). The severe decline in wild rainbow trout populations has stimulated a renewed interest in exploring the pathobiology and host-parasite interaction of *M. cerebralis* (El-Matbouli *et al.* 1999b).

Myxobolus cerebralis requires an invertebrate host to complete its life cycle and only a single species of oligochaete worm is susceptible, *Tubifex tubifex* (Markiw & Wolf 1983). The parasite alternates between two spore forms: an actinosporean (triactinomyxon) that develops in the oligochaete, and a myxosporean (*Myxobolus cerebralis*) in salmonid fish (Wolf & Markiw 1984; El-Matbouli & Hoffmann 1998; El-Matbouli *et al.* 1995). *Myxobolus cerebralis* attacks young rainbow trout before their cartilage hardens to bone, causing skeletal deformities and neurological disruption giving rise to the disease's chief symptoms of black tail, tail-chasing behavior (whirling) and cranial, jaw and opercular deformities (Schäperclaus 1931).

From a molecular biological point of view, little is known about the genetics of either triactinomyxon or Myxobolus spore stages. The sequence of the parasite's small subunit 18S ribosomal DNA gene (18S rDNA) is known, as are some other sequences coding for actins and proteases. Without the parasite's complete DNA sequence or some other point of reference, we can't know if a discovered gene even belongs to *M. cerebralis.* This paucity of information is an obstacle to researchers seeking to probe specific *M. cerebralis* genes and their functions.

Construction of a cDNA library for the parasite would therefore represent a major research advance, and is the central goal of this study. The library will establish a genetic information base for *M. cerebralis*, containing protein-encoding sequences from the genome, which can then be used by researchers to analyse functions of specific genes. The

process of constructing the cDNA library encompasses several topics of research. The library must be screened for positive *M. cerebralis* clones, from which some are selected for *in vivo* excision of the pBK-CMV phagemid vector (containing the insert) from the ZAP Express vector. Plasmid DNA containing the insert must be isolated from the vector, and then analysed to determine the sequence of the cloned cDNA. A full-length cDNA sequence can then be constructed using Rapid Amplification of cDNA Ends (RACE). Finally, bioinformatics programs are used to analyse the DNA sequences in order to predict corresponding protein sequences, which can be compared with other genes whose functions are known.

2. Literature Review

2.1 Myxozoa

2.1.1 Taxonomy of *Myxobolus cerebralis*

Myxobolus cerebralis, the parasite that causes whirling disease in salmonid fish (Hofer 1903), is the most intensively studied member of the phylum *Myxozoa* (Hedrick *et al.* 1998). It is one of more than 1,350 myxozoan parasites known to infect fish (Lom & Dykovà 1992).

Myxozoans are morphologically simple and as such were long regarded as protists; however rDNA and Hox gene sequences now indicate they are metazoans (Smothers *et al.* 1994, Anderson *et al.* 1998). In their hosts, myxozoans form plasmodia in which infective spores develop. After release into the environment, these spores attached to new hosts using penetrative filaments everted from polar capsules (El-Matbouli *et al.* 1995, Monteiro *et al.* 2002). These filaments are analogous to the cnidocycts of cnidarians (El-Matbouli *et al.* 1992; Siddall *et al.* 1995).

Within the Phylum Myxozoa, *M. cerebralis* falls within order Bivalvulidae, suborder Platysporina and genus Myxobolus – the most speciose of 52 genera (Kent *et al.* 2001). *Myxobolus cerebralis* possesses unique phenotypic and genotypic characteristics when compared with other histozoic parasites from the phylum (Hedrick & El –Matbouli 2002) and is arguably the most pathogenic *Myxobolus* species, causing significant disease in young salmonid fish (Lom & Dykovà 1992). Molecular phylogenetic comparisons of the ribosomal DNA (rDNA) of Myxozoa, demonstrate that *M. cerebralis* branches uniquely, reflecting an evolution distinct from other histozoic Myxobolus species found in fish (Hedrick & El-Matbouli 2002). Cursory genetic studies of *M. cerebralis* isolates from diverse geographic regions suggest little variation in the internal transcribed spacer (ITS) region of its rDNA, supporting the theory of its recent introduction into North America (Andree *et al.* 1999).

The discovery of the definitive oligochaete worm host of *M. cerebralis* by Wolf & Markiw (1984; El-Matbouli & Hoffmann 1989) radically changed the taxonomy of Phylum Myxozoa and provided a model for two-host life cycles for myxozoan parasites of fish. Most myxozoans are assumed to alternate between a myxospore stage developing in a fish, and an actinospore stage in an oligochaete. Myxozoans have been shown to have a number of life cycle strategies including direct transmission, alternation into annelids and other invertebrate hosts e.g. bryozoans. Several members of genus Myxobolus have been

demonstrated to utilise oligochaetes, including *Tubifex tubifex* as essential hosts (El-Matbouli *et al.* 1992; Hedrick & El-Matbouli 2002). Some of the mechanisms that underlie this complex developmental cycle, including host response (both fish and oligochaete) and resistance factors were investigated by El-Matbouli *et al.* (1995, El-Matbouli & Hoffmann 1998).

2.1.2 Development of *Myxobolus cerebralis* in its hosts

2.1.2.1 Development of myxospores in fish

A. Attachment and initial invasion: following release from the oligochaete host, waterborne actinospore stage (triactinomyxons) of *M. cerebralis* can remain viable at 7-15°C for 6-15 days (El-Matbouli *et al.* 1999b). Once a spore encounters the fish host (salmonid), attachment and penetration of the host epidermis occurs (Markiw 1992a, El-Matbouli *et al.* 1999b). Triactinomyxons can cause significant damage to the epidermis by three mechanisms: piercing of host cells by extruded polar filaments; migration of the infective sporoplasm between cells - a process facilitated by release of proteases present in the sporoplasm; and intracellular development and release of parasite daughter cells from infected host cells (Hedrick & El-Matbouli 2002). Mechanisms that control extrusion of the polar filaments appear to depend on chemical and mechanical cues, as dead or anesthetised fish or fish parts are not effective targets (El-Matbouli *et al.* 1999a). Subsequent damage to the epidermis by the attachment and invasion of large number of triactinomyxon stages in a short but heavy exposure can lead to the death of young salmonids (Markiw 1991). Attachment and penetration is a very rapid process, occurring within seconds of first contact with the fish host. By five minutes post-exposure, sporoplasms (the infective packets containing up to 64 germ cells surrounded by an enveloping cell) have egressed from the valve cells of the triactinomyxon into the epidermis of the fish. The migration of the sporoplasm packet through the epidermis may be facilitated by both mechanical damage to the epidermis and the effects of released enzymes (El-Matbouli *et al.* 1995). Enzymes such as serine and cysteine proteases influence parasite virulence factors and subvert the host defenses (Que *et al.* 2003). Cysteine and serine protease genes *Myx*CP-1 and *Myx*SP-1, respectively, were identified by Kelley *et al.* (2003; 2004) from *M. cerebralis.* These were shown to play an enzymatic role in penetration and invasion through the cellular matrix of the host tissues, as well as lysis of target cartilage.

B. Presporogonic and sporogonic stages: one hour post-exposure, individual germ cells from the sporoplasm disperse and begin to penetrate the host epidermis. After two hours, the sporoplasm cells begin to multiply in the epidermis, then migrate to the dermis and peripheral nerves (El-Matbouli *et al.* 1995). After 24 hours, there are few parasites remaining in the epidermis. By 4-24 days post-exposure, parasites can be found in the nervous tissue, initially in peripheral nerves but later migrating and replicating between nerve bundles in ganglia and the central nervous system, exploiting the nerves as paths to reach larger deposits of cartilage found in the spinal column and cranial regions (El-Matbouli *et al.* 1995). The first parasite stages appeared in the cartilage after 20 days or 35 days post exposure according to water temperature 16-17, 12-13 respectively (Halliday 1973). Presporogonic development ends and sporogony begins with autogamy: the union of two cells to form a generative cell surrounding a sporogonic cell; a phenomenon typical of myxozoans, which signals a shift from vegetative development to sporogenesis (Lom & Dykovà 1992).

The sporogonic cell gives rise to all of the cells comprising the multicellular myxospore stage. A myxospore has two cells for the polar capsules, two cells for each of the two shell valves and two cells that will later fuse to form a binucleate sporoplasm; together they make an elliptically-shaped myxospore ~10µm in diameter. More than one million myxospores may develop per fish over a period of 52-121 days depending on water temperature (Halliday 1973). The spores have thick, protective valve cells and can lie resident in the ossified fish skeleton, where cartilage was once present. Spores can exit the host through several pathways: if the fish is ingested by another fish, a fish-eating bird or another animal, spores are expelled in faeces (El-Matbouli & Hoffmann 1991b); severely crippled fish may die and decompose in the sediments directly; Nehring *et al.* (2002) found that spores of *M. cerebralis* can be released by living brown trout. Myxospores may remain viable for as long as 12 years (Schäperclaus 1954) before they infect their next host, an oligochaete worm.

2.1.2.2 Development of *Myxobolus cerebralis* spores in oligochaetes

A. Attachment and Invasion: susceptible aquatic oligochaetes, *T. tubifex,* become infected after ingestion of myxospores. Following stimuli that may be similar to those responsible for triggering the triactinomyxon stage when it encounters the fish host, the myxospore polar filaments are extruded and attach the spore to the worm gut epithelium. The spores' valves then open and the binucleate sporoplasm cell migrates between the gut epithelial cells where it will remain throughout all stages of subsequent development, in

contrast to the migratory behavior in the fish host (El-Matbouli & Hoffmann 1998, Antonio *et al.* 1999).

B. Schizogony: 5-25 days post-infection, many uni- or bi-nucleated cells which arose from the bi-nucleated amoeboid cells, undergo multiple divisions to produce numerous daughter cells. This cycle may continue throughout the development of the parasite, providing a reservoir of undifferentiated cells that can later contribute to gametogony and sporogony (El-Matbouli & Hoffmann 1998).

C. Gametogony: between 25-46 days, oval binucleate cells give rise, through multiple divisions, to pansporocysts. Pansporocysts consists of somatic cells surrounding two generative cells. The somatic cells divide to form the pansporocyst wall, while the two generative cells further divide to form gametocytes that further differentiate into haploid alpha and beta gametocytes. The mitotic divisions that give rise to the gametes are the only phase in the parasite life cycle where it exists in a haploid state (El-Matbouli *et al.* 1998). The fusion of alpha and beta gametocytes to form a zygote is the only truly sexual phase of the life cycle of *M. cerebralis* and signals the beginning of sporogony.

D. Sporogony: beginning at 50 days, fusion of gametocytes to form zygotes is evident. Eight zygotes are formed within each pansporocyst, with each zygote in turn dividing to eventually form four sporoblast cells (three surround the other). A further division of the surrounding cells makes a total of seven cells in the developing spore: three cells differentiate to form the polar capsules; three produce the valves; one undergoes multiple divisions to give rise to a cell which envelops up to 64 germ cells. After 90 days, the fully-formed triactinomyxon spores are ready to be released into the lumen; the spores are folded and deflated while present in the pansporocyst but as the pansporocyst emerges to the surface of the intestinal lumen and ruptures they are released. Once in the lumen, spores are passed towards the anus and expelled into the environment. A fully inflated triactinomyxon spore measures from 135-155µm in length and 11.5-14.5µm in width (El-Matbouli & Hoffmann, 1998).

2.2 Whirling disease

Development of the myxospore stage of *M. cerebralis* in the salmonid host gives rise to whirling disease. The disease was first observed among introduced, farmed-raised rainbow trout *Oncorhynchus mykiss*, by Bruno Hofer of the University of Munich in 1898 (Hofer 1903).

2.2.1 Symptoms and pathogenicity

The most obvious clinical sign is tail-chasing behaviour - from which the disease derives its name – which occurs 2-3 months after infection (Schäperclaus 1931). According to Hoffman *et al.* (1962) whirling can last a year after infection; however Halliday (1974) studied outbreaks of the disease where whirling initially appeared in yearlings. Whirling was at one time thought to be caused by toxins released by the parasite (Plehn 1904, 1924) but it seems more likely to be caused by erosion of the cartilage surrounding the auditory organ (Hoffman & Dunbar 1961). In addition, observations by Christensen (1966) & Halliday (1974) suggest that granulated tissue produced by the host 8-12 months after infection (Lucky 1970) puts pressure on the auditory capsule and thereby induces whirling. Halliday (1974) suggests that when this is produced in response to an earlier sub-clinical infection, it can initiate whirling in yearlings and prolong whirling in survivors.

The parasite can also infect the cartilage of the spinal column, and when this occur posterior to the 26th vertebra, it puts pressure on the caudal nerves which control pigment cells in the tail. This produces a black tail in the fish (Plehn 1904, Schäperclaus 1954, Hoffman *et al.* 1962, Hoffman 1966) and causes permanent spinal deformities in survivors (Hoffman 1966, Havelka *et al.* 1971). Other characteristic signs of the disease include cranial deformities due to interference with osteogenesis (Hoffman *et al.* 1962, Christensen 1966, Hoffman 1970), deformities of the jaws and opercula (Christensen 1966, Havelka & Volf 1970, Lucky 1970), disintegration of the fins (Havelka & Volf 1970) and opercular cysts (Taylor & Haber 1974).

The presence of abundant cartilage in the skeleton of young trout renders them highly susceptible to the disease (Schäperclaus 1986). Parasite trophozoites, or feeding stages, digest cartilage and destroy the structural framework needed for subsequent healthy bone formation, leaving the fish with permanent skeletal disfigurement (Schäperclaus 1986).

While structural deformation of cartilage due to necrosis is clear, the causes of the more acute neurological signs - whirling behaviour & black tail - have been debated. These symptoms were thought to result from destruction of cartilage and pressure on nerves of the vestibular organ (Schäperclaus 1986). Rose *et al.* (2000) found that granulomatous inflammation, associated with the parasite invasion of skull and vertebral column, extended into the perineural cerebrospinal-fluid-containing space, producing ring-like constrictions of the upper spinal cord and sometimes compressing and deforming the lower brain stem, which lead to abnormal swimming behaviour.

The cumulative effect of disease - behavioural effects and skeletal deformities - compromise the swimming and feeding functions of the fish, which may lead directly or indirectly to death of heavily infected trout (Hedrick *et al.* 1998).

2.2.2 Host susceptibility

The earliest reports of whirling disease among farmed rainbow trout in Germany documented the high susceptibility of these fish, a species recently introduced from North America, compared with indigenous brown trout (Hofer 1903). Brown trout become infected but remains asymptomatic and are thought to be a natural host for the parasite (Hoffman *et al.* 1962). Surveys and experimental studies confirmed that rainbow trout are one of the most susceptible species of salmonid to *M. cerebralis* (Hofer 1903, Hoffman & Putz 1969, O`Grodnick 1979, MacConnell & Vincent 2002). This is due in part to the independent evolution of the parasite and rainbow trout (Hedrick *et al.* 2003); it was only in the late 19th century when rainbow trout were introduced to Europe from North America, that the parasite and new host first came into contact (Hofer 1903).

Triactinomyxon spores have the ability to recognise, attach to and penetrate the epidermis of all salmonid fish tested, and to a much lesser extend some non-salmonid fish (El-Matbouli *et al.* 1999a). It is the development of the parasite after entry that varies greatly between fish species: following penetration in non-salmonid fish, the invading sporoplasm cells are destroyed within hours, but in rainbow trout active multiplication of parasite stages begins rapidly (El-Matbouli *et al.* 1995).

Hedrick *et al.* (1998) found that in controlled laboratory exposure to graded doses of the infectious stages, resistance to the disease in certain species, e.g. brown trout, can be overwhelmed by exposure to high concentrations of the infectious stages. This can explain the occurrence of disease among wild and hatchery- reared brown trout in the US and Europe respectively. Comparison between *M. cerebralis*-infected brown trout and rainbow trout, where they coexist in a Montana wild trout stream, showed that rainbow trout not only exhibited a much higher lesion severity and rate of infection than brown trout, but also that the parasite tended to concentrate in the cranial cartilage in rainbow trout versus gill arches in the brown trout (Baldwin *et al.* 2000). In a controlled laboratory exposure (Hedrick *et al.* 1999a) found rainbow trout to be much more susceptible to whirling disease infection when compared with either west slope cutthroat trout (*Oncorhynchus clarki lewisi*) or Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*).In contrast, Lake

trout *Salvelinus namaycush* and Arctic grayling *Thymallus arcticus* were found to experience only low-level disease effects.

There is a generally accepted ranking of relative susceptibilities that places rainbow trout *Oncorhynchus mykiss* as highly susceptible; Sockeye salmon *Oncorhynchus nerka,* Chinook salmon *Oncorhynchus tshawytscha*, Atlantic salmon *Salmo solar*, Cutthroat trout *Oncorhynchus clarki*, and brook trout *Salvelinus fontinalis* intermediate in their susceptibility; and brown trout *Salmo trutta* and Coho salmon *Oncorhynchus kisutch* as having low susceptibility to the disease (Sollid *et al.* 2002). Most resistant are lake trout S*almo namaycush*, which are considered refractory to infection (O'Grodnick 1979).

Recent research on whirling disease has been directed towards finding naturally acquired resistance to the parasite among strains of rainbow trout in North America. So far both field and laboratory studies have demonstrated no more than marginal resistance to the parasite among the many stocks of rainbow trout in North America (Hedrick *et al.* 1998, 1999a, b, Thompson *et* al.1999, Densmore *et al*. 2001). Laboratory studies have shown that the Hofer strain (GR) of rainbow trout reared in Bavaria, Germany have a higher resistance to whirling disease when compared with the Trout Lodge (TL) strain from North America (El-Matbouli *et al.* 2003). Hedrick *et al.* (2003) measured susceptibility of those 2 strains following exposure to triactinomyxons at different exposure doses. Severity of infection was evaluated 5 months post-exposure by presence of clinical signs, prevalence of infection, severity of microscopic lesions and spore count. It was found that the prevalence of infection, spore numbers and severity of microscopic lesions due to *M. cerebralis* among GR trout were less at all doses compared with TL. It is assumed that the German GR strain has been exposed to the parasite for up to 120 years, which is possibly sufficient time to have developed resistance to whirling disease (El-Matbouli *et al.* 2004).

2.2.3 Diagnosis

The importance of diagnosing whirling disease and detection of this sometimes cryptic parasite has grown as a result of increased outbreaks of the disease in wild trout populations in North America, and with the economic importance of commercial trout farming. For these reasons, whirling disease is listed with the International Office of Epizootics as an important disease of fresh water fishes (El-Matbouli *et al.* 1992).

Presumptive diagnosis includes the demonstration of a previous history of the disease and the presence of acute signs (active tail-chasing behaviour and caudal melanosis) or chronic pathological changes (skeletal malformation) among affected fish (Thoesen 1994).

Detection and identification of the causative agent *M. cerebralis,* is based on definitive description of spores (Lom & Hoffman 1970): the spore is 9.7µm long, 8.5µm wide, with two equally-sized polar capsules 4.2 x 3.1µm. The polar filaments make 5 to 6 turns within the polar capsules. The posterior of the spore is covered by a mucous envelope which can be visualised by negative staining with dyes such as India ink (Lom & Hoffman 1971). There is a parasutural groove visible by electron microscopy along each side of the suture line where the two protective valves join, in addition, two pores for the extrusion of the polar filaments are visible at the anterior of the spore (Hoffman & Hoffman 1972). Definitive description of spores is followed by confirmation of developmental stages or spores in cartilage of tissue sections stained with haematoxylin and eosin (H&E) (Thoesen 1994). Spores can be enumerated after extraction from host tissues; the most widely used method is the pepsin-trypsin digest or PTD (Markiw & Wolf 1974 a, b). PTD involves sequential digestion of the bony elements of the skeleton after removal of the flesh. The digested tissues are filtered and concentrated prior to examination by light microscopy for the presence of spores. An alternative method of spore enumeration is plankton centrifugation (O'Grodnick 1975). In this procedure fish heads are homogenised and the homogenate filtered through gauze. This crude filtrate is then passed through a Plankton centrifuge and the resulting pellet examined.

