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**Mycobacterium-Inducible Nramp in Striped Bass (Morone saxatilis)**

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In mammals, the natural resistance-associated macrophage protein 1 gene, *Nramp1*, plays a major role in resistance to mycobacterial infections. Chesapeake Bay striped bass (*Morone saxatilis*) is currently experiencing an epizootic of mycobacteriosis that threatens the health of this ecologically and economically important species. In the present study, we characterized an *Nramp* gene in this species and obtained evidence that there is induction following *Mycobacterium* exposure. The striped bass *Nramp* gene (*MsNramp*) and a 554-amino-acid sequence contain all the signal features of the *Nramp* family, including a topology of 12 transmembrane domains (TM), the transport protein-specific binding-protein-dependent transport system inner membrane component signature, three N-linked glycosylation sites between TM 7 and TM 8, sites of casein kinase and protein kinase C phosphorylation in the amino and carboxy termini, and a tyrosine kinase phosphorylation site between TM 6 and TM 7. Phylogenetic analysis most closely grouped *MsNramp* with other teleost *Nramp* genes and revealed high sequence similarity with mammalian *Nramp2*. *MsNramp* expression was present in all tissues assayed by reverse transcription-PCR. Within 1 day of injection of *Mycobacterium marinum*, *MsNramp* expression was highly induced (17-fold higher) in peritoneal exudate (PE) cells compared to the expression in controls. The levels of *MsNramp* were three- and sixfold higher on days 3 and 15, respectively. Injection of *Mycobacterium shottsii* resulted in two-, five-, and threefold increases in gene expression in PE cells over the time course. This report is the first report of induction of an *Nramp* gene by mycobacteria in a poikilothermic vertebrate.

Mycobacteriosis has been reported in more than 150 species of freshwater and marine fish worldwide, including striped bass (*Morone saxatilis*) (43). Chesapeake Bay is currently experiencing an epizootic of mycobacteriosis in striped bass that threatens the health of this ecologically and commercially important commercial and recreational fishery (32, 46) and has important consequences for production in aquaculture (33). The prevalence of splenic mycobacterial lesions and the prevalence of dermal mycobacterial lesions in striped bass from Chesapeake Bay tributaries have been reported to be as high as 62.7 and 28.8%, respectively (8). Diseased striped bass harbor multiple species of *Mycobacterium*, including *Mycobacterium marinum*, a known fish and human pathogen (35, 64), and *Mycobacterium shottsii* sp. nov., which is also a member of the *Mycobacterium tuberculosis* clade (47). Approximately 76% of mycobacterium-positive striped bass sampled to date harbor *M. shottsii*, either as a monooinfection or as part of a coinfection with multiple *Mycobacterium* spp. (M. W. Rhodes, H. Kator, I. Kaattari, D. Gauthier, W. Vogelbein, and C. Ottinger, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. Q-264, 2003). Gauthier et al. (21) investigated the relative pathogenicity of three *Mycobacterium* spp. isolated from wild Chesapeake Bay fish for laboratory-reared striped bass and found that *M. marinum* caused acute peritonitis and extensive granulomatous inflammation.

In some cases, a secondary phase of reactivation disease was observed. The pathology in fish inoculated with *M. shottsii* or *Mycobacterium gordoneae* was considerably less severe than the pathology in fish inoculated with *M. marinum*, and secondary disease did not occur. Both *M. gordoneae* and *M. shottsii*, however, did establish persistent infections in the spleen.

Breeding studies with *Mycobacterium*-resistant (Bcg<sup>r</sup>) and -susceptible (Bcg<sup>s</sup>) inbred mouse phenotypes resulted in identification of a single dominant, autosomal gene (termed *Bcg*) responsible for increased resistance to mycobacteria during the early stages of infection (27). Positional cloning of *Bcg* from the proximal region of mouse chromosome 1 led to the discovery of the gene for the natural resistance-associated macrophage protein (*Nramp*) (61). Vidal et al. demonstrated that *Nramp* transcripts were detected only in the reticulendothelial organs (spleen and liver) of mice and were highly expressed in purified macrophages and macrophage cell lines from these tissues. In addition, murine *Nramp1* is highly upregulated following infection with intracellular parasites (23, 26) and administration of lipopolysaccharide (LPS) and gamma interferon (25), and a strong synergistic effect is observed under the latter conditions. Transfection of the resistant, wild-type *Nramp<sup>G169D</sup>* allele in susceptible *Nramp<sup>G169D</sup>* knockout mice restored resistance to *Mycobacterium bovis* BCG and *Salmonella enterica* serovar Typhimurium in the transgenic animals (26), while overexpression of *Nramp1* by a cytomegalovirus promoter-enhancer completely inhibited intracellular replication of *S. enterica* serovar Typhimurium in normally susceptible mouse macrophages (24), indicating the crucial role of this gene in resistance to intracellular parasites.

