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A Mycobacterium-inducible Nramp in striped bass *Morone saxatilis*

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*A MYCOBACTERIUM-INDUCIBLE NRAMP IN
STRIPED BASS MORONE SAXATILIS*

A Dissertation

Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of
Doctor of Philosophy

by

Erin Jeffrey Burge

2003


APPROVAL SHEET

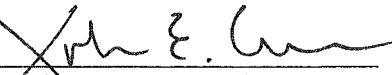
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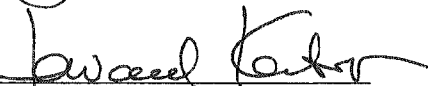
Doctor of Philosophy


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Approved, December 2003


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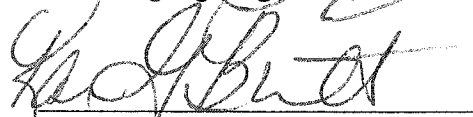

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DEDICATION

This work is dedicated in memory of my grandfathers, Robert Burge and John Houck, my grandmothers, Joan Burge, Arbeautis Rice and Bernice Houck, and to my parents. They believed I could.

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ABSTRACT

In mammals, the natural resistance-associated macrophage protein 1 gene, *Nramp1*, plays a major role in resistance to mycobacterial infections. Chesapeake Bay (USA) striped bass, *Morone saxatilis*, are currently experiencing an epizootic of mycobacteriosis that threatens the health of this ecologically and economically important species. This dissertation characterizes an *Nramp* gene in this species (*MsNramp*) and provide evidence for induction following *Mycobacterium* exposure.

The striped bass *MsNramp* gene and 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 groups *MsNramp* with other teleost *Nramps*, and exhibits high sequence similarity with mammalian *Nramp2*. *MsNramp* expression was present in all tissues assayed by RT-PCR.

Within one day of injection with *Mycobacterium marinum*, *MsNramp* expression *in vivo* was highly induced (17-fold) in peritoneal exudate cells (PE) relative to controls. Levels of *MsNramp* were increased three- and six-fold on days three and 15, respectively. Injection with *Mycobacterium shottsii* resulted in two-, five-, and three-fold increases in gene expression in PE over the time course. *In vitro*, PE expressed significantly higher levels of *MsNramp* at 4 and 24 hours post-treatment with live and heat-killed *M. marinum*. *MsNramp* response to LPS was dose-dependent in these cells, with maximum expression at 4 hr and 20 µg/ml LPS. Treatment of PE with LPS caused an increase in intracellular superoxide anion (O_2^-) levels, whereas treatment with live *M. marinum* caused a significant depression. Cultured anterior kidney cells responded to LPS with increased O_2^- and *MsNramp* production, but were uninduced or suppressed relative to controls by mycobacteria. This study represents the first report of induction of an *Nramp* gene by mycobacteria *in vivo* or *in vitro* in a poikilothermic vertebrate, and supports reports of teleost *Nramp* induction by LPS.

*A MYCOBACTERIUM-INDUCIBLE NRAMP IN
STRIPED BASS MORONE SAXATILIS*

GENERAL INTRODUCTION

Life history and economic importance of striped bass

The striped bass or rockfish (*Morone saxatilis*; Figure 1) is an anadromous teleost fish naturally distributed on the east coast of North America from Nova Scotia, Canada, to northern Florida and is one of the most commercially important species currently targeted by the U. S. coastal fishery (Austin, 1980). Relict and introduced populations are also present in several river basins of the Gulf of Mexico (Wirgin et al., 1997). Introduced populations exist on the west coast of North America from Ensenada, Mexico to British Columbia and throughout the larger reservoirs of the inland United States. Four main reproductive stocks make up the bulk of the native and introduced populations. They are known as the Atlantic coastal migratory stock, the North Carolina stock, the South Atlantic stock and the West Coast (Sacramento-San Joaquin) stock (Austin, 1980).

Striped bass growth rates are highly variable and are directly influenced by environmental and geographic factors. Habitat selection, feeding and reproduction are limited by temperatures above approximately 25°C for adult fish, with a preferred temperature range of 10 to 20°C (Coutant, 1985). Age and size at maturity are also variable, but males tend to mature (year 2+) earlier and faster than females (year 4+). Maximum age is >30 years (Secor, 2000) and an individual of 125 pounds was captured in North Carolina in 1891 (Murdy et al., 1997). Sexual dimorphism is primarily in size

only, with the largest fish almost exclusively female. Fecundity is a function of adult body size (Austin, 1980).

The striped bass is a valuable recreational target species for millions of anglers (Richards and Deuel, 1987). Due to a precipitous decline in coast-wide stocks throughout the 1970s and 1980s, the Atlantic States Marine Fisheries Commission (ASMFC)(1981) implemented an interstate fisheries management plan which resulted in a fishing moratorium. In 1984, federal oversight was increased and the states were mandated to restrict fishing and restore the striped bass population. Improved spawning success, especially in the Chesapeake Bay during the late 1980s, led to the reopening of a limited fishery in 1990 (Field, 1997). By 1995, stocks of striped bass were declared recovered and ASMFC allowed the states to expand their own fisheries. In the following years, stock abundance reached historically high levels. Commercial landings from 1990-1998 were worth more than \$40.3 million wholesale. Recreational anglers pursuing striped bass spent additional millions of dollars. Directed striped bass trips (defined as any trip where an angler targeted and/or caught a striped bass), exceeded 6.6 million in 1998 and approximately one in four saltwater trips made from Maine to North Carolina in 1997 and 1998 were specifically for striped bass (Personal communication from the National Marine Fisheries Service, Fisheries Statistics and Economics Division).

Infectious and parasitic diseases of striped bass

The utilization of striped bass as a significant recreational and commercial target species and rapidly increasing production in aquaculture for this species and its hybrids have stimulated a significant amount of research into the infectious diseases and parasites that

infect striped bass. Bacterial infections have received the greatest amount of attention as they are most often implicated in economic losses associated with aquaculture. Much less is known about the viral and parasitic diseases that affect wild and cultured fish (McAllister et al., 1987).

Viral diseases

Striped bass have been reported to be infected with several viruses that can cause mortality in other species, but the economic losses associated with aquaculture are minimal. Lymphocystis virus infection has been diagnosed from wild striped bass collected in the Chesapeake Bay (Krantz, 1970; Paperna and Zwerner, 1976) and North Carolina estuaries (Noga et al., 1991). Infectious pancreatic necrosis virus (IPNV) has been isolated as the cause of morbidity in hatchery-raised striped bass fry from Maryland (Schutz et al., 1984) but subsequently demonstrated to be nonpathogenic in experimental infections (Wechsler et al., 1986; Wechsler et al., 1987). Horizontal infection of brook trout (*Salvelinus fontinalis*) by striped bass carriers of IPNV has been documented (McAllister and McAllister, 1988).

Protozoan and metazoan infections

Reports of protozoan and metazoan infections in striped bass are widespread from both wild and cultured populations (McAllister et al., 1987), but incidents of economic losses to aquaculture have not been widely described. Paperna and Zwerner (1976) provided a comprehensive survey of the parasites of striped bass commonly occurring in the Chesapeake Bay. The authors sampled 654 one-year old and young-of-the-year striped

bass from May of 1972 until May 1973. Pathology was associated with many of the common infections, with *Ergasilus labracis*, *Philometra rubra*, *Pomphorhynchus rocci*, larval helminthes, and *Argulus bicolor* being the most prevalent infections during that time period. An epizootic of amyloodiniosis, caused by the protozoan *Amyloodinium ocellatum*, was reported in cultured striped bass hybrids (Smith et al., 1994) and has been an infrequent problem in captive, wild striped bass maintained long-term at Virginia Institute of Marine Science, Gloucester Point, Virginia, facilities (D. T. Gauthier, C. E. Harris and E. J. Burge, personal observations).

Bacterial infections

Striped bass are affected by many common freshwater and marine bacteria associated with morbidity and mortality in other fish species. Outbreaks of vibriosis (*Vibrio* spp.; usually *V. anguillarum*) in culture systems (Nedoluha and Westhoff, 1997; Toranzo et al., 1983a) and the routine isolation of *Vibrio* spp. from wild (Baya et al., 1992; MacFarlane et al., 1986b) and captive populations led to the successful application of a vibrio bacterin vaccine with efficacy in striped bass (Rogers and Xu, 1992). *Aeromonas* spp. (usually *A. hydrophila*) have been cultured from fresh- and low salinity-water striped bass and its hybrids (Hrubec et al., 1996; MacFarlane et al., 1986b; Nedoluha and Westhoff, 1995; Toranzo et al., 1983b) and implicated in disease (Hawke, 1976). Snieszko et al. (1964) identified *Pasteurella* sp. (now *Photobacterium*) associated with epizootics in white perch, *Morone americana*, and striped bass from the Chesapeake Bay. In another study, infections with *Pasteurella piscicida* (now *Photobacterium piscicida*) resulted in losses of 80% of a cultured stock in Alabama (Hawke et al., 1987). An epizootic of

Streptococcus iniae in tank-cultured hybrid striped bass in Massachusetts was the first report of this bacterial species infecting striped bass (Stoffregen et al., 1996). *S. iniae* is a cosmopolitan bacteria commonly associated with freshwater and estuarine environments and has become the focus of more intensive work in recent years (Evans et al., 2000, 2001; Sealey and Gatlin III, 2001; Shoemaker et al., 2001). *Edwardsiella tarda*, the causative agent of edwardsiellosis and commonly associated with massive mortalities of cultured channel catfish (*Ictalurus punctatus*) and European eels (*Anguilla anguilla*), was described as the causative agent of mortality in a significant die off of cultured striped bass fingerlings in Virginia (Herman and Bullock, 1986) and an epizootic in wild adult striped bass in Chesapeake Bay in Maryland (Baya et al., 1997). Fingerlings in culture have also been reported to be especially vulnerable to infection with *Cytophaga columnaris*. This disease is most commonly associated with high-density, freshwater grow-out facilities (MacFarlane et al., 1986a; Plumb, 1991). Chlamydial infections (epitheliocystis) have been implicated in gill pathology in wild striped bass (Zachary and Paperna, 1977) and in salmonid aquaculture production (Kent et al., 1998). Mycobacterioses have been reported from wild and cultured populations of striped bass from California (Hedrick et al., 1987; Sakanari et al., 1983) and wild fish from Chesapeake Bay (Cardinal, 2001). The ongoing epizootic of mycobacteriosis in the Chesapeake and the emergence of this disease as a threat to aquaculture has generated interest in the development of a vaccine prophylactic for aquatic mycobacteriosis (Pasnik et al., 2003). Additionally, one accepted new species of *Mycobacterium* (*M. shottsii*) (Rhodes et al., 2003b) and one unconfirmed new species (*M. chesapeaki*) (Heckert et al., 2001) have been isolated from wild Chesapeake Bay striped bass.