Taxonomic classification of members of genus Myxobolus to species level is difficult (Lom 1987; Yoder 1972). This has lead to misdiagnosis of whirling disease, sometimes with costly consequences (Margolis *et al.* 1996). Even when spores are detected it may take an experienced parasitologist to determine which species is present. Serologic techniques have been employed to identify *M. cerebralis* spores with more certainty (Markiw & Wolf 1978); however attempts to demonstrate the practicality of this technique have met with mixed results. Griffin & Davis (1978) demonstrated the presence of antibodies in the serum of infected fish using an indirect fluorescent antibody test (IFAT). The IFAT using polyclonal anti-sera prepared in rabbits injected with spores was also used by Markiw (1989) to show conservation of antigens between the myxosporean and actinosporean stages. Hamilton & Canning (1988) used mouse anti-*M. cerebralis* anti-sera to confirm conservation of antigens among pre-spore and spore stages. Cross-reactivity with the IFAT and direct fluorescent antibody test (DFAT) were observed between *M. cerebralis* and *M. cartilaginis* (Markiw & Wolf 1978). Enzyme linked immunosorbent assay (ELISA) was developed by (Adkison *et al.* 2005) as a non-lethal method for

detection of *M. cerebralis*-infected fish. The specificity and sensitivity of the ELISA assay in detection of infected fish is still under investigation.

Interest has increased in the use of DNA-based diagnosis for fish pathogens, particularly those that are difficult or impossible to culture. One of these approaches is polymerase chain reaction (PCR): amplification of a DNA sequence unique to *M. cerebralis.* Andree *et al.* (1998) describe a nested (two-round) PCR test for detection of *M. cerebralis* that is currently in use by several fish disease laboratories. This method involves the amplification of a 415 base pair (bp) segment of the 18S rDNA gene from *M. cerebralis*. The primary advantage of this technique over traditional testing methods is its ability to amplify to detectable levels the equivalent of a single sporoplasm of *M. cerebralis* as found in a tissue sample. It can detect the presence of the parasite in both hosts, in all known stages of its life cycle; and at lower thresholds than currently used diagnostic methods. These advantages were demonstrated by Schisler *et al.* (2001): PCR identified *M. cerebralis* significantly more often than PTD testing.

For confirmation of the identity of myxospores obtained from pepsin-trypsin digest, a single-round PCR is preferred to a nested PCR, as it minimises the risk of false positive that may result from contaminants. However, for definitive diagnosis of digest preparations containing low numbers of myxospores (or none), or where microscopic examination reveals sparse structures that may, or may not be, actual myxospores, an assay with greater sensitivity is preferred - such as the original nested procedure described by Andree *et al.* (1998) and Baldwin & Myklebust (2002), although, as is normal in PCR preparations, the cell sample is destroyed during the extraction process and consequently it is impossible to know which specific cells in a mixed cell suspension were infected (Taylor & Logan 1995).

Another set of useful techniques involve molecular hybridisation, which is especially suited for detection of all stages of *M. cerebralis*, including early developmental stages that may be present well before clinical signs are evident. *In situ* Hybridisation (ISH) is the specific annealing of labelled DNA probes complementary to the target sequence in fixed tissue or cells; followed by visualisation of the probe location. However, as it does not involve amplification of the target sequence it is not as sensitive as PCR, and is limited by the number of copies of the target sequence per cell (Nuovo 1994). A non-radioactive ISH protocol was developed by Antonio *et al.* (1998) to detect *M. cerebralis* in target tissues of subclinically and clinically infected fish, as well as tubificid oligochaetes after exposures

of these hosts to triactinomyxons and myxospores, respectively. The significant advantage of ISH over other methods of detecting *M. cerebralis* is its capability to anatomically locate all stages of the parasite, from early developmental forms to mature spore stages, at low-level infections. A unique application of the procedure is its use to detect and localise parasite stages in the oligochaete host; stages which cannot be identified by any extraction-concentration procedure used for the spore stages in fish. Additionally, ISH should be able to distinguish oligochaetes infected with *M. cerebralis* from those infected with other myxosporeans.

2.2.4 Factors influencing infection and disease

2.2.4.1 Age of fish host

Severity of infection decreases with increased age of fish (Markiw 1992b). In older fish, much of the cartilage susceptible to infection has been converted to bone, making fish more resistant to disease (Halliday 1976). Other reasons for the increased resistance of older fish may include physiological changes in the skin (Markiw 1992a) and acquired immunity (El-Matbouli *et al.* 1995). While younger fish are generally more vulnerable to disease, eggs and newly hatched sac-fry exposed to infective units do not develop infection (Putz & Hoffman 1966; Markiw 1991). Either those infected with early stages of *M. cerebralis* did not survive, or their underdeveloped organs did not provide conditions that lead to a persistent infection (Markiw 1991).

2.2.4.2 Environmental stress

Environmental stressors such as pollution, crowding, or abnormal temperatures will make fish more susceptible to the disease (Goede 1986). The parasite develops more rapidly and disease signs are more common in fish held at higher water temperature (Halliday 1973).

2.2.4.3 Infective dose

Parasitism (as measured by spore numbers) becomes more severe as fish are exposed to increasing dosages of triactinomyxon (Markiw 1992a). The myxospore burden appears to plateau at doses of 10,000-100,000 triactinomyxon/fish. In exposure tests with 2-day old rainbow trout, increasing doses of triactinomyxon resulted in increasing mortality: from 68% at 10 triactinomyxon/fish to 100% mortality at 1000 triactinomyxon/fish; 4% of uninfected control fish perished (Markiw 1991).

2.2.4.4 Fish species

Salmonid species differ in their susceptibility to whirling disease. Rainbow trout are considered among the most susceptible species while brown trout and Coho salmon have considerably more resistance (Hedrick *et al*. 1999b, O'Grodnick 1979). Adkison *et al.*

(2001) compared these 3 species in their response to the whirling disease. Microscopic examination of the rainbow trout and brown trout show significantly more sporoplasm cells in the epidermis in the first few hours post-exposure compared with Coho salmon. Later in the infection there is a reduction in the number of parasite stages and associated pathology in brown trout compared with rainbow trout. Other species that can become infected include: Snake River, greenback, Colorado River, and Rio Grande cutthroat trout

(Thompson *et al.* 1998), Yellowstone and westslope cutthroat trout (Vincent 1997), bull trout (McDowell *et al.* 1997), steelhead (Horsch 1987), arctic grayling (MacConnell *et al.* 1997), Atlantic salmon (Hoffman 1990), golden trout (Anonymous 1988), sockeye, Coho, and Chinook salmon (O'Grodnick 1978a, 1979), and mountain whitefish (Baldwin *et al.* 1997). Clinical signs of whirling disease were emerged in experimentally infected rainbow trout, brook trout, sockeye salmon, and Chinook salmon, while no clinical signs were found in brown trout, lake trout, and Coho salmon, and no spores were found in lake trout (O'Grodnick 1978a, 1979). Rainbow trout were most susceptible to disease. Brook trout, sockeye salmon, and Chinook salmon had intermediate susceptibility. Coho salmon were usually refractory to infection (but occasional spores were found), while lake trout were always refractory (O'Grodnick 1979).

Sentinel fish studies in Colorado (Thompson *et al.* 1999) found evidence of infection in brown trout, rainbow trout, and four subspecies of cutthroat trout (Colorado River, Greenback, Rio Grande, and Snake River). Whirling behaviour, a clinical sign thought to indicate more severe infection, was observed in rainbow trout in Colorado River, Greenback and Rio Grande cutthroat trout. Snake River cutthroats appeared to be somewhat more resistant to disease, while brown trout were the most resistant species (based on spores counts and clinical signs). Previous studies had found brook trout to be highly vulnerable to infection, as well (Thompson *et al.* 1997). Sentinel fish studies in Idaho found that rainbow trout and Yellowstone cutthroat trout were both vulnerable to infection with *M. cerebralis*. Based on spore counts, clinical signs, and histology, it appeared that the cutthroat trout were somewhat less affected by the parasite than rainbow trout (Elle 1997). In contrast, studies in Montana found Yellowstone cutthroat trout to be highly affected by whirling disease, as were west slope cutthroat trout and three strain of rainbow trout (DeSmet, Deschutes, Eagle Lake), brown trout were relatively unaffected (Vincent 1997). Other studies in Montana (MacConnell *et al.* 1997) and at the University of California, Davis, (McDowell *et al.* 1997) found grayling to be quite resistant to whirling disease, though the Montana studies indicated that they could become infected.

Studies in Utah evaluated the susceptibility of several salmonid hybrids. Brownbows (rainbow female x brown male), splake (lake female x lake male), brake (brown female x lake male) and tiger trout (brook female x brown male) were exposed in two reservoirs. Each type of hybrid proved capable of becoming infected with *M. cerebralis*, though a hierarchy of resistance has not yet been developed (Wilson *et al.* 1997).

2.2.4.5 Ecological factors

The cumulative effects of environmental stresses likely play an important role in influencing infection and disease. Some specific factors that have been examined include:

2.2.4.5.1 Stream productivity: infectivity of *M. cerebralis* appeared to be greater in high productivity streams (O'Grodnick 1978b). Despite the stocking of infected fish in several relatively infertile mountain streams with low trout numbers, whirling disease did not become established in wild populations of rainbow, brown, or brook trout. In contrast, infection became established among brown trout in a highly productive limestone stream to which the parasite had been introduced.

2.2.4.5.2 Sediment/organic material: more sediment and organic load can lead to greater disease because it provides more favourable habitat for the oligochaete host. Modin (1998) noted a serious outbreak of whirling disease in a California hatchery that uses a contaminated high-gradient stream as a water supply. Infection was barely detectable in fish from the stream; however fish in the hatchery reared in water that had passed through sediment-laden settling pools, suffered from severe clinical disease. Gustafson (1997) found that *T. tubifex* oligochaetes in Montana were generally found in greatest abundance in polluted sites where normal benthic community diversity had been reduced.

2.2.4.5.3 Seasonality and water temperature: water temperature can have profound effects on the development of *M. cerebralis* in *T. tubifex* worms and on the release of triactinomyxon spores. Most parasite developmental stages in the gut epithelium of *Tubifex* worms are destroyed after 24h at 30°C, after three days at 25°C and after 10 days at 20°C (El-Matbouli *et al*. 1999b). In contrast, complete development of triactinomyxon spores was observed in worms held at 5, 10, and 15°C. In worms already producing triactinomyxons, release of spores ceased within four days when worms were held at 25 and 30°C, and within 15 days at 20°C. It appears that 15°C may be optimal for production of triactinomyxon, with release continuing, albeit at a lower rate, when water temperature is lower (El-Matbouli *et al.* 1999b). Field studies have also indicated that water temperature may be an important factor. In young-of-the-year rainbow trout exposed in

sentinel cages at different times of the year, infection rates showed a seasonal pattern with significant correlation to the average water temperatures when fish were exposed. Fish began to exhibit more sever infections when they were exposed at water temperatures of 9°C, with infection peaking at about 14°C and declining when water temperature exceeded 17°C (Vincent 1998).

2.2.4.5.4 Infection "point sources": Schisler *et al.* (1997) found that the percentage of trout fry displaying clinical signs of whirling disease in the Colorado River decreased with distance downstream from Windy Gap Reservoir. They suggest that disease in wild populations may be influenced by specific "point sources" for infectivity, such as Windy Gap Reservoir.

2.2.5 Vectors for the spread of whirling disease

Movement of live fish carrying *M. cerebralis* is considered one of the major vectors for spread of whirling disease. Once established in a natural system, the parasite can spread as infected fish move up- or downstream and as waterborne triactinomyxons are carried downstream. Whirling disease spread 9.6km downstream and 500m upstream from a point of initial infection at a Michigan hatchery over a three year period (Yoder 1972). In the United States, whirling disease is likely to have been spread primarily through the transfer of live fish and by movement of infected fish within streams (Hoffman 1990). Whirling disease could also have been spread through shipments of fresh, frozen, or brined food fish infected with *M. cerebralis*. Spores remain viable when frozen at -20°C for at least three months (El-Matbouli & Hoffmann 1991b). Brined fish also retain viable spores, through hot-smoking at 66°C deactivates spores (Wolf & Markiw 1982). Predators may also spread the parasite to new waters. Spores of *M. cerebralis* survive passage through the alimentary canal of avian predators (Meyers *et al.* 1970; Taylor & Lott 1978; El-Matbouli & Hoffmann 1991b). Transfer of fish eggs is not a likely means for transfer of parasite as *M. cerebralis* is not transmitted vertically, from infected brood fish to eggs (O'Grodnick 1975a). Markiw (1991) also found that eyed eggs exposed to triactinomyxon do not become infected. The parasite could be spread, however, through contamination of egg shipments (Hoffman 1990).

2.2.6 Fish immune response to *Myxobolus cerebralis*

Trout display some immune response to infection by *M. cerebralis.* Griffin & Davis (1978) detected circulating antibodies in infected rainbow trout. Hoffmann & El-Matbouli (1996) observed parasite stages in trout subcutis become surrounded by round cells and

macrophages five days post-exposure. Apparently, parasites that have not yet reached nerve cells within five days are removed by immune cells. There was also no evidence of contact of the parasite with blood or immunocompetent cells (which could trigger an immune response) during its migration (El-Matbouli *et al.* 1995).Once in nervous tissue; the parasite is effectively shielded from attack by the immune system. Even highly susceptible rainbow trout can acquire some resistance upon re-exposure to *M. cerebralis* but this occurs only after exposure to significant numbers of triactinomyxons and after clinical signs has begun to develop as a result of the primary exposure (Ryce *et al.* 2002).

Hedrick *et al.* (1997) also observed evidence of some acquired immunity, finding that fish exposed to a high dose of triactinomyxons (1,350 per fish) developed resistance to re-infection between 24-36d after initial exposure (at 15°C). Fish exposed to a lighter dose (200 triactinomyxons per fish) did not display resistance to re-infection. Thompson *et al.* (1998) exposed two groups of sentinel rainbow trout: one spawned from wild trout in the Colorado River recruited prior to population effects of whirling disease, and the other spawned from trout recruited after whirling disease effects began to appear. The offspring of the pre-whirling disease parents had significantly higher spores loads than the progeny of post–whirling disease parents. Although survival rates were similar for the two groups, the lower spore loads in post-whirling disease trout may indicate that some level of resistance has been developed in the population of surviving fish.

2.3 Control and eradication of whirling disease

A great deal of research has been directed at developing ways to control whirling disease in fish culture settings. The worm host, *T. tubifex,* can be eradicated from ponds by allowing them to dry out; it does however have the ability to form resistant cysts, living for up to 14d in dried mud (Kaster & Bushnell 1981). Facilities can be disinfected using 0.25-1% calcium oxide or 0.5-1% potassium hydroxide (Hoffman & Hoffman 1972). Other effective reagents include: calcium hydroxide 0.5%-2.0%; sodium hypochlorite (1600ppm available chlorine); 200-800ppm Roccal (alkyl dimethylbenzylammonium chloride) (Hoffman & Putz 1969). Heat also deactivates spores: 90°C for 10 min, or 70°C for 100 min (Hoffman & Markiw 1977) or hot-smoking of fish at 66°C (Wolf & Markiw 1982) renders spores nonviable. Hoffman (1974, 1975) demonstrated that ultraviolet light (UV) treatments with 253.7nm wavelength at 35 mWs/cm^2 were effective in protecting rainbow trout from whirling disease; lower dosages reduced, but did not completely eliminate infection. Hedrick *et al.* (2000) found that a dose of 1300 mWs/cm^2 was required to

inactivate 100% of triactinomyxons held under a static collimated UV beam, as determined by vital staining. The use of medicated feed has also been proposed for controlling the disease: orally administered Fumagillin dicycloheyxlamine could prevent clinical outbreaks of whirling disease in rainbow trout and also cause distinct morphological defects in *M. cerebralis* (El-Matbouli & Hoffmann 1991a).

2.4 Polyacrylamide gel electrophoresis (PAGE) and western blot (Electroblotting)

Electrophoresis is the migration of charged molecules in solution in response to an electric field. The rate of migration depends on several factors, including: strength of the electric field, net charge, size and shape of the molecule and the viscosity and temperature of the medium in which the molecules are moving (Rybicki & Purves 1996a). The sample is run in a support matrix such as polyacrylamide gel which not only inhibits convective mixing caused by heating but also act as a sieve by retarding the movement of large molecules while allowing smaller ones to migrate freely; it can be stained and digitally scanned, or stored, providing a record of the electrophoretic run (Rybicki & Purves 1996a). In order to separate mixtures of complex proteins by electrophoresis, the protein structures have to be denatured through the use of a compound such as sodium dodecyl sulphate (SDS), an anionic detergent, which wraps around the polypeptide backbone. SDS binds to proteins quite specifically in a mass ratio of 1.4:1, allowing easy back-calculation of fragment size from the gel. It is also usually necessary to reduce disulphide bridges in the proteins before they can adopt the randomly coiled configuration necessary for separation; this can be accomplished with 2-mercaptoethanol or dithiothreitol. The separation of proteins by PAGE provides high resolution of complex protein mixtures, and has proved to be very useful in classification and identification of a range of organisms (Kersters& Ley 1980).

Blotting is a technique for the electrophoretic transfer of Protein, DNA or RNA to a suitable membrane. Transfer of proteins to membranes is widely used for creation of an “imprint” of proteins separated by SDS-PAGE; this can then be used for identification of antigens, detection of glycoprotein, detection of interacting species, or protein sequencing. The method most commonly used for electrotransfer of proteins to nitrocellulose is that reported by Towbin *et al.* (1979): proteins are first separated by mass using SDS-PAGE, and then specifically detected in an immunoassay step (see also Rybicki & Purves 1996b).

Williams & Hoole (1995) used immunoblotting, SDS-PAGE and western blotting procedures to demonstrate cross-reactivity of a polyclonal anti-carp IgM antibody with components of roach serum. This cross-reaction has been exploited in immunofluorescence

and immunogold labelling studies to localise fish host molecules on the tegumental membrane of *Ligula intestinalis* (Cestoda: *Pseudophyllidea*) freshly removed from roach fry. Monni & Cognetti-Varriale (2002) studied the antibody response of European eels (*Anguilla anguilla*) to the branchial parasites *Pseudodactylogyrus anguillae* and *P. bini* under hyperoxygenation conditions; the antigenic fractions of parasites were detected by means of electrophoretic techniques (SDS-PAGE) and by Western blot analysis. They found that the eels responded to a greater number of proteins, and that this was correlated with a decrease in the level of infestation.

2.5 Complementary DNA (cDNA) library

The genetic material of the cell is composed of nucleic acids present in two forms: deoxyribonucleic acid (DNA) which makes up the chromosomes, and ribonucleic acid (RNA) which decodes the genetic information encoded in the DNA and produces proteins for the cell. If we reverse this process and artificially build DNA from the RNA, it reveals the original protein-coding information contained in the genes; because this DNA is a copy of RNA, it is called cDNA. cDNA has two advantages over chromosomal DNA: there are no introns - non-coding sequences that often occur within eukaryotic gene sequences - so it is easier to identify and characterise genes; and cDNA only represents those genes that are being actively used by the cell, since RNA polymerase only transcribes activated genes in the first place. To synthesise cDNA from RNA, a reverse transcriptase (RT) enzyme is used.

