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Isolation of a subtilisin-like serine protease gene (*Myx*SubtSP) from spores of *Myxobolus cerebralis*, the causative agent of whirling disease

Christoph Dörfler, Mansour El-Matbouli*

Institute of Zoology, Fish Biology and Fish Diseases, University of Munich, Kaulbachstraße 37, 80539 Munich, Germany

ABSTRACT: Proteases play important roles in parasite life cycles and host–parasite interactions. They are pathogenesis factors of many pathogenic organisms and are hence potential targets for chemotherapeutic treatment of disease. We identified a subtilisin-like serine protease gene, *Myx*-SubtSP, expressed by *Myxobolus cerebralis.* After PCR with subtilisin-like serine protease primers, the gene was cloned, sequenced and aligned against the NCBI database. Its corresponding amino acid sequence included the putative conserved domains of Peptidase_S8, subtilase family and AprE, subtilisin-like serine proteases. Rapid amplification of 5′ and 3′ cDNA ends (RACE) was used to generate the full length (1385 bp) gene, with a 429 bp open reading frame. The gene encompasses coding regions for a catalytic triad formed by Asp-74, His-100 and Ser-110.

KEY WORDS: Salmonid whirling disease · *Myxobolus cerebralis* · Serine protease · Cloning

INTRODUCTION

The metazoan parasite *Myxobolus cerebralis* is the causative agent of salmonid whirling disease, which was originally described in rainbow trout from Germany (Hofer 1903). The parasite was subsequently widely disseminated through the international fish trade (Halliday 1976). In North America, the disease initially appeared in Pennsylvania and Nevada in 1958, followed by Connecticut (1961), Virginia (1965), California and Massachusetts (1966). Whirling disease is now in at least 22 states in the US and has been associated with catastrophic declines in populations of wild rainbow trout *Oncorhynchus mykiss* (Nehring & Walker 1996, Vincent 1996, Hedrick et al. 1998, Baldwin et al. 2000).

Myxobolus cerebralis has a 2-host life cycle involving the freshwater oligochaete worm *Tubifex tubifex* as primary host. After ingestion, *M. cerebralis* myxospores undergo 3-phase development in the intestinal epithelium of the oligochaete: comprising schizogony, gametogony and sporogony to generate triactinomyxon actinospores which are released into the water (El-Matbouli et al. 1998). The triactinomyxons can then infect a salmonid host by entering through the epidermis (Wolf & Markiw 1984, El-Matbouli & Hoffmann 1989).

Whirling disease of salmonids is diagnosed from 3 primary symptoms: pathological 'whirling' behaviour (Rose et al. 2000), darkening of the caudal part of the body (Schäperclaus 1990) and deformation of the head and spinal column (Hoffman et al. 1962). Although the life cycle of *Myxobolus cerebralis* has been well described, the mechanisms by which the parasite invades the fish epidermis, migrates through host tissues and promotes disruption of chondrocytes remain unresolved. It is hypothesised that these processes are facilitated by proteases generated by the parasite (Kelley et al. 2004). Proteases are known to play an important role in lesion formation and virulence of many pathogenic organisms (McKerrow 1989, McKerrow et al. 1993) and are known to enable invasion of parasites into their hosts by catalysing degradation of connective tissues. Subtilisin-like genes, which code for proteolytic enzymes, are involved in the process of host invasion for *Neospora caninum* (Louie & Conrad 1999, Louie et al. 2002), *Toxoplasma gondii* (Miller et al. 2001), and *Plasmodium falciparum* (Blackman et al. 1998, Hackett et al. 1999).

Within the host, proteases are involved in parasite metabolism and in evasion of host immune responses by degradation or activation of host immune mole-

*Corresponding author. Email: el-matbouli@lmu.de

cules. They can also facilitate degradation of host cytoskeletal proteins, activation of parasite regulatory proteins, metamorphosis of the parasite, and can affect blood coagulation and the host fibrinolytic system (McKerrow et al. 1993). Proteases are important for both parasite physiology and development (Rosenthal et al. 1988, McKerrow 1989, 1999, McKerrow et al. 1993, 1999) and contribute to parasite virulence (Que & Reed 2000, Que et al. 2002, 2003, Mackey et al. 2004); hence these enzymes are targets for potential anti-parasite treatments.