Mycobacteriosis in striped bass and other fish

The genus *Mycobacterium* is a large group (as of the year 2000 there were 82 recognized species; http://www.cdc.gov/ncidod/dastlr/TB/TB_NTM.htm) of gram-positive Actinomycete bacteria with a highly modified cell wall. They are aerobic, acid-fast staining, nonmotile rods, 0.2-0.6 µm in diameter, and 1-4 µm long. There is considerable heterogeneity in growth rates, temperature optima, nutrient requirements and cell wall organization. The cell wall is generally composed of four layers. The innermost layer is the peptidoglycan layer while the next three surface layers are composed of such lipids as mycolic acid, glycolipids, cord factor and wax D (Goodfellow and Magee, 1998). Historically, mycobacteria were divided into two non-taxonomic groups: the typical and atypical mycobacteria. The typical group comprised the obligate human intracellular pathogens (*M. tuberculosis* and *M. leprae*), while the atypical group included those ubiquitous environmental species that function as saprophytes and opportunistic pathogens. A classification scheme that separates mycobacteria species based on growth rates ('fast' vs. 'slow' growers) and chromogenic properties (Kubica et al., 1972; Lèvy-Frèbault and Portaels, 1992) largely supplanted that system. Recently the application of molecular phylogenetics to mycobacteria has led to another attempt to clarify the relationships between mycobacterial species (Rogall et al., 1990).

In 1997, the Virginia Institute of Marine Science Aquatic Animal Disease Research Laboratory (AADDL, Gloucester Point, VA) began receiving striped bass specimens with moderate to severe ulcerative dermatitis (Figure 2A). Concerned recreational fishermen and commercial watermen also began contacting the diagnostic

center to ask questions concerning the safety of consuming these fish and possible water quality issues. During the following year and a half, over 100 cases of this ulcerative dermatitis syndrome were brought to the AADDL. Standard histopathological evaluation of the skin lesions revealed a chronic granulomatous inflammatory response within the ulcer (Vogelbein et al., 1998). Visceral granulomatous inflammation, primarily within the spleen, was often found in fish displaying ulcers. Histochemical staining by the Ziehl-Neelsen method (Luna, 1968) demonstrated the presence of acid-fast bacteria inhabiting the skin lesions and spleen. Acid-fast stains are specific to the *Mycobacterium* and *Nocardia* bacterial genera because the capsule of these bacteria takes up the carbol-fuchsin stain but resists decolorizing with an acid-alcohol rinse (Sheehan and Hrapchak, 1980). After intensive culture efforts, AADDL was able to isolate *Mycobacterium* spp. from the lesions of all affected fish (Rhodes et al., 2000).

Reports of numerous striped bass with lesions from Chesapeake Bay were reported as early as 1994 in Maryland, but it was not until 1997 that mycobacteria were proved to be the etiologic agent (Baya, 1998; Vogelbein et al., 1998). Culture and subsequent characterization identified a new mycobacteria species, *M. shottsii*, that has been isolated from approximately 76% of infected striped bass of Chesapeake Bay (Rhodes et al., 2001b). *Mycobacterium shottsii* is biochemically and phylogenetically (by 16S rRNA) closely related to *M. ulcerans* (Heckert et al., 2001; Rhodes et al., 2003b), a potent human pathogen responsible for Buruli ulcer disease (Stienstra et al., 2001; van der Werf et al., 1999). Microbiological and biochemical phenotyping identified *M. marinum*, *M. peregrinum*, *M. scrofulaceum*, *M. gordonae*, *M. terrae*

complex, and isolates similar to *M. avium* (Kaattari et al., 2001; Rhodes et al., 2001a), as well as *M. shottsii* in Chesapeake Bay striped bass.

Clinical signs in fish of mycobacteriosis vary substantially by host species but can include scale loss, dermal ulcers (Snieszko, 1978), emaciation, exophthalmia, stunted growth (Nigrelli and Vogel, 1963), reduced reproductive success, ascites (Astrofsky et al., 2000), nervous system disruption, keratitis, and dermal pigment abnormalities (Noga, 1996). The disease presents itself as an internal granulomatous tissue nodule with central necrosis associated with acid-fast bacilli. A multi-layer sheath of epithelioid cells comprises the bulk of the granuloma. Internal pathology (Figure 2B) typically includes lesions of the spleen, kidney, liver and mesenteries (Noga, 1996). In the ongoing epizootic in striped bass of the Chesapeake Bay, external signs include severe, but superficial, ulcerative dermatitis characterized by scale loss, hemorrhage, hyperpigmentation of the skin, and a gritty texture. Internal granulomatous inflammation includes pale focal or nodular lesions on internal organs, predominately in the spleen (Rhodes et al., 2001b).

Mycobacteriosis has been reported from greater than 150 freshwater and marine species and was reported from captive striped bass as early as the 1960s (Nigrelli and Vogel, 1963). Previously, external lesions linked to mycobacteriosis have been reported as small, focal tubercles or shallow ulcerations in infected fish raised in intensive aquaculture facilities (Hedrick et al., 1987) or aquaria (Nigrelli and Vogel, 1963). In a study of wild caught marine fish from the San Pablo and San Francisco Bays and adjacent Pacific waters, five *Mycobacterium* spp. were identified: *M. simiae*, *M.*

scrofulaceum, *M. marinum*, *M. chelonae*, and *M. fortuitum*. Notably, striped bass in this study were found to harbor *M. marinum* (Lansdell et al., 1993).

‘Tubercular lesions’ associated with acid-fast bacilli of the liver, spleen and kidney have been described from wild populations of West Coast striped bass with prevalences as high as 25-68% (Sakanari et al., 1983). In an epizootic of cultured striped bass from California, approximately one-half of the fish suffered acute mortality and of the survivors, 80% were infected with *M. marinum* before the entire group was destroyed and the facility disinfected (Hedrick et al., 1987). The source of the infection was not determined, but the population was assayed four months prior to the outbreak and cleared for release into the wild. It was unknown whether feral brood stock brought into the facility were carriers or if the infection was acquired *in situ*. An experimentally induced infection of *Mycobacterium marinum* in 20 striped bass and 20 hybrid tilapia (*Oreochromis niloticus* x *O. mossambicus*) indicated that morbidity and mortality were significantly greater in striped bass within 8 days of infection. Injected doses were extremely high, and intended to induce acute effects. Histological examination of striped bass granulomas showed that they were significantly more numerous, larger, and less discrete when compared to tilapia. Indices of necrosis were also elevated in striped bass, indicative of a more advanced infection (Wolf and Smith, 1999). This study found that mycobacteria caused more severe pathology and disease in striped bass, relative to tilapia. Gauthier et al. (2003) examined short- and long-term experimentally induced mycobacteriosis in striped bass and reported significantly different pathology associated with three *Mycobacterium* spp. *M. marinum* produced severe pathology and underwent a secondary phase of reactivation, while *M. shottsii* and *M. gordonae* established persistent

infections, with acid-fast bacteria present until at least 45 weeks post-injection, but without eliciting granulomatous inflammation. Mycobacteriosis in European sea bass (*Dicentrarchus labrax*), another member of the family *Moronidae*, has become a significant concern to aquaculture of this species in Israel and Europe, and ongoing research into the pathology and host immunology is currently being conducted (Colomi et al., 1998).

A spatial and temporal study analyzed the histological prevalence of mycobacteriosis in 1899 striped bass from the Chesapeake tributaries Potomac, Rappahannock and York Rivers (Cardinal, 2001). Prevalence varied seasonally and by sex, with the highest incidences observed in fall-caught, male fish. Infection rates observed in spleen by histological methods varied from 31.5% to 62.7% from summer to fall, while dermal infections were observed at prevalences of 7.5% to 28.8% during the same time period. Ongoing monitoring of Chesapeake Bay tributaries continues to find mycobacterial infection in striped bass (C. Ottinger, W. Vogelbein, M. Rhodes, V. Blazer, D. Gauthier, E. Burge, unpublished data). Additionally, a large-scale temporal and spatial trawl survey project is being used to assess continuing levels of mycobacteriosis in mainstem Chesapeake Bay striped bass (C. Bonzek, R. Latour, D. Gauthier, and E. Burge, unpublished data).

Several mycobacterial fish pathogens, including *Mycobacterium marinum*, *M. fortuitum* and *M. chelonae*, can cause infections in humans. In fishes the disease is known as mycobacteriosis, but in humans, it is known alternatively as fish handlers' disease, swimming pool or fish tank granuloma (Kator and Rhodes, 1991). Recent work

has shown that other mycobacterial species found in fishes, including striped bass, pose a potential risk of zoonotic infection (Brown-Elliott et al., 2002).

Immune response to mycobacteria in mammals

Human tuberculosis (MTB) is caused by *Mycobacterium tuberculosis* (Flynn and Chan, 2001). The host immune response is generally able to contain, but not eliminate the pathogen. MTB persists intracellularly within macrophages, primarily within the lungs of infected individuals, and can be stimulated by immune suppression to reactivate to a patent infection. Granulomas characteristic of MTB include infected macrophages, giant cells, T cells, B cells, and fibroblasts. Encapsulation within the granuloma allows the bacterium to evade effectively the adaptive immune response leading to a prolonged T cell mediated cellular immune response, with little B cell antibody secretion. Recently, a novel hypothesis has been put forward predicting that *M. tuberculosis* has evolved to exploit the granuloma for its own transmission. It is predicted that MTB enhances the host granulomatous response via molecular cytokine mimicry or some as yet unknown mechanism (Doenhoff, 1997).

Resistance to mycobacterial infections in mammals is due to several physical, chemical and genetic mechanisms. Pathogenic mycobacteria are intracellular macrophage parasites and their persistence within the host is dependent on circumventing or resisting phagolysosome fusion, free-radical-based killing mechanisms, and genetically-based resistance mechanisms. Successful breaching of the primary barriers to infection (the skin and/or mucosa) by mycobacteria results in rapid migration of a variety of immune cells to the site of developing inflammation (Flynn and Chan, 2001).

Phagocytosis of invading microorganisms by polymorphonuclear leukocytes and mononuclear macrophages leads to compartmentalization within the phagosome, effectively removing them from extracellular circulation. The formation of the phagolysosome is a dynamic process that involves fusion with endocytic vacuoles containing a variety of degradative enzymes capable of digesting macromolecules and/or microorganisms. During the maturation process of the phagolysosome a progressive acidification occurs that activates various degradative acid hydrolases and other antibacterial mechanisms.