Building a 'library': to speed up the process of identifying complete genes from a cell's DNA, the genome can be divided into a 'library' of smaller fragments, each with the ability to be independently replicated when spliced into a vector and cloned in a cell. The library of fragments can be efficiently searched for the required gene, as it takes less time to search the length of a fragment, than it would to search the entire genome. There are two types of libraries:

- **Gene (Genomic) library**: a random collection of DNA fragments, typically representing the entire genome of an organism that has been inserted into a cloning vector.
- **cDNA library**: a random collection of cDNA fragments, typically representing the entire mRNA of a target tissue, that have been inserted into a cloning vector. If a particular gene sequence is required, it may be easier to find it in a cDNA library rather than a gene library, which is larger. However, the cDNA library screened

would have to be from a tissue where the gene in question was being expressed, i.e. mRNA was being transcribed.

cDNA libraries are simpler to construct, because cDNA fragments, like their parental mRNAs, are already fairly short, so an entire cDNA can be spliced into a single vector. Other advantages over genomic libraries include: there are no introns, so there is no danger of pieces of the target gene being chopped onto separate clones; the library is enriched for a target gene, since instead of one or two copies, as in the genomic library, there are as many copies as the cell could produce mRNA's for that gene (Onken 1997, Old & Primrose 1994).

Chen *et al.* (2002) constructed a cDNA library from *Schistosoma japonicum* cercariae. The primary library titre was 1.8 X 10^7 pfu /ml and the titre of amplified library was 2.5 X 10^{10} pfu/ml. The average size of inserts was 1.075kb and the recombinant efficiency was 94.4%. A lambda ZAP Express cDNA library was constructed using mRNA from *Eimeria tenella* sporulated oocysts. The library contained 6 X 10^6 clones and the titre of amplified library was 1x10^{11}pfu/ml; PCR identified that the library contained approximately 96% recombinant phages (Han *et al.* 2001). A high quality and highly representative cDNA library of *Necator americanus* third stage larvae has also been constructed, and some functional genes identified from the library by Expressed Sequence Tags (ESTs). The titre of un-amplified library was 1 x10^7 and the size of insert was about 750-3000bp. Seven out of 11 ESTs obtained from the library have a significant homology with certain functional genes (Zhan *et al.* 2000).

The aim of the present study is the construction of cDNA library from Myxobolus cerebralis, the causative agent of salmonid whirling disease.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Specimens

3.1.1.1 *Tubifex tubifex* oligochaetes worms

T. tubifex worms from our institute's SPF culture were kept in an aerated aquarium at a water temperature of 14-15°C. The bottom of the aquarium was covered with a 5cm layer of sterilised sand.

3.1.1.2 *Myxobolus cerebralis* triactinomyxon spores

Triactinomyxon spores were obtained from a population of *T. tubifex* worms that had been previously infected by the parasite.

3.1.2 Media

3.1.2.1 Solid media

3.1.2.1.1 LB agar plates

40g of LB-Agar powder (AppliChem GmbH, Darmstadt, Germany) was dissolved in 1L deionised water, the pH adjusted to 7.0, and then autoclaved at 121°C for 15min. After cooling, it was poured into Petri dishes.

3.1.2.1.2 LB top agar

32g of LB-Top Agar (Fluka ,BioChemika, Neu-Ulm, Germany) was dissolved in 1L deionised water, the pH adjusted to 7.0, and autoclaved at 121°C for 15min.

3.1.2.1.3 LB tetracycline

1L of LB-agar was prepared as described, cooled to 55°C after autoclaving, and then 1.5ml of 10mg/ml filter-sterilised tetracycline (Sigma-Aldrich chemie GmbH, Steinheim, Germany) dissolved in the agar prior to it being poured into Petri dishes, and stored in a refrigerator, 4°C, until use.

3.1.2.1.4 LB kanamycin

1L of LB-agar was prepared as described, cooled to 55°C after autoclaving, and then 6.6ml of 7.5mg/ml filter-sterilised kanamycin (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) was dissolved in the agar prior to it being poured into Petri dishes, and stored in the refrigerator until use.

3.1.2.2 Liquid media

3.1.2.2.1 LB broth

25g of LB-Medium powder (AppliChem GmbH, Darmstadt, Germany) was dissolved in 1L deionized water and the pH adjusted to 7.0, and then autoclaved at 121°C for 15min.

3.1.2.2.2 S.O.C. medium

Ready-to-use medium contained: 2% trypton, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose. (Invitrogen, Groningen, The Netherlands).

3.1.2.2.3 NZY broth

5g NaCl, 2g $MgSO_4.7H_2O$, 5g of yeast extract and 10g NZ amine (casein hydrolysate) were dissolved in 1L deionized water and the pH adjusted to 7.0, then autoclaved at 121°C for 15 min.

3.1.3 Bacterial strains

XL1-Blue MRF` strain (Stratagene, Amsterdam Zuidoost, The Netherlands)

XLOLR strain (Stratagene, Amsterdam Zuidoost, The Netherlands)

3.1.4 Antiserum

Triactinomyxon antiserum, propagated in rabbits, was kindly provided by: Dr. M.E. Markiw, U.S. Fish and Wildlife Service, National Fish Health Research Laboratory. Kearneysville, West Virginia. USA; and Prof. Ronald P. Hedrick, University of California, Davis, Department of Medicine and Epidemiology, School of Veterinary Medicine. Davis, CA 95616.

The antiserum was produced according to Markiw (1989). Briefly, the triactinomyxon spores antiserum was produced in New Zealand white rabbit that had been immunized with antigen from 1.7X 10^7 disrupted spores containing 1.6mg of soluble protein (Bradford 1976). Initial inoculation was with 1.3 X 10^7 disrupted spores in 2ml of Hanks' balanced salt solution emulsified with an equal volume of Freund's incomplete adjuvant. An initial dose of 1ml was given subcutaneously in each foreleg and intramuscularly in each hind leg. After 12 days, the rabbit was desensitized with an inoculum of 0.2ml and boosted 4h later by an intraperitoneal injection of 2ml of pooled antigen (1X10^7 disrupted and 3 X10^7 intact spores) without adjuvant. After 6 days, the rabbit was bled by cardiac puncture and 2ml aliquots of the serum were lyophilized.

3.1.5 Primer Sequences

Primer	**Sequence 5`---- 3`**
T3	AAT TAA CCC TCA CTA AAG GG
T7	GTA ATA CGA CTC ACT ATA GGG C

M13 Forward (-20)	GTA AAA CGA CGG CCA G
M13 Reverse	CAG GAA ACA GCT ATG AC
SMART II A Oligonucleotides	AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG
SMART CDS primer IIA	AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$N$_{-1}$N
PCR primer II A	AAGCAGTGGTATCAACGCAGAGT
Oligo d(T)-anchor primer	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV
PCR anchor primer	GACCACGCGTATCGATGTCGA
Oligo d (T)-linker primer	GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTT TTTTTTTTTTTTT
46-5 forward	CAAAGAAGCAGCTAAACCAAAA
46-5 reverse	GTTGACCTTGGAGACCTGGATGT
GSP1	TCCTTGGGTTTCGGGGCTTCCTTAG
GSP2	CTTCTTTGGCAGCAGGAGCAGCAGC
GSP5	TCTCCAAGGTCAACACGCTCATCAG

N.B.: N= A, C, G or T; N_{-1} = A, G, or C; V= A, C, G

3.1.6 Reagents used

Reagents	**Company**
Super SMART™ PCR cDNA synthesis kit	Clontech, Heidelberg, Germany
QIAprep Spin Miniprep Kit	QIAGEN, Hilden, Germany
Plasmid extraction Kit	QIAGEN, Hilden, Germany
MinElute ™ Gel extraction kit	QIAGEN, Hilden, Germany
cDNA synthesis kit	Stratagene, Amsterdam ,The Netherlands
Oligotex mRNA mini kit	QIAGEN, Hilden, Germany
RNeasy Mini kit	QIAGEN, Hilden, Germany
Protesilver™ silver Staining kit	Sigma-Aldrich, Steinheim,Germany

Coomassie® Brilliant blue R 250	SERVA, Heidelberg,Germany
Acrylamide/Bis 30% solution 29:1	Bio-Rad, Munich, Germany
Sodium dodecyl sulfate, SDS	Bio-Rad, Munich, Germany
Ammonium persulfate	Bio-Rad, Munich, Germany
Magnesium sulfate heptahydrate	Sigma-Aldrich, Steinheim, Germany
NZ amine , Casein hydrolysate	Sigma-Aldrich, Steinheim, Germany
Tris base	Bio-Rad, Munich, Germany
Maltose	Carl Roth. , Karlsruhe, Germany
Glycine	Bio-Rad, Munich, Germany
Non-fat dried milk	AppliChem, Darmstadt, Germany
Agarose	Carl Roth. , Karlsruhe, Germany
Tetracycline powder	Sigma-Aldrich, Steinheim, Germany
Kanamycin	Sigma-Aldrich, Steinheim, Germany
ECL western blotting detection reagents	Amersham, Freiburg, Germany
Kodak developer & fixer	Sigma-Aldrich, Steinheim, Germany
NucleoSpin® Extraction kit	Clontech, Heidelberg, Germany
Advantage® 2 PCR kit	Clontech, Heidelberg, Germany
5`/3`RACE kit	Roche, Mannheim, Germany
High pure PCR product purification kit	Roche, Mannheim, Germany
TOPO TA cloning® kit	Invitrogen, Groningen,The Netherlands
ZAP Express vector	Stratagene, Amsterdam, The Netherlands
Gigapack® III Gold packaging extract	Stratagene, Amsterdam, The Netherlands
Size-select-400 SPUN columns	Amersham, Freiburg, Germany
Escherichia Coli phage lysate	Stratagene, Amsterdam ,The Netherlands
ExAssist interference, resistant helper phage	Stratagene, Amsterdam ,The Netherlands
SDS-PAGE standards high molecular weight	Bio-Rad, Munich, Germany

SDS-PAGE pre-stained standards, broad range	Bio-Rad, Munich, Germany
SDS-PAGE pre-stained standard, broad range	BioLab, Frankfurt, Germany
Ready-load 1kb plus DNA ladder	Invitrogen, Groningen,The Netherlands
Reddy Mix PCR Master Mix	ABgene, Hamburg, Germany
AmpliTaq® DNA polymerase	Roche, Mannheim, Germany
Protran BA 85, nitrocellulose membrane	Schleicher& schuell,Dassel,Germany
Protran, nitrocellulose transfer membrane	Schleicher& schuell,Dassel,Germany
Anti-Rabbit IgG peroxides conjugate	Sigma-Aldrich, Steinheim, Germany
Hyperfilm ECL 18 x24 cm	Amersham, Freiburg, Germany
DEPC,Diethylpyrocarbonate	Carl Roth. , Karlsruhe, Germany
RNA*later*	Ambion, Cambridgeshire, U K
RnaseZAP™	Sigma-Aldrich, Steinheim, Germany
Prime RNase inhibitor	Eppendorf, Köln, Germany
X-gal	Sigma-Aldrich, Steinheim, Germany
IPTG	Sigma-Aldrich, Steinheim, Germany
Rotisol, Ethanol	Carl Roth. , Karlsruhe, Germany
Methanol	AppliChem, Darmstadt, Germany
Chloroform	Carl Roth. , Karlsruhe, Germany
DMF, dimethylformamide	Sigma-Aldrich, Steinheim, Germany
2-Mercaptoethanol	Bio-Rad, Munich, Germany
Acetic acid, Glacial	Sigma-Aldrich, Steinheim,Germany
Formaldehyde solution 37%	Sigma-Aldrich, Steinheim, Germany
Tween-20	Bio-Rad , Munich, Germany
LaemmLi sample buffer	Bio-Rad, Munich, Germany
RNA sample loading buffer	Sigma-Aldrich, Steinheim, Germany

MOPS ,formaldehyde gel buffer	Eppendorf, Köln, Germany
STE buffer	Stratagene, Amsterdam ,The Netherlands
1.5 M Tris-HCl buffer (pH 8.8)	
1 M Tris-HCl buffer (pH 6.8)	
Tris buffer saline& SM buffer	
TEMED N,N,N',N'Tetramethylethylenediamine	Bio-Rad, Munich, Germany

3.2 METHODS

3.2.1 Experimental production of triactinomyxon spores

3.2.1.1 Experimental infection of oligochaetes

Myxobolus cerebralis myxospores were obtained from the skulls of clinically diseased rainbow trout. Bone fragments were excised, homogenised in an Ultra Turax mixer, then suspended in phosphate buffer saline (PBS) and passed through successive screens with mesh sizes of 1000, 500, 250 and 100µm. The final filtrate was centrifuged and the resulting pellets containing *M. cerebralis* spores were re-suspended in PBS and spores counted (El-Matbouli *et al.* 1995). 150g (about 10,000) infection-free *T. tubifex* from the institute's SPF culture were kept in a 10L plastic aquarium, in a 5cm layer of sterilised sand covered with aerated, de-chlorinated tap water at 14-15°C, and fed Algamac-2000. The *T. tubifex* were exposed to approximately 4 million viable *M. cerebralis,* a dose of about 400 spores/worm (El-Matbouli *et al.* 1999b). The water from the culture was examined weekly for the presence of triactinomyxon spores.

3.2.1.2. Collection of triactinomyxon spores

Following detection of waterborne triactinomyxon spores approximately three months post-exposure, the water over the culture was processed as described by Soliman *et al.* (2003), briefly: water was siphoned off into a bucket, and then poured through a 20µm Nitex screen to concentrate the spores on the screen. Spores from one aquarium were suspended in 20-50ml, mixed, and 0.1ml of the suspension removed for enumeration. The total number of spores was estimated by multiplying the number obtained by 10, then by the total volume filtered. The remainder of the concentrated spore sample was put back in the refrigerator (4°C) and allowed to settle before being purified. After 2h, the clear supernatant was decanted into a fresh tube and the remainder of the sample (sediment) set aside. Spores were separated using a screen and Percoll gradient: the supernatant was first filtered through a 20µm Nitex screen; spores were then washed off the screen into a holding vial; in a 15ml centrifuge tube 10ml 20% Percoll (in distilled water) was layered carefully with the filtered triactinomyxon spores (about 2ml, Percoll-to-sample ratio about 5:1). This procedure was repeated with the sediment using an additional 10ml 20% Percoll. Both tubes were then centrifuged for 10min at 1250 rpm. At the end of run the spores were evident as a white band when the tubes were viewed in bright light against a dark background. The bands were carefully pipetted into separate clean tubes, filtered again, then washed 3-4 times to get rid of any Percoll residue. The purified triactinomyxon spores

were then concentrated and re-suspended in a small volume of distilled water and kept at -20°C until required or kept in RNAlater (for RNA preservation) for isolation of RNA.

3.2.1.3 Selection of highly triactinomyxon spores producer *Tubifex tubifex*

When the infected *T. tubifex* reached their period of peak release of triactinomyxon spores between 140-160 days, individual worms were separated into multi-well plates with de-chlorinated water (tap water leaved for 2hrs until complete evaporation of chlorin) and maintained for approximately one week at 14-15°C. This was both to determine which worms were releasing spores, and which were most productive. Worms that release high numbers of spores were selected and preserved in RNA*later* for using in RNA extraction.

3.2.2 Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting of triactinomyxon spores

Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis and western blotting analysis of the triactinomyxon spores was conducted as per the method of Soliman *et al.* (2003), described below.

3.2.2.1 SDS-PAGE

3.2.2.1.1 Protein samples

Approximately one million purified triactinomyxon spores were sonicated in a water bath sonicator (Transsonic 310, Elma, West Germany) for 5 rounds of: 2 min sonication followed by 1 min incubation on ice. An equal volume of 2x SDS sample buffer (100mM Tris-HCl, pH 6.8, 200mM 2-Mercaptoethanol, 4% SDS, 0.2 % bromophenol blue, 20% glycerol) was then added to the sonicated sample and boiled at 95°C for 5 min. The sample was then centrifuged for 2.5 min at 14,000*xg* and kept in ice until used. The supernatant, which contained the soluble proteins of triactinomyxon spores, was loaded on the prepared gel. Infection-free *T. tubifex* from our institute's SPF culture were homogenised in sterile water and centrifuged at 20,000*xg* for 5 min. The supernatant was diluted in SDS-sample buffer 1:2, and then treated as for the triactinomyxon sample (Roberts *et al.* 2003).

3.2.2.1.2 Gel Electrophoresis

The gel apparatus was set up according to the manufacture's instructions. 12 % resolving gel monomers were prepared (8ml 30% acrylamide / Bis. solution 29:1, 5ml Tris-HCl, pH 8.8, 0.2ml 10 % SDS, 3.3ml distilled water, 0.130ml 15% ammonium persulfate, 0.026ml TEMED), the gel was gently swirled, and poured into the gap between the two sandwiched glass plates, overlaid with distilled water and allowed to polymerise for 10 min. After polymerisation was complete, the distilled water was decanted off and the 5% stacking gel monomer was prepared (1ml 30% acrylamide / Bis. solution 29:1, 1.66ml Tris-HCl , pH

6.8, 0.033ml 10% SDS, 3.63ml distilled water, 0.33ml 15% ammonium persulfate, 0.018ml TEMED), poured over the resolving gel, and the comb immediately inserted between the plates. The stacking gel was allowed to polymerise for 10 min before running. All gels were run using a BioRad Mini Protean II cell (BioRad Laboratories GmbH, Munich, Germany) at 130V constant voltage, in 1x running buffer (25mM Tris, 250mM glycine pH 8.3, 0.1 % SDS). When electrophoresis was completed, one gel was subjected to staining and a replicate gel was used for western blotting.

3.2.2.1.3 Protein staining

Proteins on the gel were visualised by staining with coomassie brilliant blue R250, and with a silver staining kit according to the manufacture's instructions.

3.2.2.2 Western blotting

Freshly electrophoresed SDS-Polyacrylamide gel was dipped into transfer buffer (39mM glycine, 48mM Tris base, 0.037% SDS, 20% methanol, pH 8.3), then laid flat on pre-wetted nitrocellulose membrane supported on three layers of transfer buffer-wetted filter paper and one layer of wetted porous pad resting on the anode side of the blotting cell. The gel was overlaid with three wetted filter papers, a wetted porous pad, and then the cathode. Care had to be taken to exclude bubbles between the gel and nitrocellulose membrane, and between the nitrocellulose membrane and filter papers.

The samples were transferred to the nitrocellulose membrane using a BioRad Mini-Trans Blot Cell apparatus for 1h at a constant current of 250mA (100V). Then the assembly was dismantled and the nitrocellulose membranes soaked in blocking buffer (5% non-fat dried milk, 150mM NaCl, 10mM Tris-HCl, pH 8.0, 0.05% Tween 20) for 2h at room temperature with gentle agitation; this occupied all non-specific protein binding sites on the membrane. Membranes were then incubated with the primary antibodies - polyclonal rabbit anti-triactinomyxon antiserum - at various dilutions in the blocking buffer, and also with rabbit control antiserum, for 2h with agitation at room temperature. The membranes were washed three times each for 15 min with (TBST) Tris Buffer Saline + Tween 20, (150mM NaCl , 10mM Tris-HCl, pH 8.0, 0.05% Tween 20), to remove all of the unbounded antibodies, then incubated with the secondary antibodies - horseradish peroxidase-conjugated anti-rabbit IgGs - diluted in blocking buffer with different concentrations, for 2h at room temperature with agitation. The membranes were again washed 3 times each for 15 min with TBST. Bound antibodies were visualised using ECL™ chemiluminescent detection as follows: excess wash buffer was removed by holding the membrane vertically with forceps, and then placed in the middle of clean sheet

of polyethylene kitchen wrap. Equal volumes of detection reagent 1 and detection reagent 2 were mixed and applied to the membrane to cover it. After one minute incubation, excess detection buffer was drained off and the membrane then exposed to Hyperfilm-ECL autoradiography film, in the film cassette from 30s to 1 min. The exposed film was then developed with developing solution, washed with distilled water, and fixed**.**

3.2.3 RNA extraction

3.2.3.1 General precautions for handling RNA

Care was taken to avoid contamination with RNase enzymes which cause destruction of RNA. All glassware was baked overnight in a 180°C oven. Plastic ware was incubated with 0.1% DEPC overnight and then autoclaved for 30min. General lab equipments, pipettors and working surfaces were cleaned with RnaseZAP™ which is effective in eliminating RNase contamination. Gloves were frequently changed.