The objective of this study was to identify parasite proteases that may contribute to the pathogenicity of *Myxobolus cerebralis*. We focused on whether *M. cerebralis* possesses serine proteases, and what kinds of serine proteases are present. Herein we describe the isolation of a subtilisin-like serine protease gene, *Myx*SubtSP, from spores of *Myxobolus cerebralis.*

MATERIALS AND METHODS

Zymography. Percoll® (Sigma-Aldrich Chemie)-purified *Myxobolus cerebralis* triactinomyxon spores (1.5×10^6 ml^{-1}) were ultrasonicated for 10 min, then diluted 1:1 with Laemmli sample buffer. Sodium dodecyl sulphate (SDS) gels containing 12% acrylamide were polymerised in the presence of 1 mg ml^{-1} gelatin (Muñoz et al. 2000). Wells were loaded for each inhibitor to be tested plus a negative control. After electrophoresis, the gel was cut into individual lanes, which were then incubated in 2.5% Triton X for 1 h to remove SDS and restore proteolytic activity. The gel slices were rinsed twice in phosphate-buffered saline (PBS), then incubated overnight at room temperature in PBS baths (pH 7.4) containing each of the inhibitors: antipain (100 µg ml^{-1}), aprotinin (100 mM), E-64 (L-trans-epoxysuccinyl-leucylamidi-4-guanidino-butane) (5 µM), leupeptin (100 µg ml^{-1}); pepstatin (1 µM), phenantrolin (100 mM) (all Sigma-Aldrich Chemie), and a negative control containing PBS alone. Before they were added to the baths, the inhibitors were first dissolved in water, except phenanthrolin which was dissolved in dimethylsulphoxide (DMSO), and pepstatin which was dissolved in ethanol. After incubation, the gel slices were stained for 1 h in a 0.1% amido black solution comprising methanol (40%), acetic acid (10%) and water (50%), then de-stained in an equivalent solution without the amido black. Proteolytic compounds appeared in the gel as clear bands against a blue background, i.e. where digestion of copolymerised gelatin occurred. Any inhibitory effects resulted in disappearance of bands.

Parasite preparation and nucleic acid isolation. Triactinomyxon spores of *Myxobolus cerebralis* were filtered from an infected culture of *Tubifex tubifex* oligochaetes, enumerated in 50 µl filtered water and purified on a Percoll® gradient (Sigma-Aldrich Chemie). RNAlater (Sigma-Aldrich Chemie) was added to the purified spores which were then stored at –20°C until used for RNA extraction. Total RNA was extracted using a RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was generated using a Super SMART™ PCR cDNA Synthesis Kit (BD Biosciences). cDNA samples were stored at –20°C; extracted RNA was kept at –80°C.

Serine protease primer PCR amplification. Universal primers were used in PCR amplification of cDNA from triactinomyxon spores (Sakanari et al. 1989, Elvin et al. 1993, Blackman et al. 1998). Specific primers were then used to amplify chymotrypsin-like serine proteases (Sakanari et al. 1989): forward primer 5′-ACA GAA TTC TGG GTN GTN CAN GCN GCN CAY TG-3′, reverse primer 5′-ACA GAA TTC ARN GGN CCN CCN SWR TCN CC-3′, and to amplify subtilisin-like serine proteases (Blackman et al. 1998): forward primer 5′-CAY GGI ACI CAY GTI GCI GG-3′; reverse primer 5′-CCI GCI ACR TGI GGI GTI GCC AT-3′. All primers were synthesised by MWG-Biotech AG).