The mechanism of mycobacterial resistance due to Nramp1...
is not fully understood (4), but Nramp2 is known to take up iron from the intestinal brush border in mammals and has been linked to transferrin-independent iron transport into acidified endosomes in many different tissues (18, 31). One of the splice variants of DCT1 (Rattus norvegicus Nramp2 homolog) contains an iron-responsive element (IRE) in the 3′ untranslated region (UTR) (31). There is a very high degree of homology in all the transmembrane domains (TM) between Nramp1 and Nramp2 (44), and a mutation in Nramp2 immediately C terminal of the loss-of-function mutation in Nramp1 TM 4 is associated with microcytic anemia iron deficiency (54).

Nramp1 belongs to a small family of related proteins encoded by genes that include two known murine genes, Nramp1 and Nramp2, as well as related sequences in many other taxa (10). Nramp homologs have been found in many evolutionarily distantly related groups, such as humans (11, 37), rats (31), birds (36), fish (15), insects (48), nematodes (57), plants (5), yeast (45), and bacteria (42). Complete Nramp mRNA coding sequences for five teleosts have been published recently (12, 14, 15, 49, 52). Paralogs of Nramp seem to be present in two teleost species, Oncorhynchus mykiss (15) and Takifugu rubripes (52), while single genes are present in other teleost species, including Cyprinus carpio (49), Ictalurus punctatus (12), Danio rerio (14), and M. saxatilis (this study). Expression studies and phylogenetic analysis of fish have indicated that the nonteleost sequence similarity and tissue-specific expression patterns most closely resemble those of mammalian Nramp2. Little is known about the function of Nramp in teleosts, although in one study Chen et al. (12) demonstrated by using Northern hybridization and reverse transcription (RT)-PCR that channel catfish (I. punctatus) spleen NrampC levels were elevated in response to LPS exposure in vivo in a dose-dependent fashion. Direct evidence of induction due to exposure of fish to pathogens has not been reported previously.

The purposes of the present study were to isolate and sequence striped bass Nramp homolog(s), to characterize the coding sequence, to determine the tissue expression patterns, and to evaluate induction of the striped bass Nramp gene (MsNramp) in vivo after exposure to mycobacteria. Expression was measured in several tissues by using real-time RT-PCR (see references 7, 53, and 59 for descriptions of recent applications) following injection of M. marinum or M. shottsi into striped bass. This report is the first report of induction of an Nramp gene by an intracellular pathogen in a poikilothermic vertebrate.

**MATERIALS AND METHODS**

Experimental fish and maintenance. Striped bass (M. saxatilis) (500 to 2,000 g) were collected from the York River, Chesapeake Bay, Va. (Virginia Marine Resources permit 02-27 and VIMS Research on Animal Subjects Committee permit 0101). The tissues of these fish were used for sequencing and normal tissue expression of MsNramp. The fish were maintained in 1,160-liter tanks with flowthrough, sand-filtered water at the ambient temperature and salinity. The tanks were lit with fluorescent lights adjusted to the local photoperiod. The fish were fed daily to satiation with wild-caught small fish and crabs and were kept for more than 2 weeks prior to experimental use.

The striped bass used for the mycobacterial challenge and in vivo expression experiments were obtained as fingerlings (1 year postspawn) from the Virginia Department of Game and Inland Fisheries Vic Thomas Striped Bass Hatchery in Brookneal, Va. The fish were reared until the mean weight was approximately 200 g (2 years postspawn) in circular 1,000-liter tanks containing 21°C well water exchanged at a rate of 12 liters/min. The inflow water was degassed and oxygenated to saturation, and the tank water was treated with 1% (wt/vol) NaCl each time that the fish were handled to alleviate stress. The fish were fed trout chow (Ziegler Bros, Gardner, Pa.). Tank illumination was provided by a combination of fluorescent and natural light, with the former adjusted to the local photoperiod. Striped bass (198.4 g) were randomly assigned into three treatment groups, and moved to an isolation facility prior to infection with mycobacteria.