The samples of infected oligochaetes were preserved directly after collection in an appropriate volume of RNA*later*, a RNA stabilising agent.

3.2.3.2 Extraction of Total RNA

Total RNA, from oligochaetes producing the highest numbers of triactinomyxons, was isolated following the RNeasy Mini kit protocol for isolation of total RNA from animal tissues. Thirty milligram RNA*later*-stabilised samples were placed in liquid nitrogen, and thoroughly ground with a mortar and pestle. The tissue powder and liquid nitrogen was decanted into a liquid-nitrogen-cooled 2ml tube, and the nitrogen allowed to evaporate. Samples were then resuspended in an appropriate volume of guanidine isothiocyanate buffer, and homogenised by passing the lysate 5 times through a 20-gauge needle. 70% ethanol was then added to the lysate to create conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. The sample was then applied to the RNeasy mini–column, the RNA allowed to bind before being rinsed and then eluted in RNase-free water. The concentration of RNA was determined by measuring the absorbance at 260nm (A_{260}), while the purity of the RNA was determined by measuring the absorbance at 260nm and 280 nm (A_{260}/A_{280}) in a spectrophotometer.

3.2.3.3 Isolation of messenger RNA (mRNA) from total RNA

After extraction of RNA from the oligochaetes, mRNA was isolated from the total RNA using Oligotex mRNA spin-column protocol as follows: the volume of total RNA was adjusted to 250µl with RNase-free water, and an equal amount of OBB buffer (20mM Tris-HCl, pH 7.5, 1M NaCl, 2mM EDTA, 0.2% SDS) added with 15µl of Oligotex suspension (10% Oligotex particles, $dC_{10}T_{30}$ oligonucleotides covalently linked to the surface of

polystyrene-latex particles via a condensation reaction, 10mM Tris-HCl pH 7.5, 500mM NaCl, 1mM EDTA, 0.1 % SDS, 0.1% NaN_3). The sample was incubated at 70°C for 3 min and then at room temperature for 10 min. After centrifugation the pellet was re-suspended in buffer OW_2 (10mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA), applied to the spin column, washed once again with the buffer OW_2 before mRNA was eluted with hot (70°C) buffer OEB (5mM Tris-HCl, pH 7.5). The concentration of the mRNA was measured with spectrophotometer. Prime RNase inhibitor was added to the mRNA to protect it from RNase contamination.

3.2.3.4 Formaldehyde agarose RNA gel electrophoresis

Agarose gel (1.5%) was melted in a solution made with 10ml 10x MOPS buffer (200mM 3-[*N* –morpholino] propane-sulfonic acid, 50mM sodium acetate, 10mM EDTA, pH 6.5-7) and 85ml sterile water (121°C). The solution was cooled to 50°C and 4.5ml 37% formaldehyde solution added, before the gel was well mixed and poured into the gel support. Samples of RNA were dried in vacuum evaporator, re-suspended in 10µl formaldehyde gel loading buffer, heated at 65°C for 5-10 min, chilled on ice and then loaded. The gel was run in 1x MOPS running buffer at 5V/cm.

3.2.4 Construction of the cDNA library

The cDNA library was constructed with the ZAP Express™ cDNA synthesis kit with some modification, as noted below.

3.2.4.1 First-strand cDNA synthesis

Poly A^+ (5µg) was primed in the first strand cDNA synthesis with the oligo (dT) linker-primer that contains an *Xho* I restriction site, then transcribed using StrataScript™ reverse transcriptase and 5-methyl dCTP. A reaction mixture comprised the following reagents, added sequentially to an RNase-free microcentrifuge tube:

5µl 10x first-strand buffer

3µl first-strand methyl nucleotide mixture

2µl linker-primer (1.4µg/µl)

7.5µl DEPC-treated water

1µl RNase Block Ribonuclease Inhibitor (40U/µl)

30µl poly (A) RNA (5.37µg)

The reaction was mixed and left 10 min at room temperature, to allow the primer to anneal to the template, and then 1.5µl of Strata Script RT (50U/µl) was added to the reaction mixture which was then incubated at 42°C for 1h.

3.2.4.2 Second-strand cDNA synthesis

After 1h, the first-strand synthesis reaction was placed on ice, and the following reagents were added:

20µl 10x second-strand buffer

6µl second-strand dNTP mixture

116µl sterile distilled water

2µl RNase H (1.5U/µl)

11µl DNA polymerase I (9U/µl)

The mixture was gently mixed and incubated at 16°C for 2.5h.

3.2.4.3 Blunting the cDNA Termini

The second-strand synthesis reaction was placed immediately on ice after 2.5h, and then the following reagents added:

23µl blunting dNTP mix

2µl cloned *Pfu* DNA polymerase (2.5U/µl)

The reaction was mixed and incubated at 72°C for 30 min. After incubation, the reaction was purified to remove primers, nucleotides, polymerases, and salts, and concentrated using MinElute™ gel extraction kit protocol as per the manufacture's instructions. The DNA, after elution in 10µl distilled water, was lyophilised, using a speed vac, and resuspended in 9µl *EcoR I* adapters and incubated at 4°C for 30 min, to allow the cDNA to resuspend.

3.2.4.4 Ligating the EcoR I adapters

The following components were added to the blunted cDNA and the EcoR I adapters:

1µl 10x ligase buffer

1µl 10 mM rATP

1µl T4 DNA ligase (4U/µl)

The reaction was mixed and incubated overnight at 8°C. In the morning, the ligase was heat-inactivated at 70°C for 30 min.

3.2.4.5 Phosphorylating the EcoR I ends

After the ligase was inactivated, the reaction was cooled to room temperature for 5 min, and the adapter ends was phosphorylated by adding the following components:

1µl 10x ligase buffer

2µl 10mM rATP

6 µl sterile water

1 µl T4 polynucleotide kinase (10U/µL)

The reaction was incubated for 30 min at 37 °C.

3.2.4.6 Digesting with Xho I

The kinase was heat inactivated at 70°C for 30 min, and then left for 5 min to equilibrate to room temperature. The following reagents were then added:

28µl *Xho I* buffer supplement

3µl *Xho I* (40U/µl)

The reaction was incubated for 1.5h at 37 °C.

After 1.5h the reaction was purified to remove primers, nucleotides, polymerases, and salts, and concentrated using the MinElute™ gel extraction kit protocol as per the manufacture's instructions. The cDNA, after elution in 10µl distilled water, was lyophilised using a speed vac, and resuspended in 50µl 1x STE buffer (150mM NaCl, 10mM Tris-HCl, pH 7.4, 1mM EDTA).

3.2.4.7 Size fractionation

To remove residual oligonucleotides, restriction enzymes, and cDNAs < 400bp, which might compete or interfere with the insertion or ligation of the cDNA to the vector, the SizeSep™ 400 Spin Column, Sepharose CL-4B was used to selected the cDNA >400bp. The column was equilibrated with 1x STE buffer, and then centrifuged at 400*xg* for 2 min. The sample was applied slowly to the centre of the top of the column. cDNA was collected in a 1.5ml microcentrifuge tube after additional centrifugation at 400*xg* for 2 min. The product cDNA was concentrated using MinElute™ gel extraction kit protocol as per the manufacture's instructions, lyophilised, and then resuspended in 5µl of sterile water.

3.2.4.8 Ligating cDNA into the ZAP Express Vector

The following components were added to 5µl of resuspended cDNA:

0.6µl 10x ligase buffer

0.5µl 10mM rATP (pH 7.5)

1.0µl ZAP Express vector (1µg/µl)

0.5µl T4 DNA ligase (4U/µl)

The reaction was incubated for two days at 4°C.

3.2.4.9 Packaging of the ligated cDNA

After ligation into the ZAP Express vector, the resulting DNA was packaged *in vitro* using Gigapack III gold packaging extract. A tube of packaging extract (stored at -80°C) was quickly thawed in the hand, then the ligated DNA was added and the mixture incubated at room temperature for 2h. After 2h, 500µl SM buffer (NaCl, Tris-HCl, pH 7.5, $MgSO_4$ Gelatine) and 20µl chloroform were added and mixed gently, then the tube was centrifuged briefly to remove sediment. The supernatant containing the phage was kept at 4°C until titred.

3.2.4.10 Titration of the primary cDNA library

It is important to determine the phage titre of the library being screened in order to plate the correct number of plaque forming units per plate. A culture of XL1-Blue MRF` host strain, in LB medium, supplemented with 10mM $MgSO_4$ and 0.2% maltose, was grown to an OD_{600} of 1.0, then the bacteria was pelletised by centrifuging at 1000*xg* for 10 min. The cells were gently resuspended to half the original volume with sterile 10mM $MgSO_4$, and then diluted to a final OD_{600} of 0.5. LB top agar was melted and cooled to 55°C in water bath. 1µl of lambda phage packaging material was added to 300µl of the diluted host cells. Also, 1µl of 1:10 dilution of packaging material in SM buffer was added to 300µl of host cells. The phage and bacteria were incubated 15 min at 37°C to allow the phage to attach to cells. The following were then added:

4ml LB top agar

15µl 0.5M IPTG (in water)

50µl X-gal (250mg/ml in DMF)

The solution was mixed, and poured immediately onto LB agar plates, pre-warmed to 37 °C, and distributed evenly across the surface of the plate, before being left to solidify at room temperature. The plates were then inverted and incubated at 37°C overnight to develop the plaque colour: background plaques are blue, while recombinant plaques are white. After incubation, the plaques were counted and the ratio of blue to white calculated to determine the library titre.

3.2.5 Antibody screening of the cDNA library using ECL™ chemiluminescent detection

3.2.5.1 Absorption of the primary antibody with *Escherichia coli* phage lysate

Polyclonal primary antibody was treated with the *E.coli* phage lysate to eliminate any background or false positive results (polyclonal antibodies often contain antibodies that react with *E.coli* and phage proteins).

The *E.coli* phage lysate was diluted 1:10(v/v) in TBST. Four nitrocellulose membranes were immersed into the diluted lysate and incubated for 30 min at room temperature with occasional agitation. The membranes were then removed, air dried on Whatman 3MM paper, then washed five times for 5 min each with 50ml of TBST and excess moisture absorbed with Whatman 3MM paper. The nitrocellulose membranes were then immersed in 50ml blocking buffer (5% non-fat dried milk, 150mM NaCl, 10mM Tris-HCl, pH 8.0, 0.05% Tween 20) for 30 min at room temperature with shaking, then rinsed three times with 50ml TBST. The primary antibodies were diluted 1:5 in TBST and each nitrocellulose membrane was incubated with antibody solution at 37°C for 10 min. After removal of the last membrane, the primary antibodies were collected and used for library screening.

3.2.5.2 Preparation of 10mM IPTG-soaked nitrocellulose membranes

82mm circular nitrocellulose filters were wetted by laying them on the surface of 10mM IPTG in a Petri dish. Once wet, the filter was submerged and then the next filter added, and so on until all filters were in the solution. The filters were allowed to soak 30s to several min, before removing and blotting on Whatman paper to remove excess liquid, then being left to dry at room temperature. The filters were labelled with a pencil and stored until use.

3.2.5.3 Preparation of host bacteria

XL1-Blue MRF' glycerol stock was streaked onto LB-tetracycline agar plates and incubated at 37°C overnight. In the morning, a 50ml LB broth, supplemented with 10mM $MgSO_4$ and 0.2% (w/v) maltose, was inoculated with a single colony and grown at either 37°C with shaking for 4-6h (OD_{600} =1.0), or overnight at 30°C with shaking at 200 rpm. Cells were collected by centrifuging at 500*xg* for 10 min, and the supernatant discarded. The cells then were gently resuspended in half the original volume with sterile 10mM $MgSO_4$, and aliquots diluted to an OD_{600} of 0.5 with sterile 10mM $MgSO_4$.

3.2.5.4 Plating out the library for screening

In a sterile, 15ml tube, 300µl of OD_{600} =0.5 diluted plating cells was mixed with 1000pfu phage and incubated at 37°C for 15 min to allow the bacteria to adsorb phage. 4ml LB top agar which had been melted and cooled to 55°C, was added to the phage/bacteria mixture, then poured immediately onto a pre-warmed (37°C) 100mm LB plate and rocked until the

surface of the plate was covered. The plates were kept 10 min at room temperature to harden and then placed inverted in a 42°C incubator. After about 3.5h plaques would begin to form, and the plates were overlaid with the IPTG-soaked nitrocellulose membranes and incubated at 37°C overnight. After incubation, the orientation of the nitrocellulose membrane in relation to the plate was marked by piercing the membrane in several places with an 18-gauge needle, then the membrane was removed carefully with forceps and washed three times, 10 min each, with TBST (150mM NaCl, 10mM Tris-HCl, pH 8.0, 0.05% Tween 20) with the protein side up, to remove any remaining top agar or excess bacteria. Membranes then immersed in blocking buffer (5% non-fat dried milk, 150mM NaCl, 10mM Tris-HCl, pH 8.0, 0.05% Tween 20), with gently agitation, for 2h at 4°C. The plates were kept in a refrigerator until ready to core the plaques.

3.2.5.5 Immunoscreening using ECL reagent

After blocking any remaining protein binding sites on the nitrocellulose membranes by immersion in blocking solution, the membranes were incubated with the primary antibodies (anti-triactinomyxon antibody diluted 1:1000 in blocking solution) for 2-24h at 4°C on a horizontal shaker platform. The membranes were then washed three times with TBST, 10 min each wash, to remove any residual unbounded primary antibodies, and then incubated with the secondary antibody (anti-rabbit antibodies conjugated with alkaline phosphates, diluted in blocking buffer as per the manufacture's instructions) for 5h at 4°C on a horizontal shaker platform. The membranes were then washed with TBST again three times, 10 min each, to remove any residual unbounded conjugate.

Individual membranes were then removed with forceps, held vertically with one corner touching a small pile of Kimwipies to remove excess wash buffer, before being laid protein-side-up in the middle of a sheet of polyethylene kitchen wrap. Two millilitres of each of ECL detection solutions 1 and 2, were mixed and applied to the corners and the middle to cover the entire membrane, which was incubated for 1 min at room temperature. The membrane was again then picked up with forceps, excess detection solution drained by again holding a fresh Kimwipe to the lower edge, then placed protein-side-down onto a sheet protector and the upper sheet lowered rapidly. Excess fluid around the edges was wiped off and the air pockets were smoothed gently to let the entire sheet dry. The wrapped membrane was taken into a darkroom, placed in a film cassette, protein side up, and a sheet of autoradiography film (Hyperfilm-ECL) placed on top of the blots and exposed for 1-5 min. The exposed film was then processed according to the manufacture's

instructions. Comparison of the developed transparency with the original plate revealed the locations of positive plaques.

3.2.5.6 Purification of plaque clones of interest and PCR confirmatory test

Plaques identified as positive by antibody screening were removed from the gel as a plug, using a cut-off end of a 1000µl pipette tip. The plug was then placed into 500µl SM buffer (NaCl, Tris-HCl, pH 7.5, $MgSO_4$ Gelatine) with 20µl of chloroform. Phage was allowed to diffuse into SM buffer overnight and then the supernatant transferred to a new tube. The titre of the phage was determined, and secondary and tertiary screenings carried out as previously described. In order to minimise inclusion of inserts in the cDNA library and to avoid the false positive screening results, individual plaques were selected randomly after tertiary screening and purification and used as a template for PCR. The PCR amplified cDNA insert fragments using the T3 and T7 primers, which flank the multiple cloning site of the vector containing the insert, according to modified protocol of Kim & Jue (1990). Each PCR was performed in a 50µl final volume containing 20µM of each primer, 47µl of ReddyMix PCR Master Mix (75mM Tris-HCl, pH 8.8, 1, 25U Taq Polymerase, 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01 % Tween 20, 0.2mM each dNTPs) and seeded with 1µl of phage. PCR parameters were as follows: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 30s at 95°C, 45s at 48°C and 30s at 72°C. Final extension was carried out at 72°C for 10 min. Amplifications were performed in an Eppendorf, Mastercycler Gradient Thermal cycler (Eppendorf, Netheler-Hinz GmbH, Hamburg, Germany). Amplified products were visualised on 1% agarose gels compared against DNA molecular weight standard.

3.2.6 In vivo excision of the pBK-CMV phagemid vector containing insert from the ZAP Express vector

After cDNA clones of interest were identified in the intact Lambda ZAP Express vector by antibodies, a representative clone was selected at random (45-5) to test the specificity of the library to triactinomyxon spores. This was subjected to a process of *in vivo* biological excision of the insert and sub-cloning into a plasmid vector using the single-clone excision protocol as per the Stratagene instruction manual. A timeline of the procedure follows:

3.2.6.1 Day 1

1. The plaque of interest was cored from agar plate and transferred to a sterile microcentrifuge tube containing 500µl SM buffer and 20µl chloroform, vortexed, and incubated at 4°C overnight to release the phage particle into SM buffer.

2. A culture of XL1-Blue MRF` cells supplemented with 0.2% maltose and 10mM $MgSO_4$ was grown overnight at 30°C.

3.2.6.2 Day 2

3. The XL1-Blue MRF` cells were spun down (1000*xg*) and resuspended at OD_{600} of 1.0 in 10mM $MgSO_4$.

4. In a 50 ml Falcon tube the following were combined:

    a. 200µl of XL1-Blue MRF`cells at an OD_{600} of 1.0

    b. 250µl of phage stock (containing >1x10^5 phage particles)

    c. 1µl of ExAssist helper phage (>1 x 10^6pfu/µl

5. The Falcon tube was incubated at 37°C for 15 min.

6. 3ml of NZY broth was added to the Falcon tube and incubated at 37°C with shaking, overnight.

7. XLOLR culture in NZY broth, with out supplement, was grown overnight at 30°C with shaking.

3.2.6.3 Day 3

8. The XLOLR cells were spun down (1000*xg*) and resuspended at OD_{600} of 1.0 in 10mM $MgSO_4$.

9. The Falcon tube was heated at 65-70°C for 20 min, and then spun at 1000*xg* for 15 min.

10. The supernatant, containing the excised pBK-CMV phagemid vector packaged as filamentous phage particles, was decanted into a sterile Falcon tube.

11. 200µl of freshly grown, diluted to OD_{600} of 1.0, XLOLR cells were added to two Falcon tubes - one tube then received 100µl of the phage supernatant from step 10, the other tube received 10µl.

12. Both tubes were incubated at 37°C for 15 min.

13. 300µl of NZY broth was added to each tube and incubated at 37°C for 45 min.

14. 1µl and 200µl of the cell mixture from each tube was plated on LB- kanamycin agar plates (50mg/µl) and incubated at 37°C overnight.

3.2.7 Isolation of plasmid DNA and estimation of the cDNA insert size

The pBK-CMV plasmids were excised *in vitro* as mentioned before, and colonies appearing on the plate contained the pBK-CMV double-stranded phagemid vector with cloned DNA insert. A QIAprep Spin Miniprep kit was used to prepare the selected recombinant plasmid. One colony was cultured overnight in LB medium containing 50µg/ml kanamycin and incubated at 37°C with shaking. The cells were pelletised by centrifuging at 5000*xg* for 10 min, then resuspended in 250µl resuspension buffer P1. After complete resuspension, 250µl of lysis buffer P2, was added to the resuspended cells and the tube well mixed by inverting 4-6 times; the solution became viscous and slightly clear. 350µl of neutralisation buffer N3 was added and the tube again inverted 4-6 times; the solution became cloudy. The tube was centrifuged at 18,000*xg* for 10 min. The supernatant was transferred to the QIAprep Spin column and centrifuged at 18,000*xg* for 1 min. The spin column was washed twice with buffer PB: first wash 500µl, second wash 750µl, with centrifugation at 18,000*xg* for 1 min for both washes. The column was centrifuged for an additional minute to remove residual wash buffer. The plasmid DNA was then eluted with 50µl elution buffer EB (after standing 1 min) by centrifugation at 18,000*xg* for 1 min.