PCR was performed in 50 µl volumes, which comprised approximately 100 ng of triactinomyxon cDNA and 47.5 µl of 1.1× Reddy–Mix™ PCR master mix (ABgene): 1.25 U *Taq* DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP), and 50 pmol of each primer. Amplifications were performed in a Mastercycler gradient thermocycler (Eppendorf) using the parameters of Brown & Reece (2003): 94°C for 2 min followed by 2 cycles of 94°C for 2 min, 25°C for 1 min and 72°C for 2 min, followed by 40 cycles of 94°C for 2 min, 55°C for 2 min and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were visualised on ethidium bromide-stained 1.5% agarose gel with 1 kb DNA ladder.

Cloning, sequencing and alignment. Amplicons were excised from the gel and purified using a MinElute™ Gel Extraction Kit (Qiagen). A TOPO® TA cloning kit (Invitrogen) was used according to the manufacturer's instructions to ligate 4 µl of each amplicon into the TA cloning vector pCR®4 and to transform chemically competent *Escherichia coli*. The optical density $(OD)_{600}$ of the bacteria cultures was determined using an Eppendorf biophotometer. Cultures with an OD_{600} between 2.0 and 4.0 were used for plasmid DNA extraction with a FastPlasmid Mini Kit (Eppendorf). Plasmid DNA was sent to Sequence Laboratories Göttingen for sequencing. Sequences were aligned using the National Center for Biotechnology (NCBI) BLAST program.

5′ and 3′ rapid amplification of cDNA ends (RACE). The 5′ and 3′ ends of the subtilisin-like serine protease gene were amplified using a 5′/3′ RACE kit (Roche Molecular Biochemicals). First-strand 5′ cDNA templates were produced according to the manufacturer's protocol. We used 2 µg of total RNA with 12.5 µM of gene-specific primer 5′RACE$_{SP1}$: 5′-CCG CAA TCA CAC TCG ACA GTC TAC-3′. After purification and tailing reaction, PCR amplification of the cDNA was performed in 50 µl, comprising 5 µl dA-tailed cDNA, 1 µl oligo dT-anchor primer, 1 µl deoxynucleotide mixture, 0.5 µl *Taq* DNA polymerase (Roche), 5 µl reaction buffer (10× conc.), 36.5 µl H_2O, 1 µl (12.5 µM) of gene-specific primer 5′RACE$_{SP2}$: 5′-TTA GCC CCT GAG GCT ACT CCT ACG-3′. RACE amplification was carried out in a thermocycler using the following parameters: 94°C for 2 min, followed by 35 cycles of 94°C for 15, 60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min.

Synthesis of first-strand 3′ cDNA was carried out according to the manufacturer's protocol: 2 µg of total RNA were used in a 50 µl PCR, comprising 1 µl 3′ cDNA product, 1 µl PCR anchor primer, 1 µl deoxynucleotide mixture, 0.5 µl *Taq* DNA polymerase, 5 µl reaction buffer (10× conc.), 40.5 µl H_2O, and 12.5 µM gene-specific primer 3′RACE$_{SP5}$: 5′-ACG CTA TGC TAT TTT GTC AGG G-3′. The PCR cycle conditions were the same as for 5′ first-strand synthesis. Both 5′- and 3′-PCR samples were visualised, purified, cloned and sequenced as described above.

Phylogenetic analysis. The amino acid sequence corresponding to Nucleotides 859 to 1287 from *Myx*-SubtSP and subtilisin-like serine protease sequences from other species obtained from the NCBI database were aligned using the CLUSTAL W program (Thompson et al. 1994). Phylogenetic analysis of 100 replicates was conducted with the distance matrix method. The matrix was calculated using the Fitch-program of PHYLIP Version 3.65 (Felsenstein 2004). The tree was visualised using TreeView32 (Page 1996).

RESULTS

Zymography

Protease activity was indicated by 2 bands on the SDS-PAGE gel, molecular weights 29 and 55 kDa (Fig. 1).