Entry into host cells by pathogenic mycobacteria is achieved via multiple receptors (Armstrong and Hart, 1975; Schorey et al., 1997; Zimmerli et al., 1996) at the plasma membrane, and regardless of the receptor-mediated phagocytic mechanism, entry is dependent on plasma membrane cholesterol (Gatfield and Pieters, 2000). Typically, the phagosome with ingested microorganisms, including nonpathogenic mycobacteria, undergoes a series of fusions with lysosomes and other endocytic compartments that ultimately kill and degrade the bacteria, but in the case of pathogenic mycobacteria phagosome/lysosome fusion is suppressed. This avoidance of lysosomal killing is dependent on viable pathogenic mycobacteria, as killed organisms are rapidly degraded by phagolysosomes (Armstrong and Hart, 1971). Phagosomes that have ingested mycobacteria do acquire the late endosomal/lysosomal protein marker LAMP-1, indicating continued fusion competence of the MTB phagosome (Clemens and Horwitz, 1996), but they do not acquire components consistent with lysosomal fusion, such as clearance of MHC class I molecules, lysosome-associated glycoproteins or acid proteases like cathepsin D (Clemens and Horwitz, 1995). Molecular support for the hypothesis that

mycobacterial phagosomes do not fuse with lysosomes is provided by the discovery that *M. bovis*-infected macrophages retain the host cell protein TACO (tryptophan asparatate-containing coat), that, when present, inhibits phagosome-lysosome fusion (Ferrari et al., 1999). TACO is normally shed from phagosomes containing pathogens, allowing fusion with lysosomes.

One of the primary effector functions of macrophages in the innate immune response is the generation of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) produced during the macrophage 'respiratory burst' (Figure 3). These are potent bactericidal and bacteriostatic compounds (Goldsby et al., 2000; Nathan and Shiloh, 2000). Nitric oxide synthase type 2 (iNOS or NOS2) generates nitric oxide (NO) from the guanidine nitrogen of L-arginine (Lyons et al., 1992; Xie et al., 1992). Nitric oxide, along with spontaneous breakdown products and enzyme-catalyzed products, are a primary line of chemical host defense against pathogens (Nathan and Shiloh, 2000). Doi et al. (1993) demonstrated that intracellular growth of *M. avium* was significantly suppressed by NO, while that of *M. intracellulare* was unaffected. Differences in the efficacy of NO in suppressing growth of MTB strains has also been documented (Flesch and Kaufmann, 1987). Definitive evidence for the role of NOS2 as a protective gene against MTB was shown using NOS2^{-/-} knockout mice. Homozygous deficient individuals, and those immunosuppressed by glucocorticoid treatment, rapidly succumb to MTB. Inhibition of NOS2 by an L-arginine analog resulted in rapid progression of MTB infection in wild-type mice (MacMicking et al., 1997). Resolution or progression of infection differs significantly based on the route of infection (aerogenic

vs. intravenous inoculation) in wild-type and *NOS2^{-/-}* mice infected with laboratory and clinical strains of MTB (Scanga et al., 2001).

The ROI response of mammalian and fish macrophages and neutrophils (Figure 3) is due to the action of respiratory burst oxidase, or more appropriately, NADPH oxidase, a multisubunit cytochrome protein. This enzyme utilizes NADPH as a reducing substrate to generate superoxide anion (O_2^-) from molecular oxygen. Superoxide anion spontaneously or via enzyme-catalyzed transformation produces hydrogen peroxide (H_2O_2), hydroxyl radical (OH^\cdot), hypochlorous acid (OCI^\cdot) and peroxynitrite ($ONOO^-$). Each of these compounds, and superoxide anion, are potent antimicrobial compounds (Nathan and Shiloh, 2000). While the role of RNI in antimycobacterial activity is relatively well established, the absolute need for ROI has yet to be definitively demonstrated (Flesch and Kaufmann, 1987; Segal et al., 1999; Walker and Lowrie, 1981). The recent development of *phox^{-/-} NOS2^{-/-}* mouse strains will significantly advance understanding of the role of redundancy of ROI and RNI in mouse models of tuberculosis (Shiloh et al., 1999).

Regulation of the ROI and RNI responses is mediated by numerous cytokines, of which interferon-gamma ($IFN-\gamma$) and tumor necrosis factor-alpha ($TNF-\alpha$) play prominent roles. $IFN-\gamma$ and $TNF-\alpha$ act synergistically to activate the RNI pathway of macrophages independent of infection (Ding et al., 1988), promote tuberculostasis in MTB infection (Flesch and Kaufmann, 1990) and induce expression of inducible NOS2 in response to MTB in human monocytes and monocyte-like cell lines (Jagannath et al., 1998). $TNF-\alpha$ deficient mouse strains show enhanced necrosis within granulomas (Flynn et al., 1995) in response to MTB, while treatment with antibodies against $TNF-\alpha$ allows

regression of granulomas in mouse models of tuberculosis (Kindler et al., 1989), both indicative of reduced mycobactericidal activity and exacerbation of disease. IFN- γ and TNF- α are characteristic of a type 1 T-helper immune response (T_H1). A T_H1 response is specific to T cell/macrophage activation leading to a cell-mediated immune response, granuloma formation and the secretion of proinflammatory cytokines (Flynn and Chan, 2001). This type of immune response is considered a 'successful' response that can lead to toleration or clearance of mycobacterial disease (Mutis et al., 1993). A T_H2 profile is characteristic of T-helper activation of B cells leading to antibody secretion and an adaptive immune response (Goldsby et al., 2000). In mycobacterial infections T_H2 responses often are associated with exacerbated disease in humans (Lienhardt et al., 2002). As in most chronic diseases, there is a dynamic expression of cytokines that mediate the host response.

Genetic resistance to mycobacterial infection in mammals involves more than the direct antimicrobial gene expression events that result in cytokine expression and induction of proteins like NADPH oxidase and iNOS. Recent work has focused on the role of the Toll-like receptors and their activation of innate immunity. Certain members of the Toll protein family are known to act as signal transduction molecules that influence cytokine-mediated activation of the innate response (Stenger and Modlin, 2002). Vidal et al. (1993) cloned and sequenced a cDNA named the natural resistance-associated macrophage protein, *Nramp* (gene name is *italicized*, protein product in normal type), that encoded a novel transport protein responsible for resistance to antigenically and taxonomically distinct intracellular parasites, including *Mycobacterium* spp.

Role of *Nramp* to mammals in host resistance

Breeding studies with *Mycobacterium*- resistant (*Bcg'*) and –susceptible (*Bcg^s*) inbred mouse phenotypes identified a single dominant, autosomal gene (termed *Bcg*) responsible for increased resistance to mycobacteria during the initial, early stage of infection (Gros et al., 1981). Research with *Leishmania donovani* (Bradley et al., 1979) and *Salmonella typhimurium* (Plant and Glynn, 1976) identified closely linked or identical loci (*Lsh* and *Ity*, respectively) for resistance to these antigenically and taxonomically unrelated intracellular parasites. Positional cloning of *Bcg/Ity/Lsh* from the proximal region of mouse chromosome 1 led to the discovery of the *Nramp1* gene at that locus (Vidal et al., 1993). Evidence of the immunological role of *Nramp* was suggested by its tissue expression pattern within the mouse. *Nramp* transcripts were only detected in the reticuloendothelial organs, and highly expressed in purified macrophages and macrophage-cell lines from these tissues. *Nramp1* polymorphisms have been significantly correlated to an increased progression of tuberculosis in smear-positive patients in West Africa (Bellamy et al., 1998) and microscopy-positive individuals in Denmark (Søborg et al., 2002) and Houston, Texas (Ma et al., 2002). Segregation of *Nramp1* haplotypes in affected sibling pairs infected with leprosy was found to be significantly nonrandom (Abel et al., 1998), indicating that shared *Nramp1* alleles predispose individuals to susceptibility to *M. leprae*.

Nramp1 belongs to a small family of related proteins that includes two known mammalian genes, *Nramp1* and the ubiquitously expressed *Nramp2*, as well as related sequences in many other taxa (Cellier et al., 1996). *Nramp* homologs have been described

from many evolutionarily distant groups. Besides humans (Cellier et al., 1994; Kishi, 1994), mice (Vidal et al., 1993) and rats (Gunshin et al., 1997), *Nramp*-like sequences have been isolated from birds (Hu et al., 1995), fish (Dorschner and Phillips, 1999), insects (Rodrigues et al., 1995), nematodes (The *C. elegans* Sequencing Consortium, 1998), plants (Belouchi et al., 1995), yeast (Portnoy et al., 2000), and bacteria (Makui et al., 2000). Sequence conservation is very high between the murine paralogs, *Nramp1* and *Nramp2*, with 76.5% amino acid similarity. A *Nramp* ortholog from *Drosophila melanogaster* (*malvolio*) shares 68.8 and 73.3% amino acid similarity with the murine genes (Cellier et al., 1995), respectively. Plant sequences maintain 50-60% conservation (Canonne-Hergaux et al., 1999), and even homologs of *Nramp* from *Mycobacterium* spp. are approximately 37% identical to the murine *Nramp1* (Cellier et al., 1996).

In their analysis of mouse *Nramp1*, Vidal et al. (1993) found that the gene encodes a 548 amino acid (aa) protein with a predicted molecular mass of 60 kDa (Figure 4). Hydropathy analysis of the protein predicts 12 putative transmembrane domains (TM) and a group of N-linked glycosylation sites within a predicted cytoplasmic loop between TM 7 and 8. The polypeptide also contains a sequence motif designated as the “binding protein-dependent transport system inner membrane component signature.” This region is located in the intraphagosomal loop flanked by TM 8 and 9. It is implicated in ATP binding related to transport functions (Ames, 1986) and is found in several families of iron transporters and channels (Dassa and Hofnung, 1985). Potential phosphorylation sites related to protein kinase C are also present in the amino and carboxy termini and within the intraphagosomal loop between TM 4 and 5 (Vidal et al., 1993), corrected to TM 6 and 7 by (Cellier et al., 1995). These modifications often

indicate sites of regulatory influence. Malo et al. (1994) and Vidal et al. (1995a) sequenced *Nramp* cDNA clones from 27 *Mycobacterium*-susceptible and -resistant mouse strains and demonstrated a common difference between the two groups. A single nonconservative glycine to aspartic acid substitution within the predicted TM 4 domain of susceptible strains.