The size of the insert was checked by PCR amplification of the cDNA fragments using the T3 and T7 primers which flanking the cloning site of the vector. 20µM from each primer was added to 47.5µl of ReddyMix PCR Master Mix (75mM Tris-HCl, pH 8.8, 1.25U Taq Polymerase, 20mM $(NH_4)_2SO_4$, 1.5mM MgCl2, 0.01 % Tween 20, 0.2mM each dNTPs) and 0.5µl of the plasmid DNA was added to the mixture. PCR parameters were as follows: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 30s at 95°C, 45s at 48°C and 30s at 72°C. Final extension was carried out at 72°C for 10 min. Amplifications were performed in an Eppendorf, Mastercycler Gradient Thermal cycler (Eppendorf, Netheler-Hinz GmbH, Hamburg, Germany). Amplified products were visualised on 1% agarose gels compared against DNA molecular weight standard.

3.2.8 DNA sequencing and sequence analysis:

After isolation of the recombinant plasmid DNA and estimation of the cDNA fragment size, the purified plasmid DNA was sequenced (sequencing laboratories Göttingen GmbH, Germany). A National Centre for Biotechnology Information (NCBI) non-redundant nucleotide BLASTn search was carried out to find any similarities to the edited sequences (Altschul *et al.* 1990).

3.2.9 Primer construction from the selected sequence

After sequencing and alignment of the clone; primers 46-5 for and 46-5 rev were designed using "Oligo" software to ensure that their sequence belonged to *M. cerebralis* and not to the worm. Three other primers, GSP1, GSP2 (both 5` end) and GSP5 (3` end) were designed using the same software, for use with RACE amplification of the ends of the cDNA, to obtain the sequence of the entire clone.

3.2.10 Triactinomyxon spores and *Tubifex tubifex* cDNA synthesis

Total RNA from RNA*later*-preserved triactinomyxon spores and non-infected oligochaetes worm was extracted using an RNeasy kit as previously described. cDNA from worms and spores was synthesised using a Super SMART™ cDNA synthesis kit.

3.2.10.1 First strand cDNA synthesis

The following reagents were combined in a sterile 0.5ml reaction tube: 1µg total RNA, 7µl 3`SMART CDS primer II A (12µM), 7µl SMART II A oligonucleotides (12µM) and deionised water to 64µl total volume. The mixture was mixed, spun briefly, then incubated at 65°C for 2 min. Additional reagents were then added: 20µl 5x First-strand buffer, 2µl DTT, 10µl 50x dNTP (10mM), 5µl RNase inhibitor (20U/µl) and 5µl PowerScript reverse transcriptase. The tube was then incubated at 42°C for 90 min, before 2µl of 0.5M EDTA was added to stop the reaction.

3.2.10.2 Column chromatography

Column chromatography was used to remove unincorporated nucleotides and small (<0.1 Kb) cDNA fragments from the SMART cDNA. Three volumes of binding buffer NT2 were added to each cDNA synthesis reaction. Solutions were well mixed by pipetting, then transferred into the NucleoSpin extraction spin column and centrifuged at 14,000 rpm for 1 min. 500µl of washing buffer NT3 was then added to the spin column and centrifuged at 14,000 rpm for 1 min; this washing step was repeated twice. To elute the purified SMART cDNA; 50µl deionised water was added to the spin column, allowed to stand for 2 min with the cap open, then eluted by centrifuging at 14,000 rpm for 1 min; the elution was repeated using the same collection tube and an additional 35µl deionised water .The final elution volume was 80µl.

3.2.10.3 Amplification of cDNA by LD (long-distance) PCR

For optimisation of the PCR reaction, 2.5µl of the single stranded cDNA (sscDNA) was added to 77.5µl deionised water (end volume 80µl). A master mix was prepared, comprising: 4µl deionised water, 10µl 10x Advantage 2 PCR buffer, 2µl 50x dNTP (10mM), 2µl 5`PCR primer II A (12 µM) and 2µl 50x Advantage 2 polymerase Mix.

20µl of master mix was added to the 80µl sscDNA, and the tube placed in a pre-heated thermacycler (95°C) then subjected to the following program: 95°C for 1 min, then 15 cycles of 95°C for 15s, 65°C for 30s, and 68°C for 6 min. After the 15 cycles, 70µl was removed and kept at 4°C, and 5µl from the remaining 30µl was removed for analysis. The remaining 25 µl, was then subjected to five subsequent rounds of three cycles, with 5µl samples removed after each round. The total number of cycles was 30 cycles.

To determine the optimal number of cycles, all of the 5µl aliquots were run on an ethidium bromide-stinaed1.2 % agarose gel.

The initial 70µl (15-cycles) PCR product was then returned to the thermocycler and subjected to additional cycles required to reach optimal amplification.

3.2.10.4 Spin column purification of the PCR products

300µl of binding buffer NT2 was added to the 70µl PCR reaction and mixed by pipetting. The sample was then transferred into the NucleoSpin column and centrifuged for 1 min at 14,000 rpm. 500µl of wash buffer NT3 was added to the column and centrifuged at 14,000 rpm for 1 min; this wash step was repeated twice. The column was the spun at 14,000 rpm for 1 min to remove final traces of ethanol. 50µl of elution buffer NE was added directly onto the column and allowed to sit for 2 min before being centrifuged at 14,000 rpm for 1 min. The yield of each PCR reaction was determined by a biophotometer.

3.2.11 A verification PCR test for the cDNA sequence

To verify that the selected clone (46-5) belonged to the triactinomyxon spores, cDNA from both triactinomyxon spores and worms was amplified to detect the 511bp band generated by the 46-5for and 46-5rev primers.. The PCR mixture consisted of: 40pmol each primer, 44.5µl ReddyMix PCR Master Mix (75mM Tris-HCl, pH 8.8, 1.25U Taq Polymerase, 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01 % Tween 20, 0.2mM each dNTPs) plus 3.5µl sample cDNA (250ng). The PCR used the following reaction profile: 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 2 min. These cycles were preceded by a denaturation step of 95°C for 3 min and concluded with an elongation step of 72°C for 10 min. The PCR product was visualised on a 1.5 % agarose gel stained with ethidium bromide.

3.2.12 5`and 3`RACE amplification

After confirmation that the selected clone (46-5) belonged to the triactinomyxon, its 5` and 3` ends were amplified using 5`/3`RACE kit as described below.

3.2.12.1 5`RACE amplification

3.2.12.1.1 First –strand cDNA synthesis

Synthesis of the first-strand cDNA was performed using the gene specific primer, GSP1, and a reverse transcriptase enzyme. The reaction performed in 20ml total volume, comprising: 12.5µM GSP1 primer, 4µl 5x cDNA synthesis buffer (250mM Tris-HCl, 40mM $MgCl_2$, 150mM KCl, 5mM dithiothreitol, pH 8.3), 2µl deoxynucleotide mixture (dATP, dCTP, dGTP, dTTP 10mM each in Tris-HCl, pH 7.5), 2µg total RNA, 20U AMV reverse transcriptase and PCR grade water to 20µl. The reagents were well mixed and incubated at 55C° for 60 min, followed by 10 min at 65°C to inactivate the enzyme.

3.2.12.1.2 Purification of cDNA

The PCR product was purified using the High Pure PCR Product Purification kit, as follows: 100µl binding buffer was added to the 20µl first–strand cDNA reaction and mixed well. The sample was transferred to the high pure filter column and centrifuged for 30s at 13,000*xg*. 500µl wash buffer was added to the column and centrifuged for 30s at 13,000*xg*. Another 200µl wash buffer was added and centrifuged at 13,000*xg* for 30s. 50µl elution buffer was then applied and the column centrifuged at 13,000xg for 30s. The eluted product was stored on ice.

3.2.12.1.3 Tailing reaction

A known sequence was added to the 3`end of the first-strand cDNA using terminal transferase and ATP. 19µl of purified cDNA sample was mixed with 2.5µl 10x reaction buffer and 2.5µl dATP (2mM dATP in Tris-HCl, pH 7.5) and incubated at 94°C for 3 min, then chilled on ice. 10U terminal transferase was added to the mixture, and incubated at 37°C for 30 min. after the reaction was heat inactivated at 70°C for 10 min then kept on ice.

3.2.12.1.4 PCR amplification of the dA-tailed cDNA

The tailed cDNA was amplified by PCR using the GSP2 and dT-anchor primers in a 50µl reaction volume comprising: 7µl dA-tailed cDNA, 37.5µM oligo dT-anchor primer, 12.5µM GSP2 primer, 1µl deoxynucleotide mixture, 2.5U AmpliTaq® DNA polymerase, 5µl 10x reaction buffer and PCR grade water to 50µl. The PCR used the following cycle conditions: 94C° for 2 min, 35 cycles of 94C° for 15s, 63°C for 30s and 72C° for 1 min, with final elongation of 72°C for 7 min. 20µl of the PCR product was analysed on an 1% ethidium bromide-stained agarose gel with a corresponding DNA molecular weight ladder.

3.2.12.2 3`RACE amplification

3.2.12.2.1 First-strand cDNA synthesis

Synthesis of the first-strand cDNA was performed by using the oligo dT-anchor primer and reverse transcriptase enzyme. The reaction was performed in 20µl total volume comprising: 37.5µM oligo dT-anchor primer, 4µl 5x cDNA synthesis buffer (250mM Tris-HCl, 40mM $MgCl_2$, 150mM KCl, 5mM dithiothreitol, pH 8.3), 2µl deoxynucleotide mixture (dATP, dCTP, dGTP, dTTP, 10mM each in Tris-HCl, pH 7.5), 2µg total RNA, 20U AMV reverse transcriptase and PCR grade water to 20µl. The reagents were well mixed and incubated at 55°C for 60 min, followed by 10 min at 65°C to inactivate the enzyme.

3.2.12.2.2 PCR amplification of the cDNA

Amplification of the cDNA was conducted using the gene specific primer, GSP5, and PCR anchor primers. The PCR was performed in 50µl comprising: 1 µl cDNA product, 12.5µM each of PCR anchor primer and GSP5 primer, 1µl deoxynucleotide mixture (10mM each), 2.5U AmpliTaq® DNA polymerase, 5µl 10x reaction buffer and PCR grade water to 50µl. The PCR was performed using the following cycle conditions: 35 cycles of 15s at 94°C, 30s at 61°C, 1min at 72°C, preceded by initial denaturation at 94°C for 2 min, and finished by holding at 72°C for 7min. 20µl of the PCR product was analysed on an 1% ethidium bromide stained agarose gel with a corresponding DNA molecular weight ladder.

3.2.12.3 Purification of the 5` and 3` RACE products

Products from the amplification of the 5`RACE and 3`RACE were run on a gel and the corresponding (single) bands excised and purified using a MinElute gel extraction kit as follows: each excised cDNA fragment was incubated with 3 volumes of buffer QG at 50°C for 10min (until the gel slice was completely dissolved). One volume of isopropanol was added, and the sample mixed by inverting several times. The sample was then applied to a MinElute column and centrifuged at 10,000*xg* for 1 min. 500µl of buffer QG was added to the column and centrifuged for 1 min, then 750µl of buffer PE was added and the column allowed to stand for 5 min before centrifuging for 1 min. Finally the purified cDNA was eluted with 10µl buffer EB (10mM Tris-HCl, pH 8.5).

3.2.12.4 Cloning and sequencing of the RACE products

To facilitate sequencing, purified 5` and 3`RACE products were cloned into the pCR 2.1vector using TA cloning® kit, as follows:

3.2.12.4.1 Preparation for the cloning reaction

Fresh RACE product, 4µl, was mixed with 1µl salt solution (1.2M NaCl, 0.06M $MgCl_2$) and then 1µl vector added. The mixture was mixed gently and incubated for 5 min at room temperature, then placed on ice until needed.

3.2.12.4.2 Chemical transformation of One Shot®TOPO10 competent cells

Before starting the transformation, 40µl of 40mg/ml X-gal was spread on each of several LB-kanamycin plates which were then incubated at 37°C until ready for use. One vial of One Shot ® TOPO 10 cells was thawed on ice. 2µl of the cloning reaction was added to the vial mixed gently and incubated 30 min on ice. After incubation, the cells were heat-shocked for 30s at 42°C, without shaking, and immediately transferred back to ice. 250µl of room temperature S.O.C medium were added to the vial, and the solution incubated at 37°C with 200 rpm shaking. After 1h incubation, 50µl and 100µl of the transformation was spread on separate, pre-warmed LB-kanamycin plates and incubated overnight at 37°C and then checked for growth of white colonies (containing the cloned target).

3.2.12.4.3 Analysis of positive colonies

One white colony was cultured overnight in LB medium containing 50µg/ml kanamycin incubated at 37°C with shaking. Plasmid DNA was then isolated using QIAprep Spin Miniprep kit, as follows: cells were pelletised at 5000*xg* for 10 min and then resuspended in 250µl resuspension buffer P1. 250µl lysis buffer P2 was added to the resuspended cells and mixed well by inverting the tube 4-6 times; the solution became viscous and slightly clear. Neutralization buffer N3, 350µl, was added to the tube and mixed by inverting the tube 4-6 times; the solution became cloudy. The tube was centrifuged at 18,000*xg* for 10 min. The supernatant was transferred to a QIAprep Spin column and centrifuged at 18,000*xg* for 1 min, before being washed twice with buffer PB: once with 500µl and the second time with 750µl with centrifugation at 18,000*xg* for 1 min each wash. The column was centrifuged for an additional 1 min to remove residual wash buffer. The plasmid DNA was then eluted with 50µl elution buffer EB, after being allowed to stand for 1 min then centrifuged at 18,000*xg* for 1 min. The plasmid DNA was sequenced by a commercial sequencing laboratory (Göttingen GmbH, Germany). BLASTn and BLASTx searches were conducted, respectively, to confirm whether the sequences were unique and to search for translation of the sequence and protein similarities (Altschul *et al.* 1990).

4. Results

4.1 Experimental production and collection of triactinomyxon spores

Three months after infecting SPF *T. tubifex* culture with *M. cerebralis* myxospores, waterborne actinospores (triactinomyxons) were released. These were filtered from the water, counted, purified with Percoll, and examined microscopically to check for quality (Fig. 1). The triactinomyxons had their characteristic anchor-shape, with three leg-like appendages and an elongated style containing three polar capsules and a sporoplasm containing ~64 spherical sporozoites.

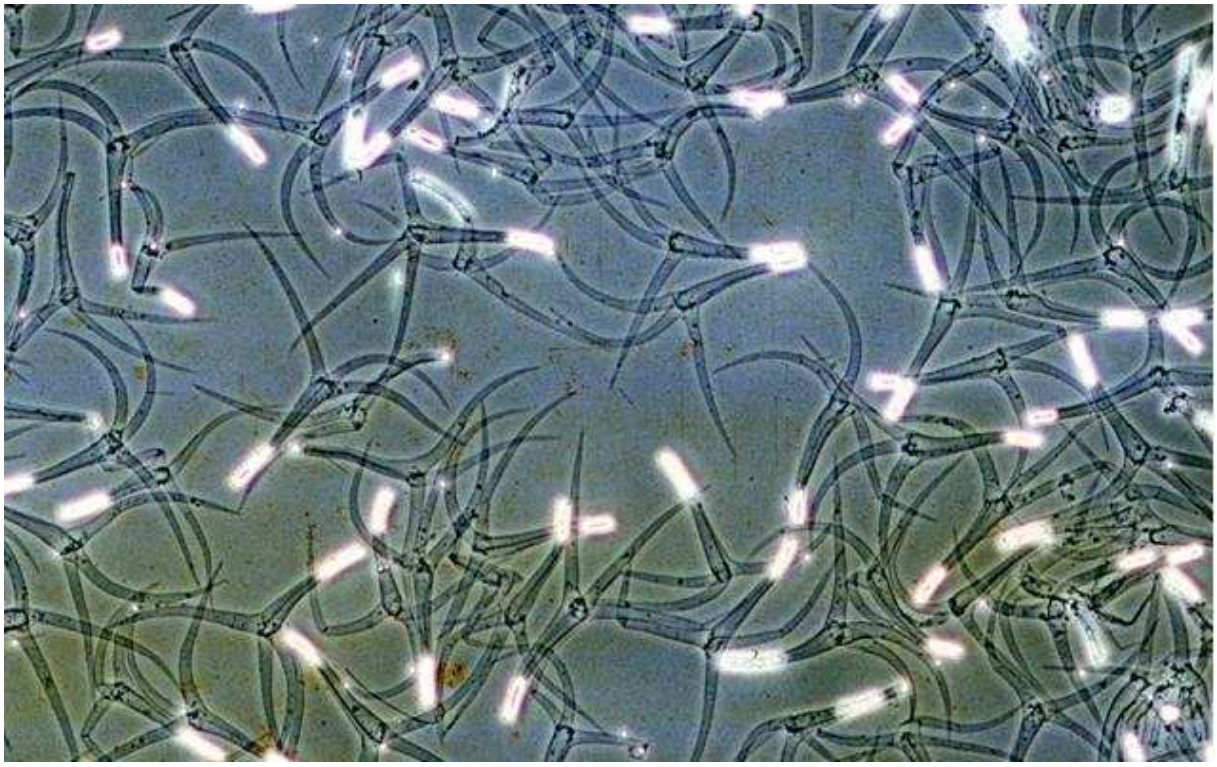


Figure 1: Mature waterborne triactinomyxon spores released from infected *T. tubifex*. Fresh unstained preparation, 250X

A portion of the collected spores was used for protein investigation using SDS-PAGE and a portion was preserved in RNA*later* for RNA isolation. Oligochaetes found to be high triactinomyxon producers, after being individually plated-out into cell well plates, were preserved in RNA*later* for RNA isolation.

4.2 SDS-PAGE and western blotting of triactinomyxons

SDS-PAGE and western blotting was conducted to demonstrate the specificity of the triactinomyxon antiserum and to determine the appropriate primary and secondary antibody dilution required for immunogenic screening of the cDNA library. Purified triactinomyxon spores and non-infected *T. tubifex* were analysed using SDS-PAGE. The *T.*

tubifex proteins were stained with the coomassie brilliant blue R 250 and produced bands with molecular weights ranging from 9-80 kDa (kilo Dalton) (Fig. 2).