The inhibitors we tested did not give a result clear enough to allow the type of protease to be determined by zymography. For antipain, a non-selective serine and cystein protease inhibitor, both bands disappeared, indicating that antipain inhibited both proteolytic activities. For aprotinin, which inhibits serine proteases including trypsin, chymotrypsin, kallikrein and plasmin, the 29 kDa band disappeared. For E-64, an effective inhibitor of cystein proteases that neither affects cystein residues in other enzymes nor reacts with low molecular weight thiols such as calpain and cathepsin B, neither band disappeared. For leupeptin, an inhibitor of serine and cystein proteases including plasmin, trypsin, papain and Cathepsin B, both bands disappeared. For pepstatin, a potent inhibitor of acid proteases, including pepsin, renin and Cathepsin D and many aspartic proteases, both bands disappeared. For phenanthrolin, a metallo protease inhibitor, only the 55 kDa band disappeared (Table 1).

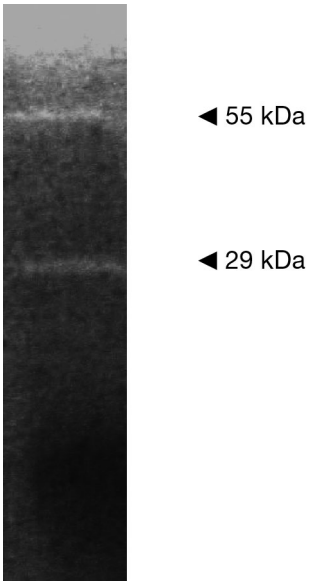

Fig. 1. *Myxobolus cerebralis.* Protease zymogram using gelatin SDS-PAGE

Table 1. Tested inhibitors and their effect on the 29 and 55 kDa protease expressed by *Myxobolus cerebralis*. +: band still visible after treatment with relevant inhibitor; –: band disappeared

Inhibitor	Type of protease(s) inhibited	29 kDa band	55 kDa band
Antipain	Serine and cystein proteases	–	–
Aprotinin	Serine proteases	–	+
E-64	Cystein proteases	+	+
Leupeptin	Serine and cystein proteases	–	–
Pepstatin	Acid and aspartic proteases	–	–
Phenanthrolin	Metallo proteases	+	–

PCR with serine protease primer

No bands were detected when chymotrypsin-like serine protease primers were used. Subtilisin-like serine protease primers generated a ~500 bp amplicon, which was excised from the gel, and cloned to yield a 503 bp sequence which we designated *Myx*SubtSP. A BLASTP search indicated putative conserved domains with Peptidase_S8, subtilase family (pfam Accession No. PF00082) and AprE, subtilisin-like serine proteases (COG Accession No. COG1404).

Rapid amplification of cDNA ends (RACE)

RACE amplification generated a ~250 bp amplicon for 3′ and >1 kb for 5′. Amplicons were excised from the gel, cloned and sequenced to yield 262 and 1122 bp respectively. The full length gene consisted of 1385 nucleotides with a 429 bp open reading frame. Start codon ATG was at Positions 859 to 861, and the termination codon TAA was at 1285 to 1287. Amino acid sequence analysis of *Myx*SubtSP showed 3 catalytic sequences (Asp-74, His-100, Ser-110) (Figs. 2 & 3). Alignment of the complete sequence using NCBI's BLASTP program confirmed the putative conserved domains.

Phylogenetic analysis

A phylogenetic tree generated with the distance matrix model of multiple subtilisin-like serine proteases, grouped *Myx*SubtSP from *Myxobolus cerebralis* with subtilisin-like serine proteases from the fungi *Metharizium anisopliae* var. *anisopliae*, *Coprinopsis cinerea, Saccharomyces cerevisiae* and *Podospora anserina*, although the bootstrap values were low (Fig. 4). This clustering was also apparent from trees generated with parsimony and maximum likelihood models (not shown).