Gene knockout experiments in mice revealed that *Nramp1* plays an important role in the early stages of parasite-macrophage interactions. Using gene-targeting technology a null allele (*Nramp1*^{-/-}) transfectant mouse line was developed that lacked the normal resistance of its parents while maintaining normal appearance and longevity. Reintroduction of the wild-type *Nramp1*^{+/+} onto that genetic background completely restored resistance to intracellular parasites (Govoni et al., 1996). Gruenheid et al. (1997) used immunofluorescence and confocal microscopy to localize the expressed Nramp1 protein to the late endocytic compartments (late endosome/lysosome) of mouse macrophages. Immunofluorescence of isolated latex-bead phagosomes showed definitively that Nramp1 is recruited to the phagosomal membrane during maturation of the microbial phagosome. The presence of 5' and 3' endocytic targeting signals in *Nramp1* transcripts (Atkinson et al., 1997) is consistent with protein recruitment directly from the Golgi apparatus to the late endosome/lysosome (Blackwell et al., 2001; Gruenheid et al., 1997). In a mycobacterial infection, the pathogen becomes localized to this compartment of the macrophage.

Govoni et al. (1995) analyzed the regulatory sequences found within the promoter region of *Nramp1* and found binding sites that were characteristic of macrophage-specific, IFN- γ -inducible gene expression and bacterial lipopolysaccharide (LPS)

responsiveness. Northern blotting experiments performed on mouse macrophage cell lines and probed with *Nramp*-specific cDNA probes showed that *Nramp1* was upregulated in the presence of IFN- γ and LPS. Further investigation revealed that *Nramp* expression in mice was substantially increased (up to 16X) by pretreatment with IFN- γ followed by LPS exposure (Govoni et al., 1997).

An early hypothesis (Vidal et al., 1993) of the mechanism of resistance linked the action of Nramp to antimycobacterial nitric oxide (NO) synthesis within the infected macrophage based on weak homology with *Aspergillus nidulans* nitrate transporter *crnA* (Unkles et al., 1991). However, this functional homology seemed unlikely given that corticosterone suppressed NO synthesis in *Bcg'* and *Bcg^s* mice, but did not affect killing of mycobacteria by *Bcg'* mice (Brown et al., 1995). Brown et al. (1997) went on to hypothesize that *Nramp1* may be responsible for mycobacterial resistance by stabilizing mRNA from genes involved in macrophage activation. Evidence for a role in metal metabolism or sequestration was suggested by data that showed influence on expression levels of Nramp polypeptide post-transcriptionally when in the presence of the iron chelator, deferoxamine (Atkinson et al., 1997).

Interestingly, the genomes of the pathogens *Mycobacterium bovis* BCG (Agranoff and Krishna, 1998), *M. tuberculosis* (Cole et al., 1998), and *M. leprae* (Cole et al., 2001) also contain a homolog of the *Nramp* family, termed *Mramp* (Agranoff et al., 1999). This discovery has resulted in several related hypotheses predicting the role of *Nramp1* in host resistance. Given that mutations in *Nramp1* impair survival of the host and that the pathogen expresses a homologous protein, it has been proposed that Nramp1 functions in resistance by removing the divalent cations critical for microbial survival, virulence or

replication in a competitive interaction with Mramp (Agranoff et al., 1999). These hypotheses were generally expressed by Gruenheid et al. (1997) prior to the report of mycobacterial *Mramp* or conclusive evidence of Nramp functionality. Jabado et al. (2000) monitored Mn^{2+} transport in *Nramp1*^{+/+} and *Nramp1*^{-/-} phagosomes and found that Nramp1-positive phagosomes accumulated less Mn^{2+} than did Nramp1-negative compartments. Additionally this transport was abrogated by a vacuolar H^+ /ATPase inhibitor. An independent study showed that overexpression of Nramp1 in a macrophage cell line also increases the efflux of radiolabelled iron (Atkinson and Barton, 1998). Research supporting the symport mechanism of Nramp1 functionality has also been conducted by Gomes and Appelberg (Gomes and Appelberg, 1998; 2002).

In contrast to these results, Zwilling et al. (1999) and Khun et al. (1999) report that *M. avium*-infected *Bcg*^r macrophages accumulate more iron (as Fe^{2+}) than susceptible *Bcg*^s phagosomes and control mycobacterial growth more effectively. They and others (Blackwell et al., 2001; Goswami et al., 2001) hypothesize that Nramp1 functions as a proton-coupled divalent cation antiporter that accumulates metals in Nramp1-expressing phagosomes. These cations are used as cofactors for the generation of toxic antimicrobial radicals that are known to inhibit growth and survival of *Mycobacterium* (Nathan and Shiloh, 2000). This hypothesis is indirectly supported by a report that disruption of *Mramp* in *M. tuberculosis* by allelic exchange mutagenesis does not alter survival of the bacteria in *Nramp1*^{+/+} or *Nramp1*^{-/-} mice (Boechat et al., 2002).

The *Nramp2* gene codes for a closely related protein in humans (Kishi and Tabuchi, 1998) and mice (Gruenheid et al., 1995). Nramp2 is known to uptake iron from the intestinal brush border in mammals and has been linked to transferrin-independent

iron transport into acidified endosomes in many different tissues (Fleming, 1998; Forbes and Gros, 2001; Gunshin et al., 1997). *Nramp2* differs significantly from *Nramp1* primarily in gene expression. *Nramp2* is expressed ubiquitously, with high levels of expression in duodenal epithelial cells (Gunshin et al., 2001), while *Nramp1* expression is primarily restricted to cells of macrophage/monocyte lineage and polymorphonuclear leukocytes (Cellier et al., 1994). One of the splice variants of *DCT1* (*Rattus norvegicus* *Nramp2* homolog) contains an iron-responsive element (IRE) in the 3' untranslated region (Gunshin et al., 1997). These stem-loop RNA structures are found in genes that are post-transcriptionally regulated by cellular iron concentrations, as appears to be the case for DCT1 (Gunshin et al., 2001). A very high degree of homology (86% amino acid identity) exists within all the TM domains between *Nramp1* and 2 (Pinner et al., 1997) and a mutation in *Nramp2* immediately C-terminal of the loss-of-function mutation in *Nramp1* TM 4 is associated with iron deficiency in rats and microcytic anemia (Su et al., 1998).

Although it is clear from numerous structural analyses, genetic screens, and cation transporter assays that *Nramp* proteins are involved in divalent metal transport, the exact mechanism by which *Nramp1* controls mycobacterial resistance is still unclear. Conflicting reports regarding the directionality of transport have resulted in two alternative hypotheses about the mechanism of resistance due to *Nramp1*. Additional research is necessary to definitively identify the role that *Nramp1* plays in resistance to intracellular parasites in mammals.

Nramp in fish

Nramp genes are known from several fish species (Chen et al., 2002; Donovan et al., 2002; Dorschner and Phillips, 1999; Saeij et al., 1999; Sibthorpe, 2002). The primary rationale for these efforts was to identify disease resistance genes that may be suitable for selective breeding for aquaculture interests (Wiegertjes et al., 1996). Dorschner and Phillips (1999) sequenced two *Nramp* homologs (*OmNramp α* and *OmNramp β*) from rainbow trout (*Oncorhynchus mykiss*) by a combination of cDNA library screening and rapid amplification of cDNA ends (RACE). The two loci were shown to possess the signature structural features of the *Nramp* family described from mammals. Phylogenetic comparisons grouped these two most closely with the *Nramp2* protein of mammals. Messenger RNA expression in juvenile trout showed that the α locus was primarily expressed in pronephros tissue and ovary, more consistent with mammalian *Nramp1*, and that the β locus expression was expressed at low levels in all tissues, but in higher amounts within the ovary, similar to *Nramp2* expression in mammals. Both loci were highly expressed in the developing ovaries of juveniles, suggesting that *Nramp* may be involved in some capacity of maturation in this species (Dorschner and Phillips, 1999). Induction of *OmNramp* was investigated by LPS treatment but the results were inconsistent (Dorschner, 1998). The author suggests that dosages may have been inadequate, immune response was depressed by stress, or that regulatory control was different in salmonid expression when compared to mammals. Previous research did show that corticosterone produced during stress depressed *Nramp* transcription in mice (Brown et al., 1995). In an attempt to investigate *Nramp* expression in the developing

ovary of trout, a possible link to endocrine disruption was investigated. This study did not detect any effect on *Nramp* transcription by polycyclic aromatic hydrocarbons (Dasmahapatra et al., 2000).

An *Nramp* gene was cloned and the full-length sequence obtained from carp (*Cyprinus carpio*) using degenerate primers and 5'RACE (Saeij et al., 1999). Carp *Nramp* also groups more closely with mammalian *Nramp2* sequences than to *Nramp1* by a phylogenetic analysis. As with the *OmNrampβ* expression patterns of rainbow trout (Dorschner, 1998), carp *Nramp* is expressed ubiquitously in the tested tissues and at relatively equal levels. Motif analysis of the predicted polypeptide showed that there was a very high degree of conservation within the predicted transmembrane domains, the glycosylation sites and the loss-of-function mutations within TM 4, relative to mammalian *Nramp*. An iron-responsive regulatory protein binding site (IRE) is present in the 3' untranslated region. Together with the tissue expression data, the authors conclude that carp *Nramp* is functionally similar to the *Nramp2* of mammals. As with the sequences reported for mammals, the greatest degree of evolutionary divergence is within the carboxy and amino terminal portions of the polypeptide. The authors made no report of a second locus and the inducibility of transcription was not described.

A study designed to identify molecular markers of disease resistance in channel catfish, *Ictalurus punctatus*, found cDNA from three alternatively spliced *Nramp* sequences, designated *NrampCa*, *NrampCb*, and *NrampCc* (Chen et al., 2002). Combinations of conserved mammalian sequence primers, cDNA library screening and RACE yielded the complete *NrampC* sequence. Each contains an identical 1650 nucleotide open reading frame with predicted polypeptide mass of 61 kDa. The

differences between the three transcripts are the result of alternative splicing and varying degrees of polyadenylation in the 3' untranslated regions. Phylogenetic analysis grouped *NrampC* with the other teleost *Nramp* sequences and *Nramp2* of mammals. The predicted polypeptide maintains the 12 TM domains and the consensus transport motif first identified in mouse *Nramp1* (Vidal et al., 1993), but differs from the mammalian and carp (Saeij et al., 1999) sequences in that it lacks an IRE, a characteristic of *Nramp2*. *NrampC* expression is present in peripheral blood lymphocytes, spleen, anterior kidney and intestine constitutively and in an activated macrophage cell line from catfish. Treatment with LPS *in vivo* and in a channel catfish monocyte cell line suggested that *Nramp* mRNA expression was induced within 5 hours of stimulation (Chen et al., 2002). The authors conclude that these results suggest that catfish *Nramp* may function similarly to *Nramp1* in resistance to intracellular parasites.

Screens of a zebrafish, *Danio rerio*, mutagenesis library identified a *DMT1* (*Nramp2* homolog) genotype as responsible for a hypochromic anemia mutant. The gene was shown to be critically important for red blood cell iron uptake in a functional assay (Donovan et al., 2002), suggesting that during certain developmental stages and in erythroid cells, teleost *Nramp* may play a fundamental role in iron acquisition.