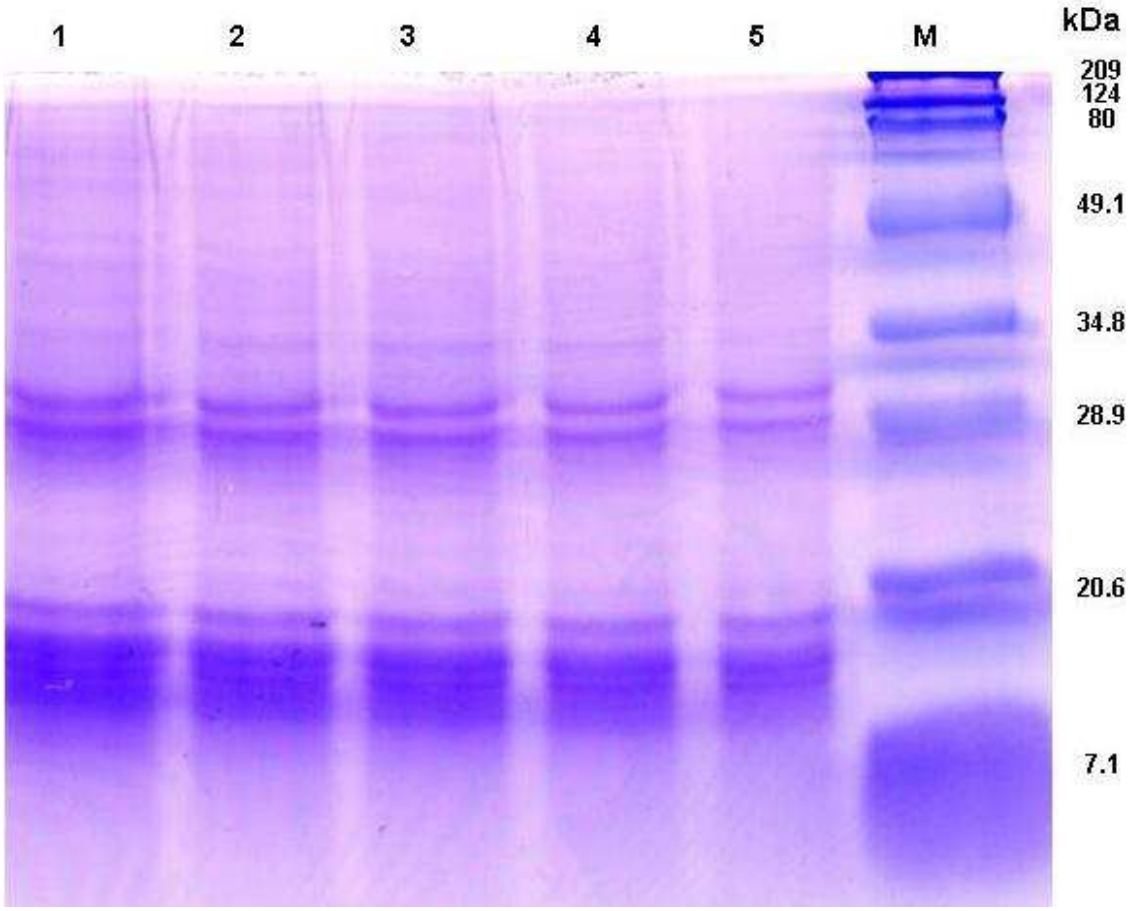


Figure 2: SDS-PAGE analysis of soluble proteins from non-infected *T. tubifex* stained with coomassie brilliant blue.

M = Prestained protein molecular weight standard (weights as marked). Lanes 1-5 = soluble worm proteins: 25, 20, 15, 10, 5µl.

When stained with coomassie blue, triactinomyxon spore proteins gave bands which were too faint, so silver stain was used. Many polypeptides with weights ranging from 8-175 kDa were detected (Fig. 3).

Western blot analysis was conducted against both *T. tubifex* and triactinomyxon spores, using different concentrations of primary antibodies, anti-triactinomyxon antiserum, and secondary antibodies, peroxidase-conjugated anti-rabbit IgGs. The primary antibodies (anti-triactinomyxon) detected only antigens of the triactinomyxon spores (Fig. 4). There was no reaction with the *T. tubifex* antigens or with the negative control. The optimal dilution of primary and secondary antibodies was 1:1000 and 1:2000 respectively. No reaction was observed between the rabbit control anti-serum and either spores or *T. tubifex* proteins.

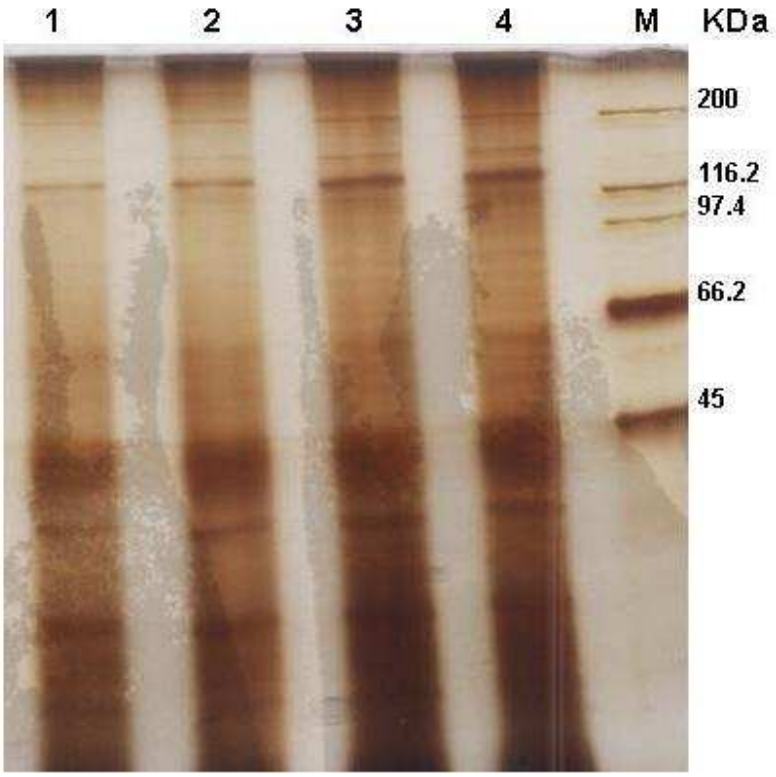


Figure 3: PAGE electropherogram of triactinomyxon soluble proteins stained with silver stain.
M = Protein molecular weight standard, KDa = kilo Dalton, Lanes 1-4 = 10, 15, 20, 25µl of spore proteins

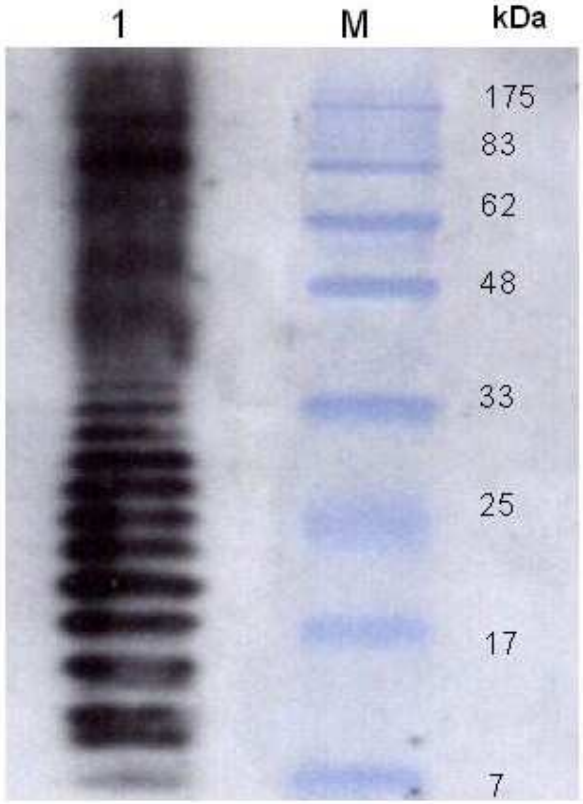


Figure 4: Detection of triactinomyxon spore antigens by western blot analysis using anti-triactinomyxon antiserum, and ECL detection reagents.
M = Prestained protein molecular weight standard, **1** = triactinomyxon protein bands

4.3 RNA from triactinomyxons, non-infected and highly-infected *T. tubifex*

Total RNA was extracted from all three sample types: triactinomyxon spores, infected worm and non-infected worm, and tested by running on 1.5% formaldehyde agarose gel: this yielded 2 bands indicating successful extraction RNA with its both 28S and 18S ribosomal RNA (Fig 5).

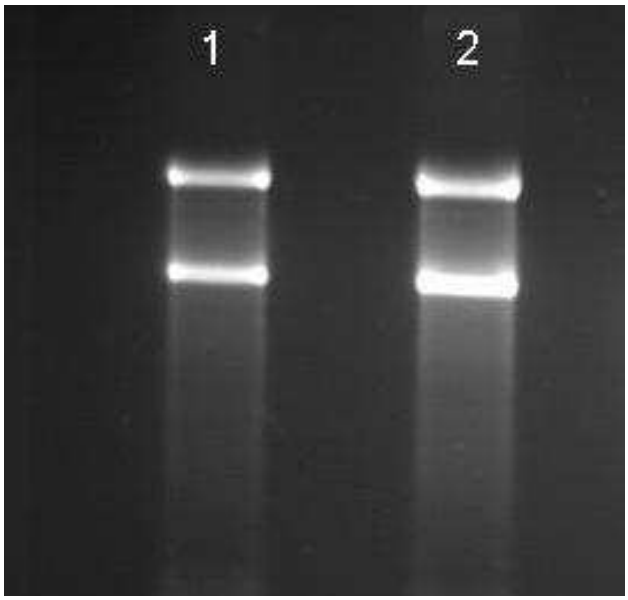


Figure 5: Total RNA extracted from triactinomyxon spores (1) and from non-infected *T. tubifex* (2) run on 1.5 % formaldehyde agarose gel.

4.4 Construction of the cDNA library

The Poly (A) mRNA, from highly triactinomyxon spores producer oligochaetes, was extracted from the total RNA measured to have a concentration of 0.179µg/µl. This was converted into double-stranded cDNA and, following ligation of an adapter and the release of an Xho I restriction site, was ligated to the *EcoRI* /Xho I cut phosphatase-treated ZAP Express vector. The resulting DNA was packaged *in vitro* using Gigapack III gold extracts and introduced into *E. coli* XL1-blue- MRF` strain. The titre of the primary library was $0.5x10^6$ and its quality estimated by performing a blue/white colour selection using IPTG (Isopropyl-ß-D-thiogalactopyranosid) and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). 97% of the library ($4.85x10^5$) produced recombinant white plaques.

4.5 Immunological screening for the triactinomyxon positive plaques

The library was screened with anti-triactinomyxon antibody, diluted 1:1000 in blocking buffer, to select only the recombinant plaques of triactinomyxon spores and not

the plaques from oligochaete worms. Using horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 in blocking buffer, positive plaques were detected by luminol-based enhanced chemiluminescence.

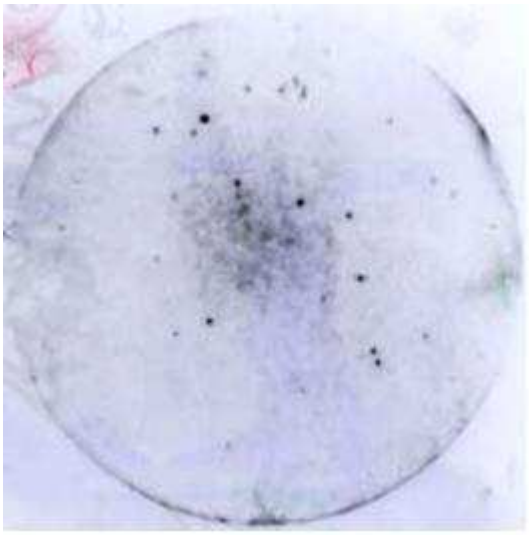


Figure 6: ECL-Hyperfilm showing primary screening for triactinomyxon positive plaques visible as black spots on the plate.

Primary screening of the library revealed only a few positives plaques out of the 1000 plaques on the plate (Fig. 6). This low primary yield was due to the high number of oligochaete plaques present. Each of the positive plaques was isolated and subjected to secondary and tertiary screening. Significantly higher yields were obtained in the secondary screening, following propagation of positive clones (Fig 7).

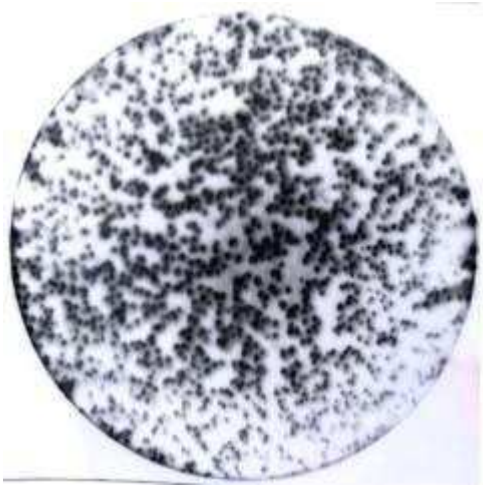


Figure 7: ECL-Hyperfilm showing the result of secondary screening.

For tertiary screening, a phage plug was removed and diluted into SM buffer before being plated at a low titre of 20–50 particles/plate which allowed single plaques to be

isolated (Fig. 8). Each positive clone was screened at least three times to avoid false positive results and contamination with negative plaques.

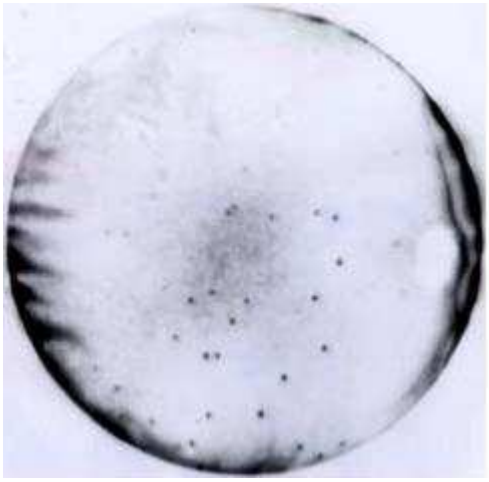


Figure 8: ECL-Hyperfilm showing the result of tertiary screening.

4.6 Purification of clones of interest and characterisation of the library inserts

More than 526 positive plaques from triactinomyxon spores were detected by immunological screening with anti-triactinomyxon antibodies. A subset of these plaques was then tested with PCR using the T3 and T7 primers, which flank the multiple cloning site of the vector, to confirm that these plaques had an insert. The PCR revealed that positive clones contained inserts with different molecular weights (Fig. 9), indicating successfully construction of the library.

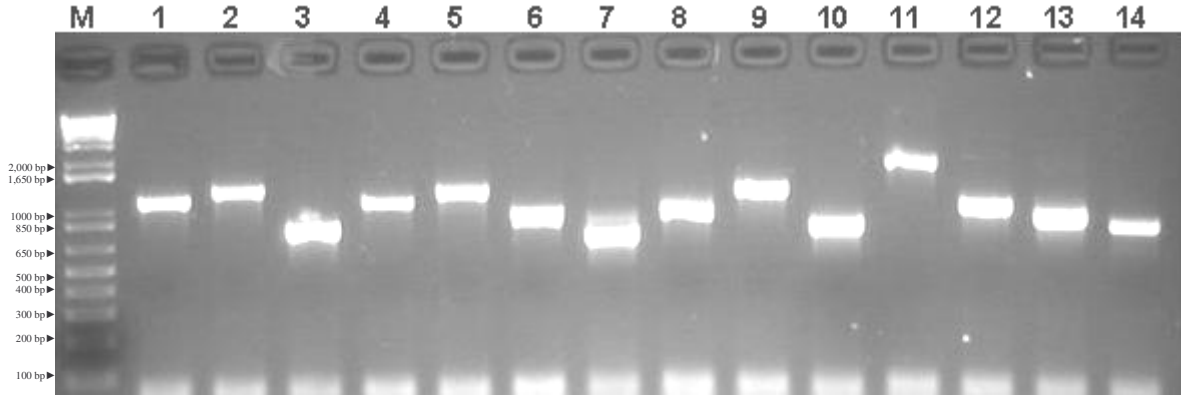


Figure 9: Gel showing results of PCR of random samples of positive plaques.

M = 1kb DNA ladder, **Lanes 1-14** = positive clones.

4.7 In vivo excision, plasmid isolation and size estimation of the selected clone

After screening the cDNA library, one clone (46-5) was randomly selected and subjected to further study. The clone's insert was excised and sub-cloned into a plasmid vector: ExAssist helper phage with XLOLR strain efficiently excised the pBK-CMV

phagemid vector from the ZAP Express vector. Colonies were grown that contained the pBK-CMV vector with the cloned DNA insert and one of these was selected, grown up and subjected to plasmid isolation and PCR amplification to estimate the size of the cDNA insert. As illustrated (Fig. 10), the PCR amplified a 750bp segment representing the cDNA insert of clone 46-5. This fragment was then sequenced.

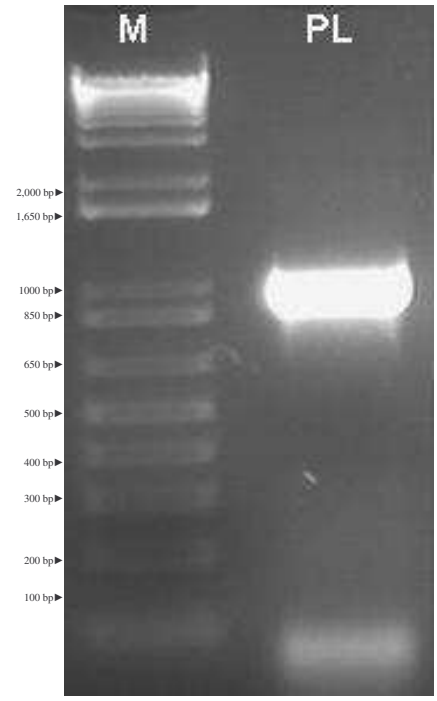


Figure 10: Gel showing amplified cDNA insert fragment.

M = 1kb DNA ladder, **PL** = cDNA

4.8 Synthesis of cDNA from triactinomyxon spores and non-infected oligochaetes

cDNA from both of non-infected *T. tubifex* and triactinomyxon spores was synthesised from the total RNA. After the first strand had been synthesised it was subjected to long distance PCR amplification (LD PCR) to obtain double-stranded cDNA. I determined 23 cycles to be the optimal number for LD PCR amplification (Fig. 11); after 24 cycles no additional PCR product was generated, and the optimal cycle number is typically regarded as one cycle fewer than this to ensure that the double-stranded cDNA (ds cDNA) remained in the exponential phase of amplification.

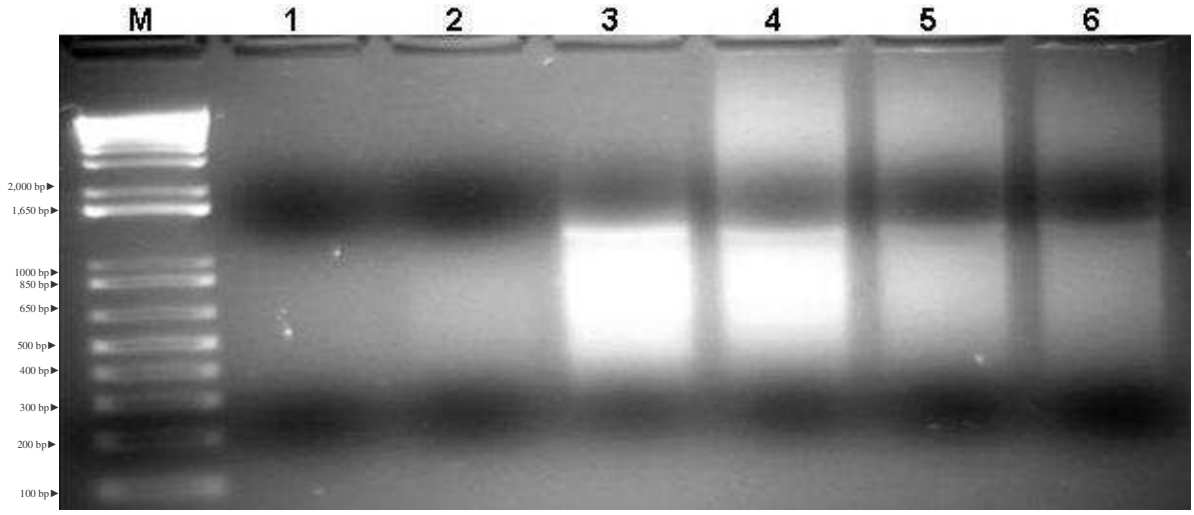


Figure 11: Gel showing effect of varying PCR cycles number; the double-stranded cDNA appears as a moderately strong smear from 500-6000bp.