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1       CTTTGCCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA
61      GATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGT
121     AGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGA
181     TAAGTCGTGTCTTACCCGGGTTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGG
241     TCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA
301     CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCG
361     GACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGG
421     GGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGA
481     TTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTT
541     TTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCT
601     GATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGA
661     ACGACCAAGGGCGAATTCGCCCTTGACCACGCGTATCGATGTCGACTTTTTTTTTTTTTT
721     TTCTCCGTAGGAGTAGCCTCAGGGGCTAAGATTGTAGCGCTCAAAGTACTTGATCAGTTG
781     GGCGAAGGTAGACTGTCGAGTGTGATTGCGGCGGTGGCGCATGTTAGCCAAAATGCTACA
841     GCGGGAGATGTCGTCAATATGAGTTTGGGACTAGATGGTACCTCCGCTGCACTAGACAGG
                          M  S  L  G  L  D  G  T  S  A  A  L  D  R          14
901     GAAGTTCAAGCAGCCGCTAACAAAGGCATTCTTTTTGCAATAGCGGCCGGCAACGATGCC
         E  V  Q  A  A  A  N  K  G  I  L  F  A  I  A  A  G  N  D  A          34
961     AAGCAAGCCAACGGATACTCGCCAGCAAGGGTCAATCATGCCAATATATTTACGGTGTCG
         K  Q  A  N  G  Y  S  P  A  R  V  N  H  A  N  I  F  T  V  S          54
1021    GCGGTAGATAGCACAGGGCGATTTGCTAGTTTCTCCAATTACGGTAACGATGTAGTAGAT
         A  V  D  S  T  G  R  F  A  S  F  S  N  Y  G  N  D  V  V  D          74
1081    GTAGCCGCCTATGGTGTCAGGGTACGTTCGACTTATTCAAACGGACGCTATGCTATTTTG
         V  A  A  Y  G  V  R  V  R  S  T  Y  S  N  G  R  Y  A  I  L          94
1141    TCAGGGTATTTTGATCACGGACAAAAAATAGCTCTGACTGTATTTTCTTTCATTAACTCA
         S  G  Y  F  D  H  G  Q  K  I  A  L  T  V  F  S  F  I  N  S         114
1201    ATAATTCCATCATGTGTTGCTTATAAGTACTTTAAAGTCGCATTTGAAGCTAGATTTTCC
         I  I  P  S  C  V  A  Y  K  Y  F  K  V  A  F  E  A  R  F  S         134
1261    AATCGATTCGATGTAGTGGTGGATTAAATTTATCTCAATGACAATTAAAACGTTTAATTA
         N  R  F  D  V  V  V  D  *                                           142
1321    AAAAATTTCTTTTAATTTTCTAACCTCTAAAAAAAAAAAAAAAAAGTCGACATCGATACGC
1381    GGGTC
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Fig. 2. Nucleotide and deduced amino acid sequence of the cloned subtilisin-like serine protease gene *Myx*SubtSP. Numbers on the left denote nucleotide sequences, those on right amino acid sequences. Amino acid sequence starts with methionine (Met-1) residue, and is represented by the single-letter code below the underlined nucleotide sequence. The catalytic triad Asp-74, His-100, Ser-110 is in **boldface**. Stop codon is indicated by an asterix (*)

Myxobolus cerebralis	VV**D**YAAYGYR	GYF---D**H**GQ	KIALTVF**S**FI
Metarhizium anisopliae var. *anisopliae*	AV**D**ILAPGSN	GTSMATP**H**IV	GLGAYLA**S**LE
Saccharomyces cerevisiae	CY**D**YFAPGIN	GTSMASP**H**YA	GILSYFL**S**LQ
Bacillus subtilis	EL**D**VMAPGVS	GTSMATP**H**VA	GAAALIL**S**--
Chloroflexus aurantiacus	KP**D**LVAPGTN	GTSMAGP**H**VA	GAVALIW**S**AN

Fig. 3. Catalytic triad (**boldface**) of *Myx*SubtSP and regions flanking active site for comparison. Amino acid sequences surrounding catalytic active site of *Myx*SubtSP (Asp-74, His-100, Ser-110) from *Myxobolus cerebralis* were aligned to the following subtilisin-like serine proteases: subtilisin-like protease PR1I (*Metarhizium anisopliae* var. *anisopliae*, CAC95043), subtilisin-like protease III (*Saccharomyces cerevisiae*, AAA35237), subtilisin (*Bacillus subtilis*, CAE18180) and subtilisin-like serine protease (*Chloroflexus aurantiacus*, ZP00357443)