The studies on *Nramp* in trout and channel catfish have suggested that the gene is induced by immunological stimuli such as LPS. This dissertation expanded upon the work of Chen et al. (2002) and Dorschner and Phillips (1999) by incorporating additional experiments designed to demonstrate whether fish *Nramp* is inducible *in vitro* and *in vivo* by *Mycobacterium* spp. In the first chapter, I present the complete nucleotide and amino acid sequence of the striped bass homolog of *Nramp*, *MsNramp*, along with

characterization and analysis. Additionally, I demonstrate for the first time in fish the highly inducible nature of *MsNramp* in response to mycobacterial challenge. The results of *MsNramp* expression *in vitro* after exposure to mycobacteria and bacterial lipopolysaccharide are the main focus of the second chapter. Data associated with biological assays of macrophage activation are also presented. The third chapter contains the results of a pilot study designed to assess mycobacterial infection rates in white perch (*Morone americana*) and striped bass captured in close spatial proximity. A preliminary analysis of the expression of *MsNramp* in both species is included.

Figure 1. Wild Chesapeake Bay striped bass (*Morone saxatilis*) from the York River estuary, Chesapeake Bay, Virginia; male, 581g, 331mm. Photo courtesy of C. E. Harris.

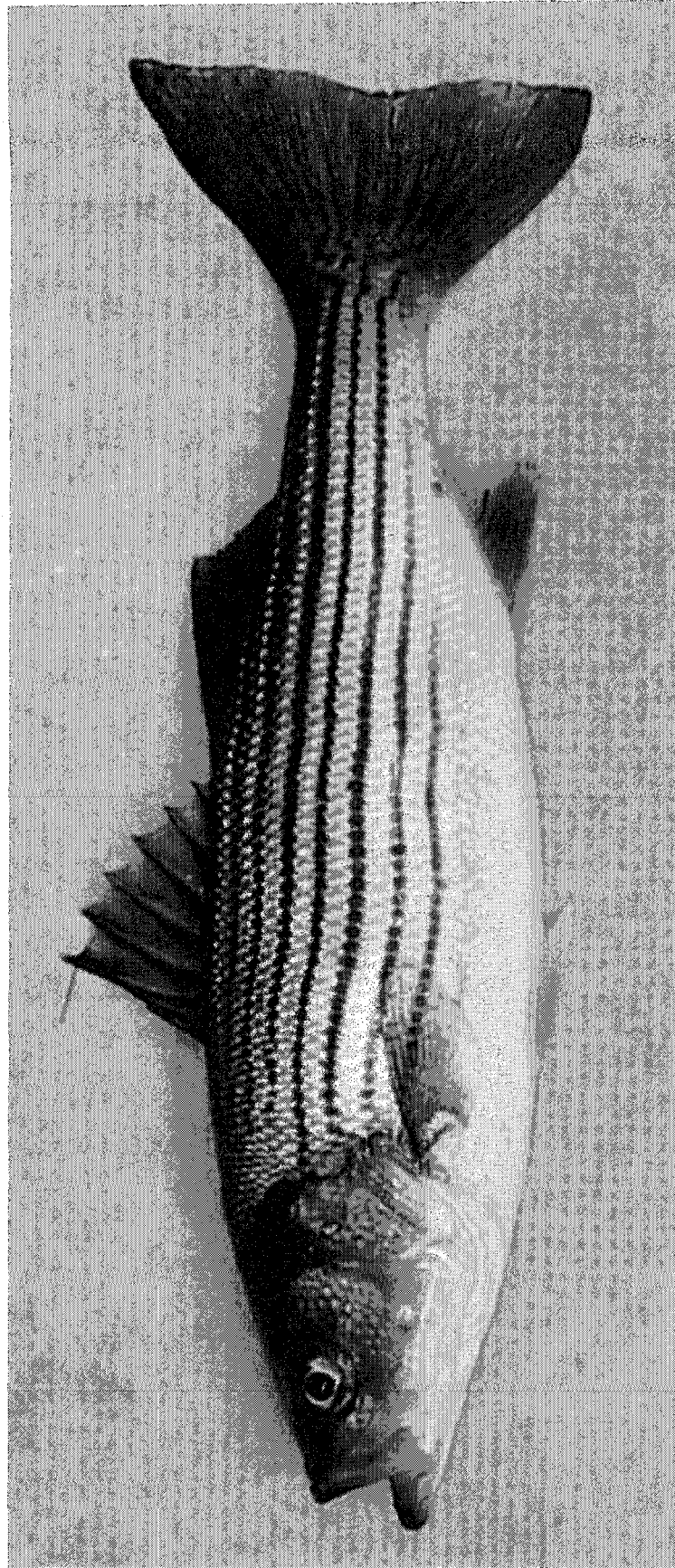
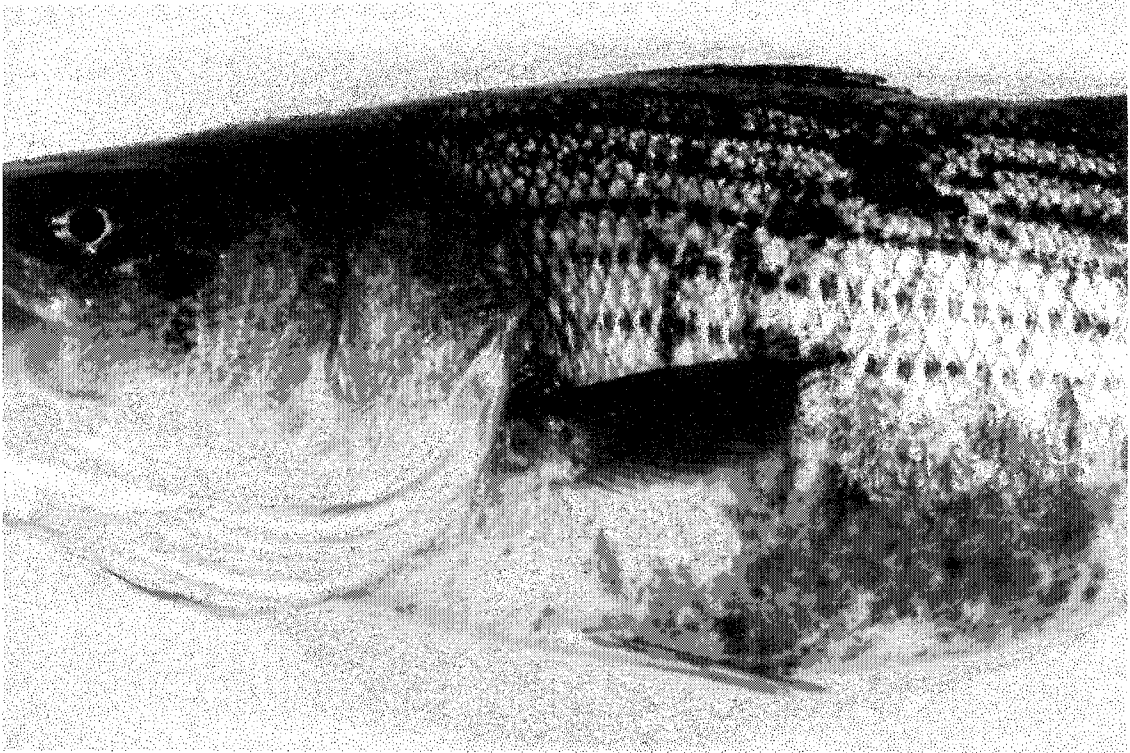


Figure 2. A) Severe ulcerative dermatitis caused by *Mycobacterium*. Photo courtesy of W. K. Vogelbein. B) Hypertrophied spleen with granulomatous inflammation from mycobacterial infection. Photo courtesy of M. W. Rhodes.

A)



B)

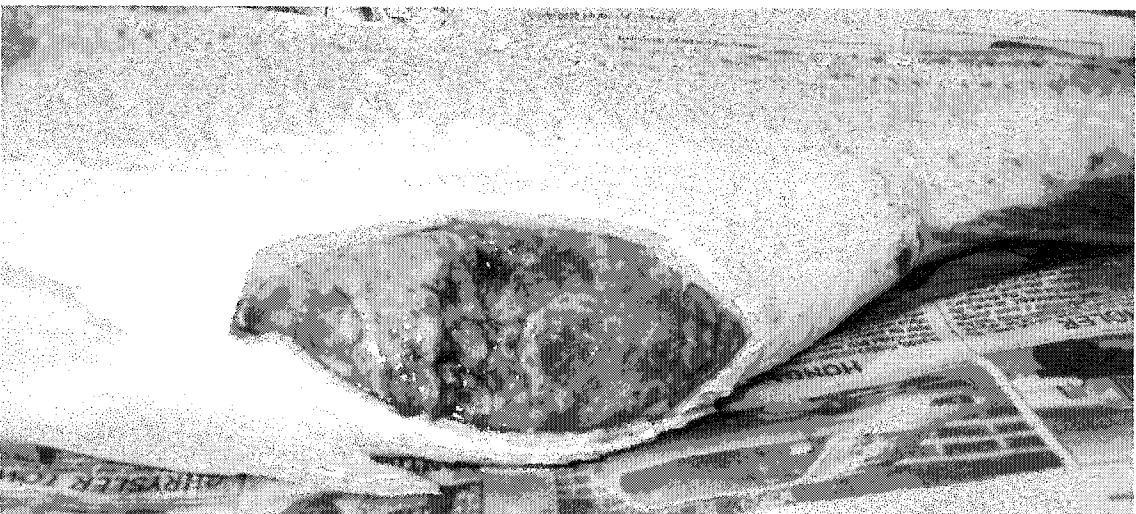


Figure 3. Biochemical pathway for the production of reactive nitrogen and reactive oxygen intermediates by phagocytes. Adapted from Nathan and Shiloh (2000), and Ortuño et al. (2000).