M = 1kb DNA ladder, **1** = 15 cycles, **2** = 18, **3** = 21, **4** = 24, **5** = 27, **6** = 30

4.9 Determination of the specificity of clone 46-5 to triactinomyxon spores

After *in vivo* excision, plasmid isolation and sequencing of clone 46-5, its specificity to triactinomyxon spores was tested by PCR using the forward and reverse primers constructed from its sequence. The primer pair amplified 511bp of cDNA from triactinomyxon spores; there was no amplification of non-infected oligochaetes or the negative control (Fig. 12). This confirmed that clone 46-5 was specific to triactinomyxon spores.

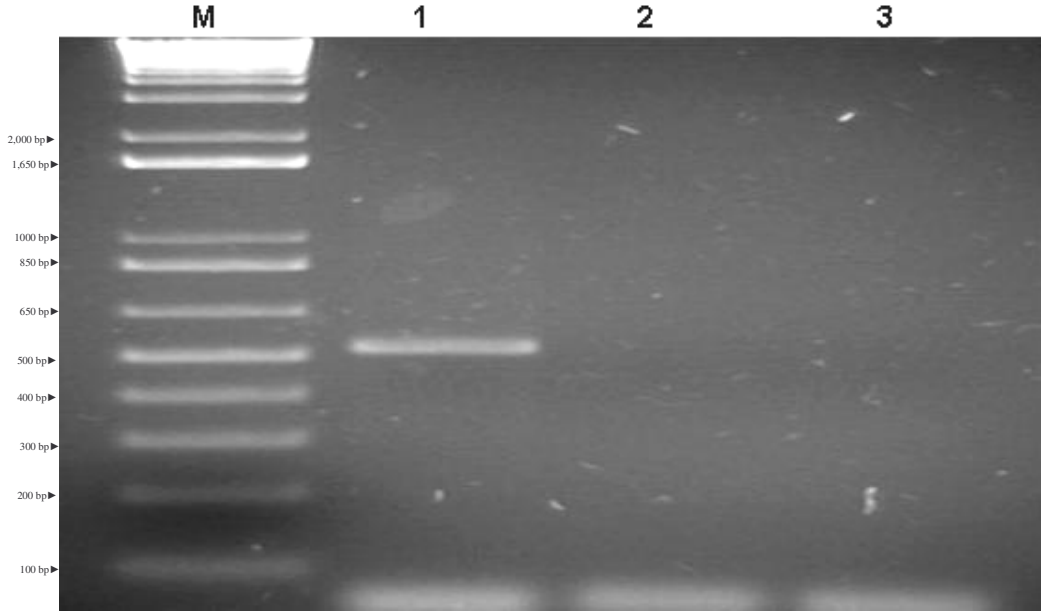


Figure 12: Gel showing that the clone is specific to triactinomyxon spores.

M = 1kb DNA ladder, 1 = cDNA from spores, 2 = cDNA from oligochaete, 3 = negative control

4.10 RACE amplification of the cDNA ends

Rapid amplification of the 5`and 3` cDNA ends (RACE) was used for obtaining a full-length cDNA from clone 46-5. Gene specific primer 1 (GSP1) was used for first-strand synthesis of the 5`RACE from the total RNA. After addition of a homo-polymeric A-tail to the cDNA, it was subjected for PCR amplification using the GSP2 primer, resulting in a 750bp fragment (Fig. 13, lane 2). The 3`RACE was conducted also, using oligo dT-anchor primer for first-strand synthesis followed by PCR amplification using primer GSP5, resulting in a 220bp fragment (Fig 13, lane 1). These fragments, 750bp & 220bp, were excised from the gel, purified, cloned in the pCR2.1 vector and transformed into competent cells; plasmid DNA was isolated from the resulting white colonies and sequenced.

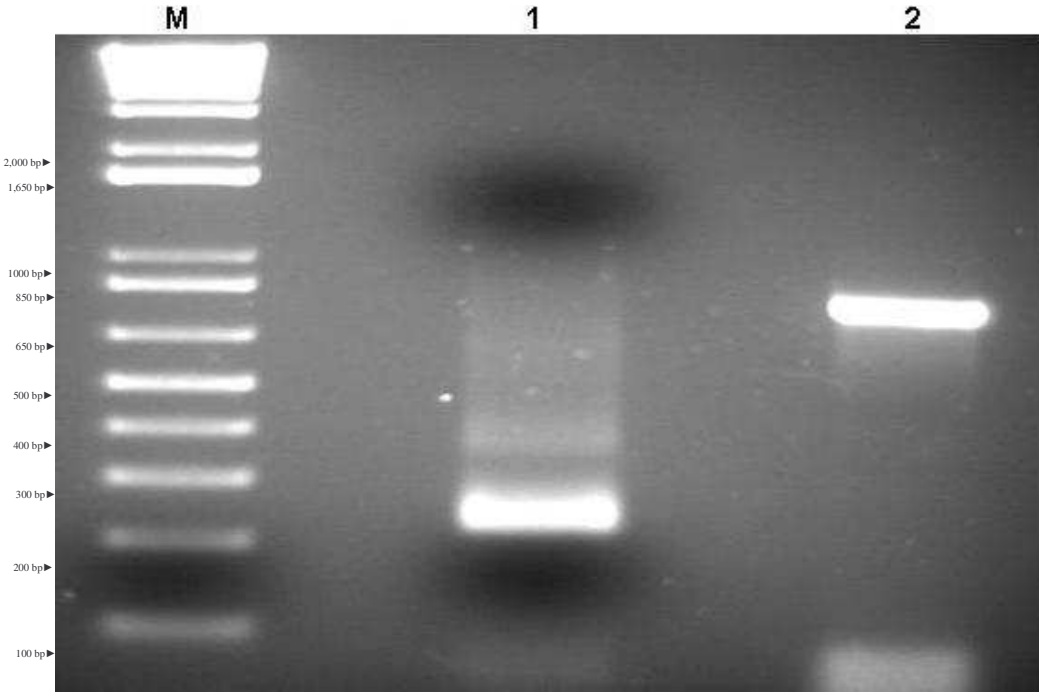


Figure 13: Gel showing RACE amplification products.

M = 1kb DNA ladder, 1 = 3`RACE, 2 = 5`RACE.

4.11 Sequence analysis of the full-length cDNA clone 46-5

The complete sequence of the clone 46-5 is 1,483bp long (Fig. 14). A nucleotide-nucleotide BLASTn search demonstrated a high percent similarity of the sequence with ribosomal protein L23a and 60S ribosomal protein L23a. GENSCAN software was used to predict the peptide sequence and coding sequences, if present. The program detected a coding sequences from 800-1375bp (Fig. 14, underlined) and predicted its peptide sequence (Fig. 15).

```
CTTCTTTGGCAGCAGGAGCAGCAGCGTTGGAGCAGTTAGGCCATtACTTTGACAAGGAAGCCTATTTTGGTGTTTGGTGC   80
ACCGCCACTGCTGCAGCGCGGCACGGCACCANTGCGGTGGGCGACTCGGTGCGAGTTACGCGTTCGTGGAAGCAAAGTAT  160
GCTCGAATTCAAAAAGCAATTGTCACGCAAATAACCGTTATATGTTATTAAATCGTTGAATTATCTAGCCTCGTACAGCT  240
CATCCTCGTCACTGCTGTCGTATGATTCGGGTTCGGATTTCGGCGGGGCCTTGGCCGCTTTACGTTTCTTGAGCTTGGGC  320
TCGACTTGACTAATCAAGTTCTCAATCAGATTCAACTGCGACTGCAAAGACCGCTGCATCACCTTCATGAGCCTCGTCAG  400
CATCGTGTGGTCCGCCACTGCGCTGGTGGTGGTGGTAGTGGTTGTCGTTGTGGTTGCTGAGCTCATTTTCGCCTTCTTGG  480
AAGAAGAAGGAGTCCCCGGCAAAGCCATGACCGTTGTGCCAGGTCCTGTGCCCGTCGCCGTTGGCTCGCGCTTCGCATCG  560
GGTTCTCTTTTAGCCTTCTGCTTCTTCTGAGCATCCAAAGACGACCCCTGGTTCACCTTGAGCTGGAAAATAATATCATC  640
CGCTACCTCCTCGTCCTCCTCGTCTCCACTATCATCGTTGCTACTCTTTCTCTTGCTGCTCTTGTCTATGTTGTTGTTGT  720
TGTTGCTGTTGTTGCTGTTGTTGTTGTTGTTGTTTTTGTTGCTAATGAACTTACCGCTACTGTTTCTATAGCTGCTC    800
TTGTCTTTGTTGCTAATGTTGCTGGTCTTGCTGCTGCTCCTGCTGCCAAAGAAGCAGCTAAACCAAAAGAGGCTCCAAAA  880
CCAAAGGAGGCACCTAAGCCTAAGGAAGCCCCGAAACCCAAGGAGGCTGCCAAGAGCAAGGATGTTGCGAAGAAGCCCGC  960
TGTTCCTAAGCCCGTTAAGACGAAGGGTGCTGAGGGTGGAAAGAAGGCACCCAGCGGTGTGCAGGCCAAGAACAAGGATA  1040
AGGCATTGAAGGCAAAGAAGGCCGTTCTGCGTGGTGTCCACGACAAGAGAAATCGCAAGATGCGGACTGCTGTTCATTTC  1120
CGTCGTCCACAGACGCTGCGACTGCCACGCACGCCTAAATATCCCCGCAAAAGCACACCAAAGCGGGTGAGGTTGGACCA  1200
GTTCAAGATCATCAAGTTCCCGCTGACGACGGAGTCTGCGATGAAGAAGATCGAGGACAACAACACGCTCGTGTTCATCG  1280
TTGACAAGCGCGCCAACAAGCCTCAGATCAAGATGGCCGTCACGAAGCTGTACAACATCCAGGTCTCCAAGGTCAACACG  1360
CTCATCAGCAATTAACAAATGCCATGTACTTTGTTTGATTGATAATTAAAAATAACGCTGAAATTTTATTCTTTTAGTTG  1440
TTTTAAAAAAAAAAAAAAAAAAGTCGACATCGATACGCGTGGTC                                       1483
```

Figure 14: Nucleotide sequence of clone 46-5.

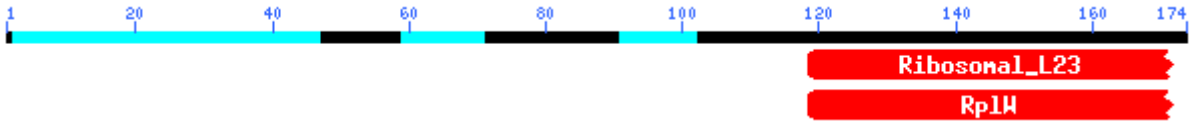
Numbers on the right denote base positions. The underlined sequence is the coding sequence (CD) detected with GENESCAN program.

```
XAAKPKEAPKPKEAPKPKEAPKPKEAAKSKDVAKKPAVPKPVKTKGAEGGKKAPSG                56
VQAKNKDKALKAKKAVLRGVHDKRNRKMRTAVHFRRPQTLRLPRTPKYPRKSTPK                111
RVRLDQFKIIKFPLTTESAMKKIEDNNTLVFIVDKRANKPQIKMAVTKLYNIQVSKVN              169
TLISN                                                                    174
```

Figure 15: GENSCAN-predicted peptide sequence of the conserved domain from the coding sequence 800-1375bp. Numbers on the right denote peptide positions.

A BLASTn search was conducted of the coding sequence and gave the same result as the search using the whole sequence. The predicted peptide sequence was subjected to a BLASTp protein-protein search which detected a putative conserved domain to ribosomal protein L23. This putative conserved domain was detected after alignment of the peptide sequence with the protein families' database of alignments and hidden Markov models

(pfam) which produce 60 % alignment with the CD of the ribosomal protein L23 family (Fig 16).



Ribosomal_L23, Ribosomal protein L23 (60% alignment)

```
Query:  118  FKIIKFPLTTESAMKKIEDNNTLVFIVDKRANKPQIKMAVTKLYNIQVSKVNTLI  172
Sbjct:    1  TDIIKYPIITEKTMNLLEKPNKYVFIVDKKANKTEIKDAVEHIFGVKVESVNTLI   55
```

Ribosomal protein L23 [Translation, ribosomal structure & biogenesis] 60% alignment:

```
Query:  118  FKIIKFPLTTESAMKKIEDNNTLVFIVDKRANKPQIKMAVTKLYNIQVSKVNTLI  172
Subject:  2  YDVIKSPVVTEKAMLLMEKENKYVFIVDPDATKPEIKAAVEELFGVKVEKVNTLN   55
```

Figure 16: **Putative conserved domain as detected by BLASTp search, with protein CD alignment with the pfam.**

The BLASTp search using the peptide sequence revealed alignments with a high percent similarity (60-85%) to ribosomal protein L23 and 60S ribosomal proteins of different organisms. These results strongly suggest that the triactinomyxon sequence 46-5, codes for a functional protein belonging to the same family as ribosomal protein L23.

5. Discussion

Whirling disease, caused by the myxozoan parasite *M. cerebralis*, has contributed to severe population declines in both wild and farmed salmonid fish (Baldwin *et al.* 2000, Hedrick *et al.* 1998). It has also been documented as a predominant cause of recruitment failures and subsequent rainbow trout population decline in North America (Nehring & Walker 1996, Thompson *et al.* 2002). The parasite has a complex lifecycle involving alternating spore stages: a *Myxobolus* spore stage developing in salmonids and a triactinomyxon spore stage developing in *Tubifex tubifex* oligochaete worms. This study involved construction of a cDNA library from triactinomyxon spores, which can be searched for genes expressed in the triactinomyxon spore stage.

Triactinomyxon spores were collected and purified with Percoll and then denatured by sonication in a buffer containing Mercaptoethanol and SDS; both of which aid denaturation of the proteins into discrete polypeptide subunits (Sambrook *et al.* 1989). These subunits were then electrophoresed, transferred to nitrocellulose membranes and analysed by Western Blotting which produced strong signals with the triactinomyxon proteins.

For the library to be successfully screened, it is essential to use high quality polyclonal antibodies to recognise the denatured proteins (Sambrook & Russell 2001). The SDS-PAGE and western blotting experiments were preliminary steps for establishing the bases; specificity, sensitivity, quality and the concentration of antibodies for optimal screening of the library. The antibody used in this experiment was polyclonal antiserum which reacts with many different epitopes and thereby facilitates detection of cDNA clones which express protein fragments of interest (Sambrook & Russell 2001). A Western blotting experiment confirmed that the chosen antibody efficiently recognised triactinomyxon proteins with no reaction to host oligochaete proteins. Western blotting was also used to determine the optimal antibody concentration for screening: a dilution of 1:1000 for anti-triactinomyxon primary antibodies and 1:2000 for secondary antibodies. Using ECL detection reagent we could detect less than 1pg of triactinomyxon antigen (Soliman *et al.* 2003), which indicates the sensitivity of the immunogenic screening of the constructed cDNA library with polyclonal antibodies.

The quality and quantity of the mRNA is of fundamental importance to the construction of a large, representative cDNA library, because the library can't be better than the mRNA from which it is derived. Also, the higher the concentration of sequences

of interest in the starting mRNA, the easier the task of isolating relevant cDNA clones (Sambrook & Russell 2001). It is important, therefore, to stabilise the RNA in tissue immediately after harvesting, and avoid changes in subsequent gene-expression patterns due to degradation of the RNA. RNA*later* was deemed a suitable stabilising agent (QIAGEN RNeasy mini kit manual 2001). Total RNA was extracted from the highly triactinomyxon spores producer worms (about 120 days post exposure to *M. cerebralis* spores)) to increase the chance of obtaining cDNA clones from nearly all developmental stages of the parasite in the oligochaetes.

An essential step when preparing the mRNA was to separate it from the transfer RNA (tRNA) and ribosomal RNA (rRNA) fractions of total RNA. The amount of rRNA and tRNA is vastly greater than mRNA and will decrease the efficiency of the reverse transcription reaction; which is also why cDNA is constructed from mRNA not from total RNA. Fortunately, the bulk of mRNA carries strings of poly-As at their 3` termini, and can be separated from total RNA on an oligo (dT) cellulose column (Aviv & Leder 1972; Chomczyniski & Sacchi1987). Following purification, mRNA was primed in the first-strand synthesis with the hybrid oligo (dT) linker primer, and transcribed using Strata™ Script reverse transcriptase and 5-methyl dCTP. The oligo (dT) linker primer "5`-GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTT TTTTTTTTTTTTT-3`" was designed with a "GAGA" sequence to protect the *Xho*I restriction enzyme recognition site "**CTCGAG"** and 18-base poly (dT) sequence. The restriction site allows the finished cDNA to be inserted into the ZAP Express vector in a sense orientation (*Eco*R I- *Xho* I) with respect to the *lac Z* promoter (Short *et al.* 1988); while the 18-base poly (dT) region binds to the 3`poly (A) region of the mRNA template. The Strata™ Script enzyme is a genetically engineered Moloney Murine leukaemia virus reverse transcriptase without any detectable RNase H activity. The total yield of first-strand cDNA is substantially higher with this enzyme than with non-engineered reverse transcriptase and the proportion of full-length cDNAs is significantly greater (Kotewicz *et al.* 1988; Gerard & D'Alessio 1993; Telesnitsky & Goff 1993).

Many procedures required that the double–stranded cDNA be digested with restriction enzyme(s) before cloning, yet this step can be risky because the cDNA of interest itself may be cleaved into two or more fragments which become separated during the cloning process. To protect cDNA from digestion, 5-methyl dCTP was used during the first-strand synthesis; i.e. the nucleotide mixture for the first-strand synthesis contained normal dATP, dGTP, dTTP plus the analogue 5-methyl dCTP. The completes first–strands

then have a methyl group on each cytosine base which protects the cDNA from restriction enzymes used in subsequent cloning steps (Han & Rutter 1988; Huse & Hansen 1988). Only the unmethylated site within the linker-primer was cleaved by *Xho* I.

The product of the first-strand synthesis, a cDNA-mRNA hybrid, was used for second-strand synthesis. RNase H produced nicks and gaps in the mRNA strand of the hybrid, creating a multitude of fragments which serve as primers that are used by DNA polymerase I during the synthesis of the second cDNA strand (Okayama & Berg 1982; Gubler & Hoffman 1983). The second strand nucleotide mixture was supplemented with additional dCTP to reduce the probability of residual 5-methyl dCTP becoming incorporated in the second strand, ensuring that the restriction sites in the linker-primer will be susceptible to future restriction enzyme digestion. Uneven termini of the double–stranded cDNA were 'nibbled' or filled in with cloned ***Pfu*** DNA polymerase to create a blunted ends for *Eco*R I adapters' ligation. The adapters composed of 10-mer "3`-GCCGTGCTCC**p**-5`" and 14-mer oligonucleotides "5`-**OH**-AATTCGGC ACGAGG-3`"which are complementary to each other with an *Eco*R I cohesive end (AATTC). The 10-mer oligonucleotide was phosphorylated (**p**) which allowed it to ligate to blunt termini of cDNA and other adapters; while the 14-mer oligonucleotides was kept dephosphorylated (**OH**) to prevent ligation to other cohesive ends.

After ligation of the adapters and inactivation of the ligase enzyme, the 14-mer oligonucleotide was phosphorylated to enable its ligation to dephosphorylated vector arms. The ligation reaction was carried out in a small volume to maintain high concentration of adapters in order to minimise blunt-end ligation of the cDNA (Sambrook & Russell 2001). *Xho* I digestion released the *Eco*R I adapter and residual linker-primer from the 3`end of the cDNA and thereby prepared it for insertion into the vector. This strategy greatly improved the efficiency of the ligation step in cDNA cloning and eliminated the need to digest the cDNA with restriction enzyme before insertion into the vector (Yang *et al.* 1986; Elledge *et al.* 1991). Before insertion, the cDNA was fractionated by gel filtration to remove unused adapters and residual linker-primer created by the *Xho* I enzyme digestion. This process significantly increases the number of recombinants that contain cDNA (Sambrook & Russell 2001). Fractionation also enabled fragments less than 400bp to be discarded; these were unwanted products of incomplete first-and/or second-strand synthesis. Discarding small fragments also reduces the number of recombinants that must be screened, and increases the chance of isolating full-length cDNAs corresponding to rare mRNAs encoding large proteins (Sambrook& Russell 2001).