**RNA extraction and RT for cDNA: sequencing and tissue expression.** Peritoneal exudate (PE) cells were isolated from wild striped bass by a modification of standard techniques (51). Cells were elicited to the peritoneal cavity by injection of a 1% (wt/vol) solution of complete medium to induce exudation (54). Neobacterium cytophagum (ATCC 13067) was used to challenge PE cells. Anesthetized fish were inoculated intraperitoneally with 10 ml of ice-cold Leibowitz’s L-15 medium containing 100 U of penicillin-streptomycin per ml and 100 ml of sodium heparin per ml. After 10 min, lavage fluid was withdrawn through a ventral incision (51). Anterior kidney, brain, heart, gill, gonad, intestine, liver, muscle, and spleen samples (approximately 100 mg each) were dissected from the fish and either stored in RNA later (Ambion) or extracted immediately. Total RNA was isolated with TRIzol (Invitrogen) used according to the manufacturer’s protocol. The integrity of the total RNA was assessed by electrophoresis in 1% denaturing formaldehyde–agarose gels. The RNA quality and concentration were determined by UV spectrophotometry at 260 and 280 nm, with background correction for protein contamination at 230 nm. The total RNA was resuspended in RNA Storage Solution (Ambion) and stored at −80°C until it was used. RT of 5 μg of RNA was accomplished by using SuperScript II RNase H− reverse transcriptase and oligo(dT)12-18 (Invitrogen) priming according to the manufacturer’s recommendations.

**Amplification of MsNramp cDNA.** Primers and hybridization probes used in standard PCR, RT-PCR, RNA ligase-mediated rapid amplification of cDNA ends (RACE), and sequencing analyses are listed in Table 1. An initial 262-bp fragment of striped bass MsNramp was obtained by using primers NrampA and NrampB, which were derived from consensus mammalian sequences (12), and striped bass PE cDNA. Fragments 5′ and 3′ of this initial fragment were obtained by using combinations of striped bass-specific MsNramp primers (MsNramp736 and MsNramp1020), which were developed by sequencing RT-PCR products, and primers developed for O. mykiss Nramp (MDNM4/1F, MDNM4/2F, OmNramp1263, OmNramp1463) (15). PCR parameters were empirically determined for each primer set, and the PCRs were performed with thermocyclers from MJ Research, Inc. The PCR mixtures (final volume, 50 μl) contained (final concentrations) 1.0 μU of Platinum Taq High Fidelity DNA polymerase, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 2 mM MgSO4, 1× PCR buffer (Invitrogen), each primer at a concentration of 0.2 μM, and 1 to 2 μl of cDNA template. A total of 1,242 bp of MsNramp sequence was generated in this manner. Tissue expression of MsNramp was shown by amplification of cDNA from a variety of tissues (see above) by using primer sets (NrampA plus MDNM4, MDNM4/1F plus OmNramp1463, and MsNramp736 plus MsNramp1020). MsNramp-positive tissues were visualized by 1% agarose gel electrophoresis and ethidium bromide staining.

**RNA ligase-mediated RACE.** The 5′ and 3′ ends of MsNramp cDNA were amplified by RACE, based on procedures developed by Frohman et al. (20). The 5′ and 3′ ends of MsNramp were isolated by using a GeneRacer kit (Invitrogen). For the 5′ end, 5 μg of RNA from mycobacterium-inoculated striped bass PE cells was dephosphorylated with calf intestinal phosphatase, and the 5′ cap structure was removed by using tobacco acid pyrophosphatase. An RNA oligonucleotide sequence was ligated to the dephosphorylated, decapped 5′ end of striped bass mRNA, and the hybrid molecule was reverse transcribed by using SuperScript II RT. RACE-ready 3′ cDNA was obtained by RT of 5 μg of PE cells RNA by using the GeneRacer Oligo dT primer, a modified oligo(dT) primer with a 3′-nucleotide tail of full-length mRNA. RACE-ready first-strand cDNA was treated with RNase H to remove the RNA template.

The RACE PCR mixture for 5′ MsNramp consisted of 1 μl of RACE-ready 5′ cDNA, 0.6 μM GeneRacer 5′ primer (complementary to the GeneRacer RNA oligonucleotide ligated to 5′ cDNA), 0.2 μM gene-specific primer 5RACE1, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1× PCR buffer, 2 mM MgSO4, and 2.5 U of Platinum Taq DNA polymerase. The cycling parameters for a touchdown PCR program were as follows: 94°C for 2 min, 94°C for 0.5 min, and 72°C for 1 min for five cycles; 94°C for 0.5 min and 70°C for 1 min for five cycles; 94°C for 0.5 min and 68°C for 1.5 min for 25 cycles; and 68°C for 10 min.