DISCUSSION

Proteases play an integral role in interactions between parasites and their hosts, and underlie the pathogenicity of many organisms (McKerrow 1989, McKerrow et al. 1993). These enzymes have also been described in *Myxobolus cerebralis* (Kelley et al. 2003, 2004) and are possibly a key pathogenesis factor of this parasite, which causes salmonid whirling disease. Introduced to the USA in the 1950s, whirling disease spread rapidly and devastated both wild trout and hatchery populations. Recently, rainbow trout resistant to the disease (Strain H) was discovered in Germany (Hedrick et al. 2003). The mechanisms which underlie the greater resistance of the H strain are currently unknown. We hypothesise that differences in the way this German strain and a susceptible strain from North America (Strain TL) respond to parasite proteases contributes to their different susceptibility to the disease.

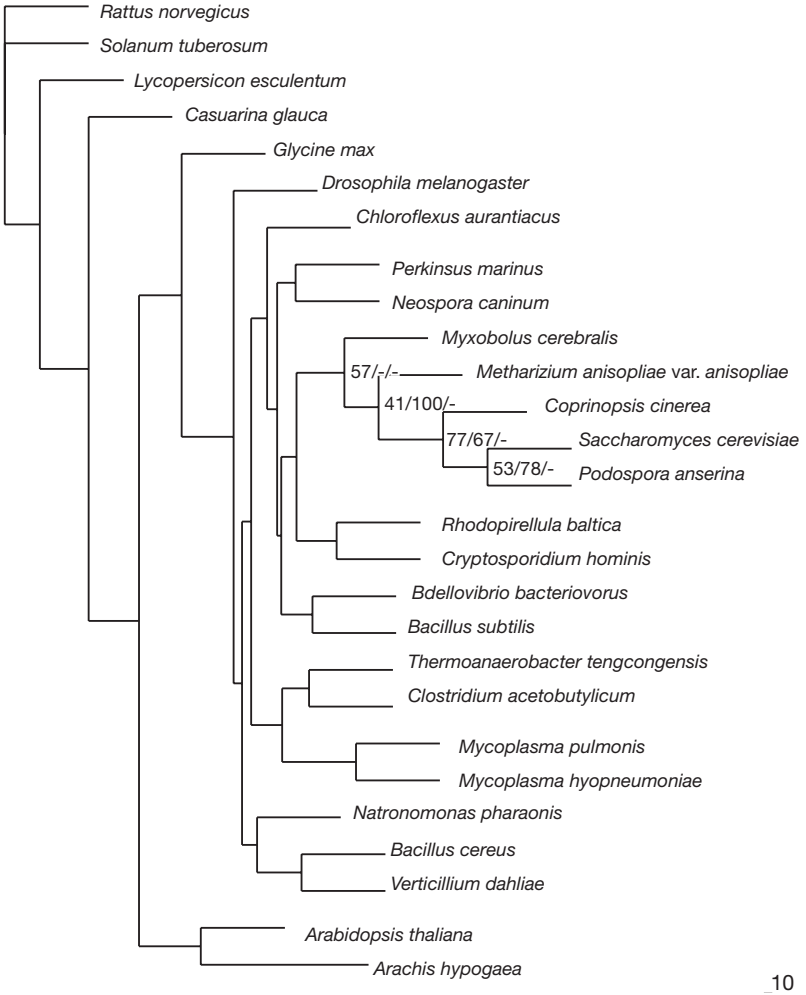

Fig. 4. Phylogenetic distance matrix tree generated following CLUSTAL W (Thompson et al. 1994) alignment of the *Myx*SubtSP sequence determined from *Myxobolus cerebralis* and following subtilisin-like serine protease sequences: AAA61987, *Rattus norvegicus*, AAF04257, *Neospora caninum*; AAQ54746, *Perkinsus marinus*; CAE18180, *Bacillus subtilis*; CAE80099, *Bdellovibrio bacteriovorus*; BAC78619, *Coprinopsis cinerea*; AAC03564, *Podospora anserina*; CAC95043, *Metharizium anisopliae* var. *anisopliae*; AAA35237, *Saccharomyces cerevisiae*; ZP_00357443, *Chloroflexus aurantiacus*, AAY63882: *Solanum tuberosum*, CAA07001, *Lycopersicon esculentum*; AAO62352, *Casuarina glauca*; AAD02075, *Glycine max*; BAB70678, *Arabidopsis thaliana*; AAY54007, *Arachis hypogaea*; AAM24081, *Thermoanaerobacter tengcongensis*; AAU15651, *Bacillus cereus*; CAI50405, *Natronomonas pharaonis*; AAS45251, *Verticillium dahliae*; NP_350134, *Clostridium acetobutylicum*; YP_287727, *Mycoplasma hyopneumoniae*; CAD71780, *Rhodopirellula baltica*; AAC28563, *Drosophila melanogaster*; CAC13828, *Mycoplasma pulmonis*; XP_667589, *Cryptosporidium hominis*. Numbers at nodes indicate bootstrap values for distance matrix, parsimony and maximum likelihood. Values below 50 are not shown