The size fractionated cDNA was next ligated to the ZAP Express vector which increases both cloning capacity and the number of unique lambda cloning sites (Jerpseth *et al.* 1992). Bacteriophage lambda was used as the ZAP Express vector as it is highly efficient at becoming infected by phage particles packaged *in vitro* (Promega technical bulletin 2001). Inserts cloned into the vector can be rapidly excised out of the phage in the form of the kanamycin-resistant pBK-CMV phagemid vector allowing insert characterization in a plasmid system (Short & Sorge 1992; Alting-Mees *et al.* 1992; Short et at. 1988). In addition, clones in the vector can be screened with either DNA probes or antibody probes.

The lambda library was then packaged *in vitro* by adding the recombinant lambda DNA to an *E. coli* extract containing assembly proteins and precursors required for encapsulating lambda DNA to produce infectious recombinant lambda phage (Becker *et al.* 1977). I used the highly efficient Gigapack III Gold packaging system (Kretz *et al.* 1994). The library was plated using an *E. coli* host strain, XL1-Blue MRF`, deficient in the mcr system (modified cytosine restriction) which normally cleaves DNA at methylcytosine residues (Raleigh *et al.* 1988; Woodcock *et al.* 1989). XL1-Blue MRF` was also selected as it has F` episome which serves three purposes. First, is required for the ß-galactosidase-based non-recombinant selection strategy through the *lac Z* gene present in the F` episome; when cDNA is present in polylinker, expression from the *lac Z* gene is disrupted and white plaques are produced. Without an insert the amino terminus of ß-galactosidase is expressed and hence non-recombinants appear as blue plaques. Second, the F` episome is required for *in vivo* excision of the pBK-CMV phagemid vector from the ZAP Express vector, as the F` episome expresses genes that create the F` pili found on the surface of bacteria, without which the filamentous phage infection could not occur for the *in vivo* excision. Third, the F` episome contains the *lac* repressor (*lacI*^{q} gene), which blocks transcription from the *lac Z* promoter in the absence of the inducer (IPTG) (ZAP Express cDNA synthesis kit manual 2000). Propagation medium contained 0.2% maltose to promote substantial induction of maltose operon including the lamb gene, which encodes the cell surface receptor to which bacteriophage lambda binds (Schwartz 1967).

The primary library titre was 0.5 x 10^6 with 97% recombinant plaques; a result comparable with Stürzenbaum *et al.* (2003) who constructed an earthworm cDNA library using the same protocol, and achieved a titre of 0.5 x 10^6 recombinant plaques. Similarly, a cDNA expression library constructed for sheep louse *Bovicola ovis* had a primary titre of 5 x 10^5, with 97% recombinant plaques (Nattrass & James 2001).

After construction, cDNA libraries are typically amplified to provide a near limitless source of cDNA clones, this was not done for the triactinomyxon cDNA library in order to preserve accurate representation of the mRNA in the cDNA library (Sambrook & Russell 2001). As the library was constructed from mRNA extracted from infected worms, it contains clones of both triactinomyxon spores and the host. Even a single round of amplification could distort the representation of cDNA clones through variations in growth rate of recombinants skewing the proportions of cDNA in the library. After titrating, the library was subjected to immunological screening using the polyclonal anti-triactinomyxon antibody at a dilution of 1:1000, as determined by western blotting, and 1:2000 secondary antibodies, conjugated anti-rabbit antibodies peroxidase. Chemiluminescence detection method was used to select 526 positive plaques which contained the cloned triactinomyxon mRNA. These plaques were screened three times to screen out any contamination by negative plaques. In the primary screening, the antiserum selected only a few positive plaques as the proportion of triactinomyxon plaques was originally low compared with oligochaete plaques. In the second screening, after selective propagation of plaques from round one, the number of positive plaques was much higher. In tertiary screening the cultures were diluted to reduce the number of plaques on the plate to allow easy selection of only single plaques. Positive clones were tested by PCR, which showed that these clones contained inserts with different molecular weights, eliminating the probability of selecting a false positive plaque that has no insert and indicating the successful construction of the library.

To test whether the positive plaques were expressing genes specific to triactinomyxon spores, one plaque was selected at random for further investigation. Plaque designated 46-5 was chosen from the 526 positives, and subjected to *in vivo* excision of the insert, which was then sub-cloned into a plasmid vector in one step (Jerpseth *et al.* 1992; Short *et al.* 1988). The sample DNA was inserted into the lambda phage genome in the presence of filamentous bacteriophage-derived proteins (ExAssist helper phage proteins) by simultaneously infecting a strain XL1-Blue MRF` of *E. coli* with both the lambda vector and ExAssist helper phage (Short *et al.* 1988). Within the XL1-Blue MRF` strain, the ExAssist helper proteins recognised the initiator DNA at the lambda vector and produced a ‘nick’ in the strand. DNA synthesis began at the nick and duplicated the lambda vector ”downstream” (3`) of the nicked site. Synthesis of new single-strand DNA (ssDNA) continued along the cloned insert until it encountered the termination signal within the lambda vector. The ssDNA was then circularised by the gene II product from

the ExAssist helper phage, forming a molecule which contained: the DNA that was between the initiator nick and terminator signal, the pBK-CMV phagemid vector and the target insert. This process is considered a sub-cloning step because sequences associated with normal lambda vectors are located outside the region defined by the initiator and terminator signals, and hance are excluded from the circularised DNA; circularisation of the DNA also automatically recreates a functional f1 origin as found in f1 bacteriophage or phagemids (Short & Sorge 1992). 'Packaging' signals were linked to the f1 origin sequence to permit the circularised ssDNA to be packaged into phagemid particles and secreted from the XL1-Blue MRF` strain. The *E. coli* strain XLOLR was then infected with the phagemid and plated out on kanamycin-LB plates to give colonies which contained the sub-cloned DNA (Short *et al.* 1988). Co-infection with the helper phage was prevented because the ExAssist helper contains an amber mutation that prevents replication of its genome in a non-suppressing *E. coli* strain, such as XLOLR. This also means that single- stranded rescue cannot be performed with XLOLR using the ExAssist phage. XLOLR cells are also resistant to lambda infection, preventing lambda DNA contamination after excision (ZAP Express cDNA synthesis kit Manual 2001).

After *in vivo* excision the XLOLR plasmid DNA containing the insert was extracted and sequenced. Specific primers were designed to amplify only triactinomyxon cDNA and not DNA from the oligochaete host. To obtain the full length of the 46-5 cDNA, rapid amplification was used on the mRNA template between a defined internal site, using gene-specific primers constructed from the 46-5 clone, and unknown sequences at either the 3` or 5` end of the mRNA (Frohmann 1994). Sequencing revealed that 46-5 coded for ribosomal protein L23, and comparison with the database showed that sequence homology compared with different organisms ranged from 60-85%: 85% with *Oncorhynchus mykiss*, 82% with *Argopecten irradians* 78% with *Branchiostoma belcheri,* 73% with *Danio rerio*, 65% with *Homo sapiens*, and 60 % with *Spincia deracea.*

Ribosomes are large ribonucleoprotein complexes present in all living cells, which are responsible for manufacturing proteins translated from mRNA blueprints (Maguire & Zimmermann 2001). Ribosomes are divided into large- and small-subunits (Öhman *et al.* 2003); clone 46-5 coded for ribosomal protein L23 (denoted as L25 in some eukaryotes) which is a component of the large-subunit (Öhman *et al.* 2003). The function of L23 has been recently identified as the anchor point for the signal recognition particle, positioning the particle in such a way that it can read the signal sequence of the nascent polypeptide (Pool *et al.* 2002). Kramer *et al.* (2002) show that L23 can have an additional function to

provide a docking site for the Trigger Factor chaperone which assists the folding process of newly synthesised polypeptides. As ribosomal proteins are located near the opening of the exit tunnel it suggests a possible role in protein secretion, since such ribosomal proteins are in a position to associate with the translocon of the endoplasmic reticulum during protein secretion (Nissen *et al.* 2000). L23 ribosomal protein is essential for the growth of *E. coli* and the association of Trigger Factor with the ribosome; mutation of an exposed glutamate in L23 prevents Trigger Factor from interacting with ribosomes and nascent chains, and leads to protein aggregation and conditional lethality in cells which lack the protein-repair function of the DnaK chaperone (Kramer *et al.* 2002). Eukaryotic ribosomal subunits L23a and L35, which are close to the nascent-chain exit site, have been shown to comprise the ribosome attachment site for signal recognition particle 54, SPR54 (Pool *et al.* 2002). SPR54 is therefore strategically positioned to scan emerging polypeptides for the presence of a signal sequence (Willem *et al*. 2003). In *E. coli*, L23 (a homologue of L23a) seems to have an even more intricate role, functioning as an attachment site not only for the SPR (Gu *et al.* 2003; Ullers *et al.* 2003) but also for the chaperone and prolyl-isomerase trigger factor (Kramer *et al.* 2002).

A conserved domain to ribosomal protein L23 was detected during the alignment process of the triactinomyxon predicted peptide sequence in GENBANK (Marchler-Bauer *et al.* 2003) with nearly 60% homology with the L23 ribosomal protein in the protein families' database (pfam). Given the universal requirement for ribosome function, it is not surprising that the ribosomes of all organisms, both eukaryotic and prokaryotic, are highly similar. Accordingly, some ribosomal constituents have structural features that are highly conserved between species and across kingdoms (McIntosh & Bonham-Smith 2001). Further research would be required to investigate the specific function of triactinomyxon ribosomal protein L23.

In conclusion, triactinomyxon spores cDNA library was successfully constructed with titre of $0.5x10^6$ pfu; 97 % of the library was recombinant plaques. 526 Plaques were selected as positive by immunoscreening. One positive plaque was selected and subjected to further identification. The selected positive plaque was found to be coded for triactinomyxon ribosomal protein L23.

For further study, a short sequences run of one or both ends of the 526 positive clones can be done to generate an Expressed sequence Tags (EST) which provide the researchers with, a quick and inexpensive route for discovering new genes, obtaining data on gene regulation and expression, and for construction genome maps.

6. Summary

Construction and screening of an expression cDNA library from triactinomyxon spores of *Myxobolus cerebralis*, the causative agent of salmonid whirling disease

In the initial stage of the investigation, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis of triactinomyxon spores were performed to confirm the quality and specificity of the antiserum, and to determine a suitable dilution for the primary and secondary antibodies to be used to immunoscreen the cDNA library. Spores were sonicated and boiled in SDS sample buffer to dissociate their amino acids, and then electrophoresed on a 12% polyacrylamide gel. This fractionated the spore proteins which were then transferred onto nitrocellulose membranes, blocked with skim milk, then incubated with the primary antibodies (anti-triactinomyxon antibodies) at different dilutions. After several washings, the membranes were incubated with the secondary antibodies at different dilutions. The primary anti-triactinomyxon antibodies reacted with spore protein at a dilution of 1:1000, and the secondary antibodies reacted at a dilution of 1:2000. There was no reaction between the antiserum and the control *Tubifex tubifex* oligochaete worm protein, which confirmed specificity of the antibodies to triactinomyxon spores, and hence their suitability for immunoscreening the library.

The ZAP Express cDNA library was constructed using mRNA extracted from the triactinomyxon spores. First-strand cDNA was synthesised using an oligo (dT) linker-primer that contained an *Xho* I restriction site with MMLV reverse transcriptase. Following second-strand cDNA synthesis by RNase H and DNA polymerase I, the uneven termini of the double stranded cDNA were filled in with cloned Pfu DNA polymerase and EcoR I adapters which were ligated to the blunt ends. The double-stranded cDNA was then digested with *Xho* I restriction enzyme, cDNA fragments less than 400bp were removed and the remaining cDNA was ligated with the lambda ZAP Express vector. The recombinants were packaged in vitro using Gigapack III gold packaging extract. An aliquot of the packaged phage was used to infect *E. coli* XL1-Blue MRF` strain for titration, which revealed that the primary cDNA library contained 0.5 X 10^6 clones, with 97% recombinant and only 3% non-recombinant.

The cDNA library was then screened using the anti-triactinomyxon antibodies to facilitate selection of triactinomyxon clones from oligochaetes clones. The recombinant phage was adsorbed onto the *E.coli* host XL1-Blue MRF` strain, then plated on LB agar

and incubated at 42°C for ~3.5 hrs. The plates were then overlaid with 82mm circular IPTG-soaked nitrocellulose membranes and incubated at 37°C overnight. The membranes were then blocked with 5% skim milk and incubated with primary anti-triactinomyxon antibodies, washed, then incubated with secondary anti-rabbit peroxidase conjugated antibodies. Positive clones were selected and re-screened twice more to give a final selection of 526 plaques.

One clone (46-5) was selected and subjected to *in viv*o excision of the pBK-CMV phagemid from the ZAP express vector. A primer was then constructed using this sequence information and tested against cDNA from both triactinomyxons and oligochaetes. The primer bound to triactinomyxon cDNA but not oligochaete, which confirmed the clone represented triactinomyxon spores. The sequence of the entire clone was obtained using rapid amplification of the cDNA ends. A search of the clone sequence against GenBank revealed that it related to ribosomal protein L23 and it had a high percentage similarity to this protein from different species. A conserved domain for L23 was also identified in the clone sequence.

7. Zusammenfassung

Konstruktion und Skreening einer Expression cDNA Bibliothek aus Triactinomyxon Sporen von *Myxobolus cerebralis*, dem Auslöser der Drehkrankheit der Salmoniden

Zu Beginn der Untersuchung wurden eine SDS-Polyacrylamidgelelektrophorese (SDS-PAGE) und eine Western Blot Analyse der Triactinomyxonsporen durchgeführt, um die Qualität und Spezifizität des Antiserums zu untersuchen. Darüberhinaus wurden die optimalen Verdünnungen der primären und sekundären Antikörper bestimmt, um anschließend die exprimierten Klone aus einer cDNA-Bibliothek zu selektieren, die mit dem Antiserum reagierten. Die Sporen bzw. die Sporen Schalen wurden in einem Ultraschall Wasserbad aufgebrochen, mit dem SDS-Probenpuffer gekocht und dann einer Elektrophorese auf einem 12 %igem Polyacrylamidgel unterzogen. Das auf diese Weise fraktionierte Protein wurde auf eine Nitrocellulosemembran transferiert, mit Magermilch geblockt und anschließend mit den primären Antikörpern (anti-Triactinomyxon-Antikörper) in unterschiedlichen Verdünnungen inkubiert. Nach einigen Waschschritten wurde die Membran mit Sekundärantikörpern in verschiedenen Verdünnungen inkubiert. Die primären anti-Triactinomyxon-Antikörper reagierten mit Sporenprotein bei einer Verdünnung von 1:1000. Die optimale Verdünnung der sekundären Antikörper war 1:2000. Es gab keine Reaktion zwischen dem Antiserum und aufgetrenntem Extraktprotein des Oligochaeten *Tubifex tubifex*. Damit zeigte sich, dass die der Antikörper für Triactinomyxon Sporen spezifisch und für die Untersuchung einer Bibliothek geeignet ist. Die ZAP Express® cDNA Bibliothek wurde anhand von mRNA, die von Triactinomyxonsporen extrahiert wurde, konstruiert. Der erste Strang cDNA wurde mit einem Oligo-(dT)-primer, der eine *Xho* I Restriktionsstelle enthält, mit MMLV reverser Transkriptase synthetisiert. Nach der Synthese des zweiten cDNA Strangs durch RNase H und DNA Polymerase I, wurden die ungleichen Enden der Doppelstrang cDNA mit klonierter *Pfu* DNA Polymerase aufgefüllt und EcoR I Adapter in die abgestumpften Enden ligiert. Anschließend wurde die doppelsträngige cDNA mit dem *Xho* I Restriktionsenzym verdaut und ein cDNA Fragment, das kleiner als 400 bp war isoliert; die übrige cDNA wurde mit dem Lambda ZAP Express Vektor ligiert.

Die Rekombinanten wurden mittels Gigapack III® Gold Packaging Extrakts *in vitro* verpackt. Ein Aliquot des verpackten Phagen wurde verwendet, um den *E.coli*- Stamm XL1-Blue MRF' für eine Titration zu infizieren. Dabei zeigte sich, dass die primäre cDNA Bibliothek 0,5 x 10^6 Klone enthält; davon waren 97% rekombinant und nur 3% nicht-

rekombinant. Die cDNA-Bibliothek wurde dann mit dem anti-Triactinomyxon Antikörper untersucht, um die Triactinomyxonklone von den Oligochaetenklonen zu selektieren. Der rekombinante Phage wurde an den *E.coli* Wirtsstamm XL1-Blue MRF' adsorbiert und bei 42°C ca. 3,5 Stunden lang auf Agarplatten inkubiert. Die Platten wurden mit runden, IPTG-getränkten Nitrozellulosemembranen überdeckt und bei 37°C über Nacht inkubiert. Die Nitrozellulosemembranen wurden dann in 5% Magermilch geblockt, erst mit primären anti-Triactinomyxon Antikörpern und dann mit dem Sekundärantikörper inkubiert. Insgesamt konnten 526 positive Phagenplaques gezählt werden. Ein Klon (46-5) wurde selektiert und einer *in vivo* Exzision des pBK-CMV Phagemids vom ZAP Express Vektor unterzogen. Anhand der erhaltenen Sequenz des Klons wurde ein Primer konstruiert und sowohl an cDNA aus Triactinomyxonsporen als auch an cDNA aus Oligochaeten getestet. Der Primer reagierte nur mit Triactinomyxon- jedoch nicht mit *Tubifex tubifex*-cDNA. Somit wurde bestätigt, dass der Klon aus dem mRNA Pool der Triactinomyxonsporen stammt. Eine RACE-PCR ergab die komplette Sequenz, die dem ribosomalen Protein L23 verwandt ist. Auch wurde eine conserved domain für das Ribosomale Protein L23 in den Klonsequenzen identifiziert.

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Publications:

1. **Soliman H., Geisslar K., El-Matbouli M**. **(2003)** SDS-PAGE and western blot analysis of triactinomyxon spores of *Myxobolus cerebralis*, the cause of whirling disease in salmonid fish. *Journal of fish Diseases* **26**, 621-625.
2. **Soliman H., El-Matbouli M. (2005)** An Inexpensive and Rapid Diagnostic method of the Koi Herpesvirus (KHV) Infection by a Loop-Mediated Isothermal Amplification Method. *Virology Journal* (accepted)

3. **Soliman H., El-Matbouli M. (2005)** Reverse transcription loop mediated isothermal amplification (RT-LAMP) for rapid detection of viral hemorrhagic septicaemia virus (VHS). *Veterinary Microbiology* (submitted).
4. **Soliman H., El-Matbouli M. (2005)** Nested Polymerase Chain Reaction Assay Improving Koi Herpesvirus DNA Detection in Infected Fish Tissues. *Journal of Fish pathology*. (accepted).
5. **El-Matbouli M., Soliman H**. **(2005)** Rapid diagnosis of Tetracapsuloides bryosalmonae, the causative agent of proliferative kidney disease (PKD) in salmonid fish by a novel DNA amplification method, loop-mediated isothermal amplification (LAMP). *Parasitology Research* **96**, 277-284.
6. **El-Matbouli M., Soliman H. (2005)** Construction and screening of cDNA Library from the Triactinomyxon spores of *Myxobolus cerebralis*, the causative agent of salmonid Whirling Diseases. *Parasitology* (submitted).
7. **El-Matbouli M., Soliman H**. **(2005)** Development of a rapid assay for diagnosis of whirling disease in fish and oligochaetes using Loop-mediated isothermal amplification. *Journal of fish diseases* (accepted)