The 5′ RACE PCR mixture for MsNramp consisted of reaction components similar to those in the 5′ RACE PCR mixture, with the following exceptions: 1 μl of RACE-ready 3′ cDNA, 0.6 μM GeneRacer 3′ primer [complementary to the 3′-nucleotide tail of the oligo(dT) primer], and 0.2 μM primer 3RACE1; 0.2
μM primer 3RACE2, or 0.2 μM primer 3RACE4. The cycling parameters for primer 3RACE1 were as follows: 94°C for 2 min; 94°C for 0.5 min and 72°C for 2 min for five cycles; 94°C for 0.5 min and 70°C for 2 min for five cycles; 94°C for 0.5 min, 65°C for 0.5 min, and 68°C for 2 min for 25 cycles; and 68°C for 10 min.

Multiple products were obtained in the reaction initiated with primer 3RACE1, so a nested PCR was performed by using the standard PCR components along with 0.2 μM GeneRacer 3′ nested primer, 0.2 μM primer 3RACE2, and 1 μl of the 3RACE1-amplified products. The conditions for this reaction were optimized as follows: 94°C for 2 min; 94°C for 0.5 min, 65°C for 0.5 min, and 68°C for 2 min for 25 cycles; and 68°C for 10 min. Primer 3RACE4 was used to confirm that a full-length sequence was obtained after 3RACE2 products were sequenced. The reaction conditions and cycling parameters were identical to those used for primer 3RACE1.

Cloning. Putative internal MsNrramp fragments were blunt-end cloned into pST-Blue-1 by using T4 DNA ligase and were transformed into E. coli (Invitrogen). Insert-containing vector molecules were transformed into Escherichia coli formants were grown on Luria broth (LB) agar plates with kanamycin selection used for primer 3RACE1. Sequencing reaction (LI-COR Biosciences). At least 10 clones were sequenced.

GeneRacer 3′ primer
GeneRacer 3′ nested primer
Msf 3′ nested primer
M13R 1R Dye 700

Sequence analysis. MsNrramp fragments were bidirectionally determined with a LiCor 4000L DNA sequencer by the dye chain termination method by using a ThermoSequence cycle sequencing kit according to the manufacturer’s instructions (Amersham Biosciences). Plasmid DNA (1 to 2 μg) and 3 pmol of the fluorescent primers M13F (forward) and M13R (reverse) were used in the sequencing reaction (LI-COR Biosciences). At least 10 clones were sequenced for each fragment.

Sequence analysis. MsNrramp fragments were aligned and edited in Sequencer (version 4.1; Gene Codes Corp.). Full-length cDNA nucleotide and deduced amino acid sequences were analyzed to determine similarity to previously published sequences by using GenBank resources (http://www.ncbi.nlm.nih.gov/GenBank/index.html). Searches for similar sequences were performed by using the Basic Local Alignment Tool (BLAST) algorithms (1). Multiple-sequence alignment was performed by using ClustalX (version 1.81) (58). Potential microsatellite sequences were detected with the Tandem Repeats Finder software (version 3.23) (6), and polyadenylation signals were analyzed by using polya55 (59). The amino acid sequences of the following proteins were used in the alignment and phylogenetic analyses: Bos taurus Nrramp1 (GenBank accession number U12852), C. carpio Nrramp (AF529267), Drosophila melanogaster malvoglio (U23948), Gallus gallus Nrramp1 (U40598), Homo sapiens Nrramp1 (L32185), H. sapiens Nrramp2 (NP_000608), I. punctatus NrrampC (AF400108), Macaca fascicularis (AF152739), M. musculus MsNrramp (AY008876), Mus musculus Nrramp1 (AAA39838), M. musculus Nrramp2 (AAC24051), O. mykiss Nrramp (AF048760), O. mykiss Nrramp (AF048761), Ovis aries Nrramp (U70255), Pinus palustris Nrramp (AF190773), R. norvegicus DCT1 (A535319), T. rubripes Nrramp (AJ496549), and T. rubripes Nrramp (AJ496550). TM were predicted by using HMMTOP (version 2.0) (60), and a motif analysis was performed by using the PROSITE reference library (34).

Phylogenetic analysis. A phylogenetic analysis was conducted by using the MEGA software (version 2.1) (35). An optimal tree was constructed by using the pairwise distance model and neighbor joining (50). Indels were removed from the multiple-sequence alignment, and the reliability of the trees was assessed by examining 10,000 bootstrap replicates. Drosophila malvoglio (48) was used as an outgroup.