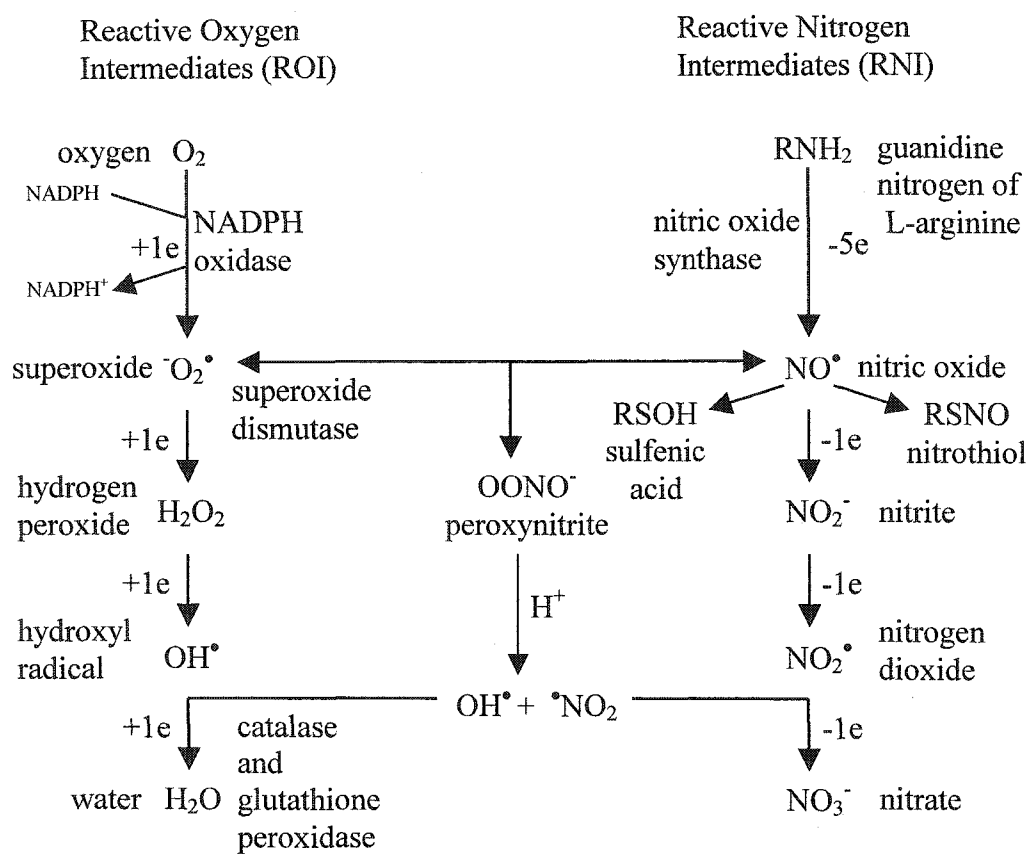
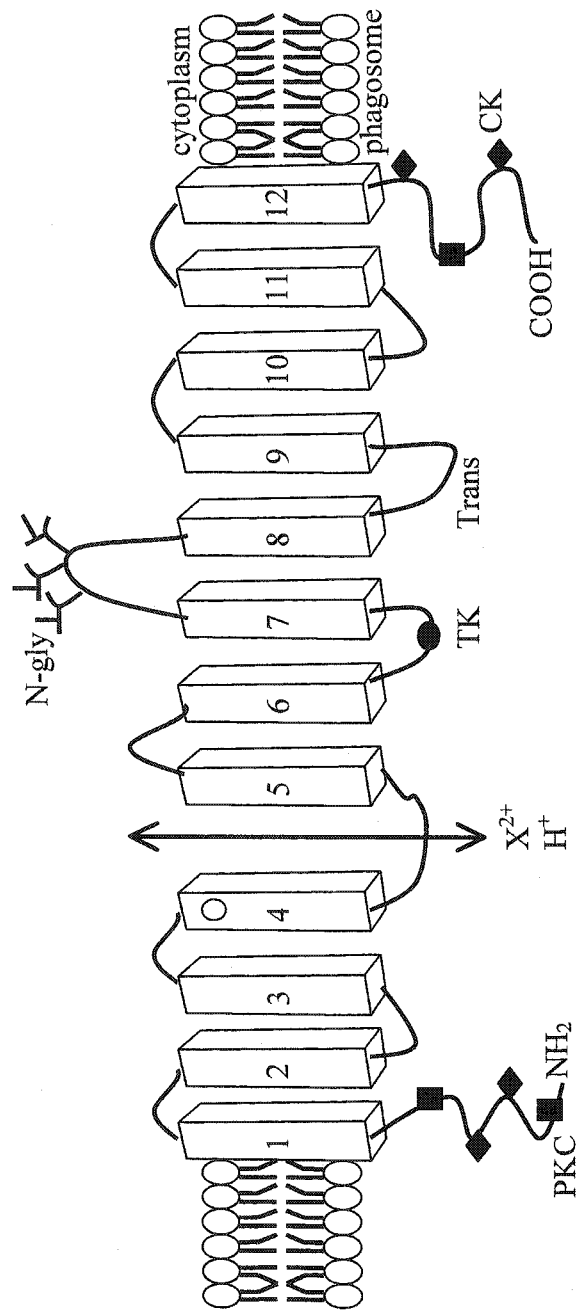


Figure 4. Domain model of *MsNramp* showing topological orientation, transmembrane domains (numbered), phosphorylation sites (CK, PKC, TK), glycosylation (N-gly) and the conserved transport binding motif (Trans). TM 4 contains a loss of function mutation that causes susceptibility to mycobacterial infection (Nramp1) and microcytic anemia (Nramp2) in mice. Number of kinase active sites and glycosylation signatures differ slightly between striped bass and mice. Adapted from Saeij et al. (1999).



CHAPTER ONE. A *MYCOBACTERIUM*-INDUCIBLE *NRAMP* IN
STRIPED BASS *MORONE SAXATILIS*

ABSTRACT

In mammals, the natural resistance-associated macrophage protein 1 gene, *Nramp1*, plays a major role in resistance to mycobacterial infections. Chesapeake Bay (USA) striped bass, *Morone saxatilis*, are currently experiencing an epizootic of mycobacteriosis that threatens the health of this ecologically and economically important species. In the present study, we characterize an *Nramp* gene in this species and provide evidence for induction following *Mycobacterium* exposure. The striped bass *Nramp* gene (*MsNramp*) and 554 amino acid gene product 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 groups *MsNramp* with other teleost Nramps, and reveals high sequence similarity with mammalian *Nramp2*. In this study, *MsNramp* expression was present in all tissues assayed by RT-PCR. Within one day of injection with *Mycobacterium marinum*, *MsNramp* expression was highly induced (17-fold higher) in peritoneal exudate cells (PE) relative to controls. Levels of *MsNramp* were increased three- and six-fold on days three and 15, respectively. Injection with *Mycobacterium shottsii* resulted in two-, five-, and three-fold increases in gene expression in PE over the time course. This study represents the first report of induction of an *Nramp* gene by mycobacteria in a poikilothermic vertebrate.

INTRODUCTION

Mycobacteriosis has been reported in greater than 150 species of freshwater and marine fishes worldwide, including striped bass, *Morone saxatilis* (Nigrelli and Vogel, 1963). Chesapeake Bay (USA) is currently experiencing an epizootic of mycobacteriosis in striped bass that threatens the health of an economically important commercial and recreational fishery (Heckert et al., 2001; Rhodes et al., 2001b) and has important consequences for production in aquaculture (Hedrick et al., 1987). Prevalences of splenic and dermal mycobacterial lesions in striped bass of Chesapeake Bay tributaries have been reported as high as 62.7% and 28.8%, respectively (Cardinal, 2001). Diseased striped bass harbor multiple species of *Mycobacterium*, including *M. marinum*, a known fish and human pathogen (Hoyt et al., 1989; Zeligman, 1972), and *M. shottsii*, also within the *M. tuberculosis* clade (Rhodes et al., 2003b). Approximately 76% of mycobacteria-positive striped bass sampled to date harbor *M. shottsii*, either as a single isolate or as part of a polymicrobial infection (Rhodes et al., 2003a). Gauthier et al. (2003) investigated the relative pathogenicity of three *Mycobacterium* spp. isolated from wild Chesapeake Bay fish on 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. Pathology in fish injected with *M. shottsii* or *M. gordonae* was considerably less severe than that seen in *M. marinum*-injected fish, and

secondary disease did not occur. Both *M. gordonae* and *M. shottsii*, however, did establish persistent infections in the spleen.

Breeding studies with *Mycobacterium*-resistant (*Bcg^r*) and -susceptible (*Bcg^s*) inbred mouse phenotypes identified a single dominant, autosomal gene (termed *Bcg*) responsible for increased resistance to mycobacteria during the early stages of infection (Gros et al., 1981). 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 (*Nramp1*) (Vidal et al., 1993). *Nramp1* transcripts were only detected in the reticuloendothelial organs (spleen and liver) of mice, and highly expressed in purified macrophages and macrophage-cell lines from these tissues. In addition, murine *Nramp1* was highly upregulated following infection with intracellular parasites (Gomes and Appelberg, 2002; Govoni et al., 1996) and LPS/IFN- γ administration (Govoni et al., 1997), with a strong synergistic effect noted in the latter. Transfection of the resistant, wild-type *Nramp1*^{G169} allele to susceptible *Nramp1*^{G169D} knockout mice restored resistance to *M. bovis* BCG and *Salmonella typhimurium* in the transgenic animal (Govoni et al., 1996), while overexpression of *Nramp1* by a cytomegalovirus promoter/enhancer completely inhibited intracellular replication of *S. typhimurium* in normally susceptible mouse macrophages (Govoni et al., 1999), indicating the crucial role of this gene in resistance to intracellular parasites.

The mechanism of mycobacterial resistance due to *Nramp1* is not fully understood (Bellamy, 2003), but *Nramp2* is known to uptake iron from the intestinal brush border in mammals and has been linked to transferrin-independent iron transport into acidified endosomes in many different tissues (Forbes and Gros, 2001; Gunshin et

al., 1997). One of the splice variants of *DCT1* (*Rattus norvegicus* *Nramp2* homolog) contains an iron-responsive element (IRE) in the 3' untranslated region (Gunshin et al., 1997). A very high degree of homology exists within all the TM domains between *Nramp1* and 2 (Pinner et al., 1997) 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 (Su et al., 1998).

Nramp1 belongs to a small family of related proteins that includes two murine genes, *Nramp1* and *Nramp2*, as well as related sequences in many other taxa (Cellier et al., 1996). *Nramp* homologs have been described from many evolutionarily distant groups, such as humans (Cellier et al., 1994; Kishi, 1994), rats (Gunshin et al., 1997), birds (Hu et al., 1995), fish (Dorschner and Phillips, 1999), insects (Rodrigues et al., 1995), nematodes (The *C. elegans* Sequencing Consortium, 1998), plants (Belouchi et al., 1995), yeast (Portnoy et al., 2000), and bacteria (Makui et al., 2000). Complete *Nramp* mRNA coding sequences for five teleosts have been recently published (Chen et al., 2002; Donovan et al., 2002; Dorschner and Phillips, 1999; Saeij et al., 1999; Sibthorpe, 2002). Paralogues of *Nramp* seem to exist in two teleost species, *Oncorhynchus mykiss* (Dorschner and Phillips, 1999) and *Takifugu rubripes* (Sibthorpe, 2002), while single genes are present in *Cyprinus carpio* (Saeij et al., 1999), *Ictalurus punctatus* (Chen et al., 2002), *Danio rerio* (Donovan et al., 2002), and *Morone saxatilis* (Burge et al., 2004). Expression studies and phylogenetic analysis in fish indicate that sequence similarity and tissue-specific expression patterns most closely resembles mammalian *Nramp2*. Little is known of the function of *Nramp* in teleosts, although in one study, Chen et al. (2002) demonstrated via Northern hybridization and RT-PCR that channel catfish (*Ictalurus*

punctatus) spleen *Nramp* levels were elevated in response to bacterial lipopolysaccharide (LPS) exposure *in vivo* in a dose dependent fashion. Direct evidence of induction from exposure to pathogens in fish has not been previously reported.