In the present study, zymography suggested the presence of serine proteases in *Myxobolus cerebralis*, which corresponds to the studies of Kelley et al. (2004), who were the first to identify and characterise a serine protease gene from *M. cerebralis*. Of the 6 inhibitors tested, 3 serine protease inhibitors, antipain, aprotinin, and leupeptin, affected parasite protease activity. To amplify the putative protease gene, we used generic primers for chymotrypsin-like and subtilisin-like serine proteases, given the widespread occurrence of both enzyme types in nature (Barret et al. 1998). Although *M. cerebralis* has a protease with a catalytic residue arrangement similar to that of the chymotrypsin family of serine proteases (Kelley et al. 2004), we could not detect a band with the degenerate chymotrypsin-like serine protease primers that we used. With the subtilisin-like serine protease primers we obtained an amplicon designated *Myx*SubtSP.

Serine proteases are the largest family of proteolytic enzymes (McKerrow et al. 1993) and are grouped into 6 clans (SA, SB, SC, SE, SF, SG) with 27 families, denoted S1 to S27 (Rawlings & Barrett 1994). Subtilases belong to the superfamily of subtilisin-like serine proteases. Over 200 subtilases are presently known and complete amino acid sequences for more than 170 have been elucidated (Siezen & Leunissen 1997). Alignment of *Myx*SubtSP to the database of known protease sequences revealed a putative conserved domain with Peptidase_S8, subtilase family.

The common catalytic apparatus of serine proteases consists of a nucleophilic serine residue adjacent to histidine, with aspartate in an unconserved position to complete a catalytic triad (Perona & Craik 1995). In *Myx*SubtSP, we determined that the 3 sequences Asp-74, His-100 and Ser-110 comprised the catalytic triad. The linear arrangement of these catalytic residues places *Myx*SubtSP in the subtilisin clan (SB) (Rawlings & Barrett 1993, 1994).

In the phylogenetic analysis, the subtilisin-like serine protease from rat was the clear outgroup. High similarities were found between *Myx*SubtSP and the subtilisin-like serine proteases of fungi *Coprinopsis cinerea*, *Podospora anserina*, *Metharizium anisopliae* var. *anisopliae* and *Saccharomyces cerevisiae*. Although these clustered in the phylogenetic tree, bootstrap values were quite low, which was most likely due to the use of short sequences with significantly variable regions.

The isolation of *Myx*SubtSP allows for analysis of the subtilisin-like serine protease in the infection process in susceptible and non-susceptible trout, for instance by means of quantitative transcription studies during experimental infection of the H and the TL strains.

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