Mycoptera. M. marinus (Virginia Institute of Marine Science strain M30) (M. W. Rhodes, I. Kaattari, S. Kotob, H. Kator, W. K. Vogelbein, E. Shotts, and S. Kaattari, Fish Health Sect. Am. Fish. Soc. Annu. Conf., abst. Q-423, 2000) and M. shottsii (Virginia Institute of Marine Science strain M175 [= ATCC 700981]) (46) were isolated from splenic tissue of Chesapeake Bay striped bass and grown as described by Gauthier et al. (21). Briefly, mycoptera were inoculated into Middlebrook 7H9 medium with oleate-albumin-dextrose-catalase enrichment and 0.05% polysineethylenesorbitan monosolate (Tween 80) and grown until the log phase (10 days). Cultures were pelleted by centrifugation at 12,000 × g for 20 min and washed once in phosphate-buffered saline (PBS) with 0.05% Tween 80 (PB). Washed cultures were resuspended in PB, vortexed vigorously with glass beads (diameter, 50 μm) for 2 min, and filtered through Whatman no. 1 paper to reduce clumping and obtain a homogeneous suspension. The absorbance at 590 nm was adjusted to PB with 0.05 (concentration, approximately 107 CFU/ml), and the preparation was diluted 10-fold prior to injection with PBS. Efluorant water from the isolation facility was treated for a minimum contact time of 20 min with hypochlorite maintained at a diluted final concentration of 100 mg/liter after the fish were infected with mycoptera.
Infection. Immediately before introduction of striped bass into the isolation facility, fish were separated into three groups (10 fish each), anesthetized by using 100 mg of Finquel (MS-222; Argent Chemical) per liter, weighed, and inoculated intraperitoneally with 1.5 ml of a diluted mycobacterial suspension or sterile PBS. Group 1 fish received 1.5 ml of PBS; group 2 fish received 1.4 × 10^6 CFU of M. marinum; and group 3 fish received 0.93 × 10^6 CFU of M. shottsii. In order to model mycobacterial infections as they might appear in a wild population (i.e., a long-term, chronic condition with low initial doses), the mycobacterial doses were adjusted to ensure that fish received a sublethal challenge that corresponded to approximately 5,000 CFU/g. Previous work indicated that the doses used were sublethal and would establish chronic infections (21). The doses injected were calculated by plating mycobacteria on Middlebrook agar.

Sampling. Three fish from each group were randomly selected 1, 3, and 15 days postinoculation, anesthetized with a lethal dose of Finquel (500 mg/liter), and dissected to remove tissues for measurement of MsNramp. All media and reagents used for sample preparation and storage were obtained from Sigma Chemical unless indicated otherwise. Samples (100 to 200 mg) of anterior kidney, spleen, and white muscle were removed from groups 1 and 3, and 100 to 200 mg of PM were removed from group 2. Tissues were homogenized and processed for electron microscopy (EM) and RNA extraction. Samples were stored in RNAlater by dilution with 1 volume of HBSS and centrifugation at 4,000 rpm for 30 min. Anterior kidney, spleen, and white muscle samples were shipped on dry ice and stored at −80°C.

RNA extraction, washed once in HBSS, and resuspended in RNAlater. An aliquot containing 2 × 10^6 cells in HBSS was adhered to individual glass slides by using 100 mg of Finquel (MS-222; Argent Chemical) per liter, weighed, and fixated in 1.5% glutaraldehyde for 1 h for EM analysis. The remaining cells were processed for sample preparation and storage. Glutaraldehyde-fixed cells were post fixed in 1.5% osmium tetroxide and stained with uranyl acetate and lead citrate for 7 min. Stained sections were examined in a transmission electron microscope (TEM) equipped with a SIT charge-coupled device camera (Olympus). The TEM was operated at 80 kV.

RESULTS

MsNramp is a 3,530-bp gene encoding a 554-amino-acid protein. M. x. PE cell cDNA for MsNramp was isolated by using combinations of consensus mammal-, trout-, and striped bass-specific primers (Table 1). Internal coding region sequences were obtained by using primers NramPA and NramB, primers NramPA and MDNM1P, and primers MDNM1P and OmNm1463. A total of 1,242 bp of the internal coding region was sequenced in this manner. The internal fragments were used to design primers 5RACE1, 3RACE1, 3RACE2, and 3RACE4 for use in 5' and 3' RACE-PCR. 5' RACE, performed with primers 5RACE1 and the GeneRacer 5' primer (Invitrogen), produced a 620-bp product that included a 183-bp 5' UTR and the translation start codon at position 184. 3' RACE fragments contained a TAG termination codon at position 1848 and a 1,682-bp 3' UTR. Compilation and alignment of all the fragments produced by RT-PCR and RACE demonstrated that MsNramp is a 3,530-bp gene with a 1,665-nucleotide single open reading frame encoding a 554-amino-acid polypeptide (Fig. 1). There are three