The purpose of the present study was to isolate and sequence striped bass *Nramp* homolog(s), characterize the coding sequence, determine tissue expression patterns and to evaluate the induction of *MsNramp* *in vivo* after exposure to mycobacteria. Expression was measured in several tissues using real-time RT-PCR (see (Blaschke et al., 2000; Stordeur et al., 2002; Torres et al., 2000) for recent applications) following injection of striped bass with *M. marinum* or *M. shottsii*. This represents 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, *Morone saxatilis*, (500-2000g) were collected from the York River, Chesapeake Bay, VA (Virginia Marine Resources permit # 02-27 and VIMS Research on Animal Subjects Committee permit #0101). Nucleic acids were isolated from tissues of these fish for sequencing and examination of the normal tissue expression of *MsNramp*. The fish were maintained in 1,160 l tanks with flow-through, sand filtered water at ambient temperature and salinity. Tanks were lit with fluorescent lighting adjusted to local photoperiod. Fish were fed daily to satiation with wild-caught small fish and crabs and held greater than two weeks prior to experimental use.

Striped bass used for the mycobacterial challenge and *in vivo* expression experiments were obtained as fingerlings (1 year post-spawn) from the Virginia Department of Game and Inland Fisheries, Vic Thomas Striped Bass Hatchery in Brookneal, Virginia (USA). Fish were reared to approximately 200 g mean weight (2 years post-spawn) in circular 1,000 l tanks containing 21°C well water exchanged at a rate of 12 l/min. Inflow water was degassed and oxygenated to saturation and the tank water was treated with 1% NaCl (w/v) each time fish were handled to alleviate stress. 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 local photoperiod. Thirty striped bass (198.1 g \pm 67.4 g) were randomly selected, separated into three treatment groups and moved to an

isolation facility prior to infection with mycobacteria. Mycobacterial exposures were conducted at the United States Geological Survey, National Fish Health Research Laboratory, in Kearneysville, West Virginia.

RNA extraction and reverse transcription for cDNA: sequencing and tissue expression. Peritoneal exudate cells (PE) were isolated from wild striped bass by a modification of standard techniques (Secombes, 1990). Cells were recruited to the peritoneal cavity by adjuvant injection of 100 μ l Freund's Incomplete Medium 7-10 days prior to harvesting. Anesthetized fish were injected intraperitoneally with 10 ml ice-cold Leibowitz's L-15 medium containing 100 U/ml penicillin-streptomycin (P-S) and 100 U/ml sodium heparin. After 10 minutes, lavage fluid was withdrawn through a ventral incision (Secombes, 1990). Anterior kidney, brain, heart, gill, gonad, intestine, liver, muscle, and spleen (approximately 100 mg each) were dissected from striped bass and either stored in RNAlater™ (Ambion, Austin, TX) or immediately extracted. Total RNA was isolated with TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Integrity of total RNA was assessed by electrophoresis in 1% denaturing formaldehyde-agarose gels. RNA quality and concentration was determined by UV spectrophotometry at 260/280 nm, with background correction for protein contamination at 320 nm. Total RNA was resuspended in RNA Storage Solution (Ambion, Austin, TX) and stored at -80°C until use. Reverse transcription of 5 μ g RNA was accomplished using SuperScript™ II RNase H⁻ reverse transcriptase and oligo dT₁₂₋₁₈ (Invitrogen, Carlsbad, CA) priming according to the manufacturer's recommendations.

Amplification of *MsNramp* cDNA. Primers and hybridization probes used in standard PCR, RT-PCR, RLM-RACE and sequencing are listed in Table 1. An initial 262 bp fragment of striped bass *MsNramp* was obtained using primers NrampA and NrampB, derived from consensus mammalian *Nramp1* sequences (Chen et al., 2002), and striped bass PE cDNA. Fragments 5' and 3' of this initial fragment were obtained using combinations of striped bass specific *MsNramp* primers (MsNramp736 and MsNramp1020), developed by sequencing RT-PCR products, and primers developed for *Oncorhynchus mykiss* *Nramp* (MDNMP1F, MDNMP4, OmNramp1263, OmNramp1463) (Dorschner and Phillips, 1999). PCR parameters were empirically determined for each primer set and executed on MJ Research, Inc. thermocyclers. PCR reactions contained final concentrations of 1.0 U of Platinum® *Taq* DNA polymerase High Fidelity, 0.2 mM dNTPs, 2 mM MgSO₄, 1x PCR Buffer (Invitrogen, Carlsbad, CA), 0.2 µM for each primer, and 1-2 µL cDNA template in a final volume of 50 µL. A total of 1242 bp of *MsNramp* sequence were generated in this manner. Tissue expression of *MsNramp* was shown by amplification of cDNA from a variety of tissues (see above) using primer sets NrampA/MDNMP4, MDNMP1F/ OmNramp1463, and MsNramp736/MsNramp1020. *MsNramp* positive tissues were visualized by 1% agarose gel electrophoresis and ethidium bromide staining.

RNA-ligase mediated rapid amplification of cDNA ends (RLM-RACE). The 5' and 3' ends of *MsNramp* cDNA were amplified using rapid amplification of cDNA ends (RACE), based on procedures developed by Frohman, et al. (1988). Isolation of the 5' and 3' ends of *MsNramp* was accomplished using the GeneRacer™ kit (Figure 11)

(Invitrogen, Carlsbad, CA). For the 5' end, 5 µg RNA from mycobacteria-injected striped bass PE were dephosphorylated with calf intestinal phosphatase and the 5' cap structure removed by tobacco acid pyrophosphatase. An RNA oligonucleotide sequence was ligated to the dephosphorylated, decapped 5' end of striped bass mRNA and this hybrid molecule reverse-transcribed using SuperScript II RTTM. RACE-ready 3' cDNA was obtained by reverse transcription of 5 µg of PE RNA using the GeneRacer Oligo dT Primer, a modified oligo dT primer with a 36 nucleotide tail, complementary for the 3' polyA tail of full-length mRNA. RACE-ready first-strand cDNA was treated with RNase H to remove the RNA template.

RACE PCR for 5' *MsNramp* consisted of 1 µL RACE-ready 5' cDNA, 0.6 µM GeneRacer 5' Primer (complementary to the GeneRacer RNA oligo ligated onto 5' cDNA), 0.2 µM of gene-specific primer 5RACE1, 0.2 mM dNTPs, 1x PCR buffer, 2 mM MgSO₄ and 2.5 U Platinum *Taq* DNA polymerase. Cycling parameters consisted of a touchdown PCR program under the following conditions: 94°C for 2 min; 94°C for 0.5 min and 72°C for 1 min, for 5 cycles; 94°C for 0.5 min and 70°C for 1 min, for 5 cycles; 94°C for 0.5 min and 68°C for 1.5 min, for 25 cycles; 68°C for 10 min.

3' RACE PCR of *MsNramp* consisted of similar reaction components to 5' RACE PCR with the following exceptions: 1 µL RACE-ready 3' cDNA, 0.6 µM GeneRacer 3' Primer (complementary to the 36 nucleotide tail of the oligo dT primer), 0.2 µM of primer 3RACE1, 3RACE2 or 3RACE4. Cycling parameters for the primer 3RACE1 consisted of: 94°C for 2 min; 94°C for 0.5 min and 72°C for 2 min, for 5 cycles; 94°C for 0.5 min and 70°C for 2 min, for 5 cycles; 94°C for 0.5 min, 65°C for 0.5 min, and 68°C for 2 min for 25 cycles; 68°C for 10 min. Multiple products were obtained for the

reaction initiated with primer 3RACE1, so a nested PCR reaction consisting of the standard PCR components with 0.2 μ M GeneRacer 3' Nested Primer, 0.2 μ M 3RACE2 primer and 1 μ L of the 3RACE1 amplified products. 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; 68°C for 10 min. Primer 3RACE4 was used for confirmation that full length 3' sequence was obtained after sequencing 3RACE2 products. Reaction conditions and cycling parameters were identical to those used for primer 3RACE1.

Cloning. Putative internal *MsNramp* fragments were blunt-end cloned into pSTBlue-1 by T4 DNA ligase and transformed into *Escherichia coli* strain NovaBlue competent cells for blue/white screening (BD Biosciences Clontech, Palo Alto, CA). Transformants were grown on Luria Broth (LB) agar plates with kanamycin selection for plasmid uptake and X-gal, IPTG screening for transformation. Insert-containing white colonies were amplified in LB and their plasmids isolated using PERFECTprep® spin columns (Eppendorf, 5'→3', Inc., Boulder, CO). Screening of clones was accomplished by *MsNramp*-specific PCR or vector primers that yielded products of the expected insert size. RACE PCR products were cloned into pCR®4-TOPO by topoisomerase I, using single deoxyadenosine residues added by *Taq* polymerase during amplification and the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). Insert-containing vector molecules were transformed into TOP10 *E. coli*, thereby disrupting the lethal gene *ccdB*. Insert-containing transformants were screened for insert size by vector specific PCR. Transformants containing full 5' and 3' inserts were amplified in LB and plasmids isolated using the Qiaprep Spin miniprep kit (Qiagen, Inc., Valencia, CA).

Sequencing. The sequence of *MsNramp* fragments was bidirectionally determined on a LiCor 4000L DNASequencer by the dideoxy chain termination method using the ThermoSequenase™ Cycle Sequencing kit according to the manufacturer's instructions (Amersham Biosciences Corp., Piscataway, NJ). Plasmid DNA (1-2 µg) and 3 pmol of the fluorescent primers M13F (forward) and M13R (reverse) were used in the sequencing reaction (LI-COR Biosciences, Lincoln, NE). A minimum of ten clones was sequenced for each fragment.