### Table 2. Tissue-specific constitutive expression of MsNramp

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cp for controls (avg ± SEM)f</th>
<th>PCRb</th>
<th>Fold difference compared with white musclec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior kidney</td>
<td>21.01 ± 0.29</td>
<td>1.72</td>
<td>85</td>
</tr>
<tr>
<td>PE cells</td>
<td>23.54 ± 0.18</td>
<td>1.80</td>
<td>28</td>
</tr>
<tr>
<td>Spleen</td>
<td>18.90 ± 0.32</td>
<td>1.70</td>
<td>240</td>
</tr>
<tr>
<td>White muscle</td>
<td>29.20 ± 0.32</td>
<td>1.72</td>
<td>NA</td>
</tr>
</tbody>
</table>

a The average crossing point (Cp) was calculated from nine control fish (three fish per time point); the crossing point is the cycle during amplification (45 total cycles) at which fluorescence rises above the background level. The values for all tissues types are significantly different (P < 0.01).

b PCR is the efficiency of the PCR for each tissue type.

c The fold difference was calculated by using PCRn, PCRp, NA, not applicable.

### REFERENCES


regions of tandem repeats in the 3′ UTR, at positions 1876 to 2090 (consensus pattern TTCCTCT), 2050 to 2071 (AATCA GAA), and 2949 to 2971 (GTGTGATAAAAT). Two prospective, atypical polyadenylation signals are present at position 3425 (nonamer) and position 3436 (hexamer) and are followed closely by a poly(dA) tail that is at least 32 nucleotides long.

Striped bass MsNramp has all the important motifs and regulatory elements of mammalian Nramp. The deduced amino acid sequence of striped bass MsNramp shows that this protein has a minimum molecular mass of 61,157 and contains 12 putative membrane-spanning domains composed of highly hydrophobic amino acids (Fig. 2). Analysis of the protein topology predicted that the amino and carboxy termini exist below the membrane with alternating internal and external loops of hydrophilic amino acids. MsNramp contains three potential amino-linked glycosylation sites in the external loop between TM 7 and TM 8. Two potential phosphorylation sites related to protein kinase C, along with two casein kinase phosphorylation signatures, are located in the amino terminus, and a single protein kinase C site and two additional casein kinase

![FIG. 1. Striped bass MsNramp nucleotide and MsNramp amino acid sequences (GenBank accession number AY008746). Included are the 183-bp 5′ UTR, the 1,665-bp open reading frame, and the 1,682-bp 3′ UTR. The numbers on the right indicate nucleotide and amino acid positions. The brackets indicate the ATG translation start codon (position 184) and the TAG termination codon (position 1848). Single-letter amino acid designations are located under the second nucleotide of the corresponding codons. The arrows indicate primer binding (see Table 1 and 2 for sequences). The motifs include potential microsatellite tandem repeats, the shaded areas indicate polyadenylation signals, and the double box indicates the poly(dA) tail.](http://iai.asm.org/)

![Figure 1](http://iai.asm.org/)

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FIG. 2. ClustalX amino acid alignment of selected Nramp homologs. Abbreviations: Ms, *M. saxatilis* Nramp; Tr, *T. rubripes* Nramp/H9252; Dr, *D. rerio* Nramp; Cc, *C. carpio* Nramp; Ip, *I. punctatus* Nramp; Om, *O. mykiss* Nramp/H9252; Hs, *H. sapiens* Nramp2; Mm, *M. musculus* Nramp2. Accession numbers are listed in Materials and Methods. Periods indicate amino acid identity compared to the striped bass sequence. The residues surrounded by thick lines are potential protein kinase C (PKC) phosphorylation sites, and the areas in boxes are casein kinase (CK) motifs. The polypeptides that are in double boxes and shaded are putative TM domains. The polypeptide that is indicated by white text on a black background is the transport system inner membrane component signature (transport).
motifs are present in the carboxy-terminal end. A single tyrosine kinase site is located between TM 6 and TM 7. Each of the potential phosphorylation sites is located within hydrophilic amino acid subsequences predicted to be intracellular. A highly conserved binding-protein-dependent transport system inner membrane component signature, a prominent feature of murine Nramp1 and the proteins encoded by members of several other iron transporter and channel gene families (13), is located in the intracellular region between TM 8 and TM 9. Unlike the C. carpio homolog (49) and other Nramp2 isoforms (31, 40) but similar to channel catfish NrampC (12), no identifiable iron-responsive regulatory-binding-protein site (IRE) consensus sequence (CNNNNNCAGUG) (9) was identified in the striped bass 3' UTR.

Alignment and phylogenetic analysis group striped bass MsNramp with other teleost and mammalian Nramp2 proteins. The striped bass MsNramp nucleotide and MsNramp amino acid sequences were aligned with the sequences of other vertebrate homologs (Nramp1 and Nramp2) (Fig. 2) in order to examine potentially important distinguishing characteristics. Three distinct clades were evident in the phylogenetic analysis of Nramp nucleotide and Nramp amino acid sequences. Vertebrate Nramp1 clustered in one subgroup, while all teleost sequences clustered with mammalian Nramp2 proteins. Nramp sequences of the Cyprinidae and both paralogs from rainbow trout formed a separate clade within the teleost sequences. MsNramp was phylogenetically most similar to T. rubripes Nrampβ (Fig. 3).

MsNramp mRNA is expressed in several tissues but at variable levels. Anterior kidney, brain, heart, gill, female and male gonad, intestine, liver, muscle, PE, peripheral blood leukocyte, and spleen samples were positive for MsNramp mRNA transcripts as determined by qualitative RT-PCR with three primer sets for three male and female striped bass (data not shown). PCR performed with total RNA prior to cDNA generation confirmed that no genomic DNA contamination was present in the mRNA samples. For each tissue 5 μg of RNA was analyzed. Constitutive expression of MsNramp in striped bass tissues was variable, as determined by real-time semiquantitative RT-PCR. The average crossing points for the different tissue types of control striped bass demonstrated that there were large constitutive differences in MsNramp expression. Comparison of constitutive expression (Table 2) in different tissues revealed that expression of MsNramp was lowest in white muscle. The levels of expression were approximately 28-, 85-, and 240-fold higher in PE, anterior kidney, and spleen samples, respectively. Analysis of variance and Tukey’s multiple-comparison test showed that each tissue type was significantly different from each of the other tissue types (P < 0.01).

Cytology and ultrastructure confirmed that infection of PE cells occur within 1 day of injection of mycobacteria. To confirm that striped bass exposed to Mycobacterium harbored mycobacteria intracellularly within 1 day after infection, light microscopy and transmission electron microscopy were used to examine PE cells. All infected and control fish survived to the end of the experiment (15 days), and no outward manifestations of disease were apparent. Fish inoculated with M. marinum had gross inflammation of visceral fat and mesenteries at 15 days postinfection, whereas sham-inoculated fish and M. shottsii-inoculated fish displayed no gross inflammation.
Wright-Giemsa-stained cytospin preparations showed that peritoneal lavages were composed primarily of macrophages (>50%), along with various numbers of lymphocytes and thrombocytes and low numbers of granulocytes. Ziehl-Neelsen staining indicated that both *M. marinum* and *M. shottsii* were phagocytosed by PE cells 1 day after injection. Electron microscopy revealed mycobacteria within membrane-limited phagosome macrophages (Fig. 4).

**MsNramp in *M. marinum*-inoculated striped bass is highly induced.** A primary objective of this study was to document the induction of *MsNramp* in fish exposed to *M. marinum* and *M. shottsii* (Fig. 5). The expression of *MsNramp* in PE cells of striped bass infected with *M. marinum* (Fig. 5B) was 1,701.0% ± 14.02% higher than the expression in control PE cells within 1 day after injection. The levels of *MsNramp* in PE cells continued to be elevated after 3 and 15 days, and the levels of expression were 412.46% ± 10.00% and 623.47% ± 66.66% of the level of expression in the control, respectively. Anterior kidney (Fig. 5A) *MsNramp* expression in fish exposed to *M. marinum* did not differ significantly from control expression until 15 days, when the anterior kidney level of expression was 130.31% ± 10.58% of the level of expression in the control. No significant increases were seen in spleen or white muscle samples on any day (data not shown).

**M. shottsii induces expression of MsNramp in striped bass PE cells.** PE cells of striped bass inoculated with *M. shottsii* expressed *MsNramp* at levels that were 216.47% ± 9.86%, 461.34% ± 20.79%, and 311.89% ± 21.45% of the control levels on days 1, 3, and 15, respectively (Fig. 5B). No significant induction of *MsNramp* was seen in anterior kidney samples (Fig. 5A) at any time after injection. The expression in the spleen was depressed at 3 days (*P < 0.05*) compared with the expression in day 3 controls, but by 15 days the levels of *MsNramp* in spleen samples were statistically similar for control and *M. shottsii*-inoculated fish. The results for white muscle were more variable than the results for the other tissues, although the values were much lower, and depression of *MsNramp* transcription was observed at day 1 (*P < 0.01*) (data not shown).

**DISCUSSION**

In this study, we examined the in vivo expression of the *M. saxatilis* homolog of the Nramp gene (*MsNramp*) during exposure to mycobacteria. We found that striped bass *MsNramp* transcription is significantly upregulated (17-fold) in PE cells following infection with *M. marinum, M. shottsii* also induced expression, although not to the degree seen with *M. marinum*. Induction of expression was rapid (less than 24 h) and long lasting (more than 15 days) in PE cells. The long-lasting nature of the induction may have been the result of mycobacterial replication, additional infection of previously naive PE cells, and/or long-lasting induction within individual PE cells. Stimulation of mouse bone marrow-derived macrophages with LPS and gamma interferon has shown that there is rapid induction of Nramp1, which peaks at 12 h (3), while functional alleles of murine *Nramp1* have a bacteriostatic effect on *Mycobacterium avium* containing phagosomes for at least 10 days (19). In the murine model, Nramp1 is recruited to the membrane of the phagolysosome during the initial stages of mycobacterial infection (29), where it maintains the fusiogenic properties of this compartment (19). It is likely, based on the high sequence similarity between mammalian and striped bass homologs and on the pattern of induction seen for mycobacterium-infected PE cells, that striped bass uses the gene product in a similar fashion.

PE cells of fishes consist of an enriched population of highly activated phagocytes that serve as important mediators of the immune response to infection within the peritoneal cavity (16). Assuming that the elevated levels of *MsNramp* expression in peritoneal preparations is of macrophage origin, lower total levels of *MsNramp* in anterior kidney and spleen samples from fish inoculated with mycobacteria would be expected as the proportion of macrophages in these tissues is significantly lower than the proportion of PE cells. Trafficking of mycobacteria by infected PE cells may account for the late increase in *MsNramp* expression observed in anterior kidney samples. Previous work has shown that well-developed granulomas are not present histologically in anterior kidney samples 2 weeks after...
intraperitoneal injection of mycobacteria (21). In the same study the workers did observe small numbers of acid-fast mycobacteria within inflammatory foci of anterior kidney and spleen samples 2 weeks after intraperitoneal injection. Longer-term analysis of MsNramp expression may indicate that as infection occurs within the anterior kidney and spleen, differentiation and activation of resident macrophages are accompanied by upregulation of this gene.

The conserved features of MsNramp include a topology of 12 TM, N- and C-terminal phosphorylation sites, extracytoplasmic glycosylation, and the binding protein-dependent transport system inner membrane component signature. The transport signature is implicated in ATP-binding related to transport functions (2) and is found in several gene families whose members encode iron transporters and channels (13). Alternative splicing has been identified in several Nramp2 homologs, including human (40), mouse and rat (56), macaque (65), and channel catfish (12) homologs. In the mammalian Nramp studies, alternative splicing was shown to correspond to alternate C-terminal amino acids, distinctive subcellular or tissue expression, and the presence or absence of an IRE. IREs are stem-loop RNA structures often found in genes that are posttranscriptionally regulated by cellular iron concentrations, as Nramp2 (DCT1) appears to be in rats (30). Channel catfish splice variants resulted in a single, non-IRE-encoding open reading frame, regardless of which transcript was translated. No genetic evidence for a second locus or alternative splicing was found for the striped bass homolog, and preliminary work with polyclonal antisera directed against the C-terminal 20 amino acids of channel catfish NrampC resulted in identification of a single band in striped bass but two separate bands in channel catfish (Charles Rice, Clemson University, personal communication).

In summary, isolation of important disease resistance loci and characterization of gene products are important preliminary steps toward a greater understanding of disease resistance in economically valuable finfish (17). Genes responsible for innate resistance to intracellular pathogens are likely candidates for selective breeding in aquaculture (62) and enhance our understanding of the evolution of innate immunity in vertebrates. This study demonstrated that striped bass contain a highly conserved natural resistance-associated macrophage protein, MsNramp, that has a high level of homology to mammalian Nramp2 and has all the hallmark features of the proteins encoded by the Nramp gene family described for humans (37, 38) and mice (28, 61). MsNramp is induced in vivo in PE cells within 1 day of injection of Mycobacterium spp. This is the
first report of induction of an Nramp gene from fish exposed to intracellular pathogens.

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