Sequence analysis. *MsNramp* fragments were aligned and edited in Sequencher v. 4.1 (Gene Codes Corp., Ann Arbor, MI). Full length cDNA nucleotide and deduced amino acid sequences were analyzed for similarity to published sequences using GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>) resources. Searches for similar sequences were performed using the Basic Local Alignment Tool (BLAST) algorithms (Altschul et al., 1990), nucleotide percent identity calculated by the FASTA program (Pearson and Lipman, 1988), and amino acid percent identity and similarity calculated by PSI-BLAST (Altschul et al., 1997). Multiple alignments were performed using ClustalX v. 1.81 (Thompson, 1997). Pairwise gap costs were set at 10.0 for gap opening and 0.10 for gap extension. The Gonnet 250 protein weight matrix was used. Multiple alignment parameters were a gap opening penalty of 10.0, gap extension 0.20, and divergent sequences were delayed 30%. Potential microsatellite sequences were detected by Tandem Repeats Finder software v. 3.21 (Benson, 1999) and polyadenylation signals were analyzed by polyadq (Tabaska and Zhang, 1999). The following GenBank amino acid sequences were used in alignment and phylogenetic analysis: *Bos taurus* Nramp1

accession number U12852, *Cyprinus carpio* NRAMP AJ133735, *Danio rerio* DMT1 AF529267, *Drosophila melanogaster* malvolio U23948, *Gallus gallus* NRAMP1 U40598, *Homo sapiens* NRAMP1 L32185, *Homo sapiens* 2 NP_000608, *Ictalurus punctatus* NrampC AF400108, *Macaca fascicularis* AF153279, *Morone saxatilis* MsNramp AY008746, *Mus musculus* Nramp1 AAA39838, *Mus musculus* 2 AAC42051, *Oncorhynchus mykiss* Nramp α AF048760, *Oncorhynchus mykiss* β AF048761, *Ovis aries* NRAMP U70255, *Pimephales promelas* Nramp AF190773, *Rattus norvegicus* DCT1 AAC53319, *Takifugu rubripes* Nramp α AJ496549, *Takifugu rubripes* β AJ496550. Transmembrane domains (TM) domains were predicted by HMMTOP v. 2.0 (Tusnady and Simon, 2001), and motif analysis was conducted using the PROSITE reference library (Hofmann et al., 1999).

Nucleotide and amino acid accession number. *Morone saxatilis* MsNramp nucleotide and deduced amino acid sequences have been submitted to GenBank and assigned accession number AY008746.

Phylogenetic analysis. Phylogenetic analysis was conducted using MEGA v. 2.1 software (Kumar et al., 2001). An optimal tree was constructed using the pairwise-distance model and neighbor-joining (Saitou and Nei, 1987). Indels were removed from the multiple alignment and the reliability of the trees assessed by 10,000 bootstrapping replicates. *Drosophila melanogaster* malvolio (Rodrigues et al., 1995), an Nramp homolog from that species, served as an outgroup.

Mycobacteria. *Mycobacterium marinum* (Virginia Institute of Marine Science strain M30) (Rhodes et al., 2000) and *M. shottsii* (VIMS strain M175, ATCC 700981) (Rhodes et al., 2001b) used in these studies were isolated from splenic tissue of Chesapeake Bay striped bass, and grown as described in Gauthier et al. (2003). Briefly, mycobacteria were inoculated into Middlebrook 7H9 medium with OADC enrichment and 0.05% polyoxyethylene-sorbitan monooleate (Tween 80) (MDB) and grown until log-phase (10 days). Cultures were pelleted by centrifugation at $12,000 \times g$ for 20 min and washed once in phosphate-buffered saline with 0.05% Tween 80 (PB). Washed cultures were resuspended in PB, vortexed vigorously with glass beads (500 μm diameter) for 2 minutes and filtered through Whatman No. 1 paper to reduce clumping and obtain a homogeneous suspension. Absorbance at 590 nm was adjusted with PB to 0.05, or approximately 10^7 CFU/ml, and diluted tenfold prior to injection with phosphate-buffered saline (PBS). Effluent 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/l after the fish were infected with mycobacteria.

Infection. Immediately before introduction of striped bass to the isolation facility, fish were separated into three groups of ten fish each, anaesthetized using 100 mg/l Finquel (MS-222, Argent Chemical, Redmond, WA), weighed, and injected intraperitoneally with 1.5 ml of diluted mycobacterial suspension or sterile PBS. Group 1 fish received an injection of 1.5 ml PBS. Group 2 fish received 1.4×10^6 CFU of *M. marinum*. 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 dosages) mycobacterial dosages were adjusted to ensure that fish received a sublethal challenge that corresponded to approximately 5000 CFU/g. Previous work indicated that these inoculations were sublethal and would establish chronic infections (Gauthier et al., 2003). Injected dosages were enumerated by plating mycobacteria on Middlebrook's Agar.

Sampling. Three fish from each group were randomly selected one-, three-, and 15-days post-injection (p. i.), anesthetized with a lethal dose of Finquel (500 mg/l) and dissected to remove tissues for measurement of *MsNramp* transcripts. All media and reagents used in sample preparation and storage were from Sigma Chemical (St. Louis, MO) unless otherwise noted. Samples (100-200 mg) of anterior kidney (AK), spleen (SPL), and white muscle (WM) were removed, rinsed once in phenol-red free Hank's Balanced Salt Solution (HBSS) and stored in RNeasy lysis buffer. Samples in RNA storage buffer were held overnight at 4°C, and stored at -20°C, as per the manufacturer's instructions. PE were isolated as described above, without the use of Freund's Incomplete Medium. PE were washed once in L-15 containing 2% fetal bovine serum (Invitrogen, Carlsbad, CA), P-S, 10 U/ml sodium heparin and counted on a Reichert Brightline hemacytometer. Viability assessed by trypan blue exclusion was greater than 95% for all fish sampled. An aliquot of 2×10^7 PE cells was removed for RNA extraction, washed once in HBSS and resuspended in RNeasy lysis buffer. An aliquot of 5×10^6 cells in HBSS was adhered to individual glass slides using a cytospin (Shandon, Inc., Pittsburgh, PA) at 700 x g for 7 min. Cytospin slides were either fixed in methanol (10 sec) and Wright/Giemsa stained or fixed in 1% paraformaldehyde (10 min) and stained with the Ziehl-Neelsen acid-fast

technique (Luna, 1968). The remaining cells were fixed in 1.5% glutaraldehyde/0.1 M sodium cacodylate/0.15 M sucrose (pH 7.2, 1 hr) for electron microscopy.

Electron microscopy. Glutaraldehyde-fixed cells were post-fixed for one hour in 1% OsO₄/0.1 M sodium cacodylate. Cells were dehydrated through graded ethanols (10-100%) at 15 min per step, with a one hour *en bloc* staining with saturated uranyl acetate at the 70% step. Dehydration was followed with three 30 min incubations in 100% propylene oxide, and cells were embedded in Spurr's resin. Ultrathin sections (90 nm) were prepared on a Reichert-Jung ultramicrotome, mounted on Formvar-coated copper grids, and stained with Reynold's lead citrate for 7 min. Stained sections were examined on a Zeiss CEM902 transmission electron microscope.

RNA extraction for induction of *MsNramp* *in vivo*. PE were removed from RNAlater™ by dilution with 1 vol HBSS and centrifugation at 4000 x g for 5 min. AK, SPL, and WM were removed from storage buffer and a 100 mg subsample taken just prior to extraction. Total RNA was isolated and evaluated as previously described. Integrity and quality of total RNA was assessed as previously described.

Real-time semiquantitative reverse transcription-PCR. Two gene-specific primers and two gene-specific hybridization probes were used to measure PCR product formation in real time (Table 2) (Wittwer et al., 1997). This procedure was performed on the Roche Molecular Biochemicals LightCycler System and the appropriate primers and hybridization probes developed using LightCycler Probe Design v. 1.0 software (Idaho

Technology, Inc, Salt Lake City, UT). All reagents were prepared at 4°C in low light to minimize nonspecific amplification and fluorophore degradation.

The PCR reaction consisted of a master mix of water, manganese acetate (final concentration 4.25 mM), hybridization probes (0.2 μ M), primers (0.5 μ M) and LightCycler RNA Master Hybridization Probes enzyme mixture. To initiate the reaction, 500 ng of sample RNA was added to each capillary and LightCycler cycling immediately begun. RNA samples were quantified immediately before use by spectrophotometric detection at 260 and 280 nm, and corrected for protein concentration at 320 nm. RNA sample concentrations calculated by spectrophotometry were reproducible within 5%.

Reverse transcription was performed at 61°C for 20 min, followed by primary denaturation of the RNA:cDNA hybrid at 95°C for 30 sec. Amplification consisted of 45 cycles of denaturation (95°C for 1 sec), annealing/hybridization (54°C for 15 sec), and elongation (72°C for 11 sec). Each cycle was followed by fluorescence monitoring by the LightCycler at 640 nm. Two amplification reactions were performed for each RNA sample. Data collection and preliminary analyses were conducted using LightCycler Data Analysis software v. 3.3.

qRT-PCR analysis. *MsNramp* expression was quantified by calculation of percent increase or decrease in transcript number in mycobacteria-infected tissues or cells compared to sham-injected controls. Six replicates of each of five RNA concentrations (1000, 500, 250, 100, 50 ng RNA) were amplified two to three times for each tissue type and a mean efficiency of PCR (PCR_E) calculated (Table 5). The PCR_E was calculated as:

$$PCR_E = 10^{-1/\text{slope}}$$

$$\text{where } 1 \leq PCR_E \leq 2$$

Measurement of the slopes for AK, PE, SPL, and WM was accomplished by linear regression of the crossing points of the six replicates against the RNA concentration. The crossing point (Cp) of the qRT-PCR is the point during amplification at which fluorescence of a sample rises above the background. That point on the amplification curve is proportional to the amount of starting template mRNA (*MsNramp*) in the sample. Percent difference is then calculated as follows:

$$\% \text{ difference} = (PCR_E^{\Delta Cp} \times 100) - 100 \text{ (Gentle et al., 2001)}$$

where $\Delta Cp = (\text{control sample crossing point} - \text{experimental sample crossing point})$

Statistical analysis. For calculation of crossing points and slope for PCR_E , linear regression was performed by LightCycler software v. 3.3. Intra- and inter-assay variation were analyzed by single-factor ANOVA ($\alpha=0.05$), linear regression, Student's *t*-test and power analysis of this experimental system (Gentle et al., 2001). Each time point sample (one-, three- and 15-days post-injection) was analyzed by single-factor analysis of variance (ANOVA) and multiple comparisons performed using Tukey's Multiple Comparison ($\alpha=0.05$ and 0.01) in SAS v. 8.0 (SAS Institute Inc., Cary, NC) with Kramer's modification for unequal samples sizes where appropriate.

RESULTS

***MsNramp* is a 3530 bp gene encoding a 554 aa protein.** Complementary DNA for *MsNramp* was isolated from *Morone saxatilis* peritoneal exudate cells (PE) using combinations of consensus mammalian, trout, and striped bass specific primers (Table 1). Internal coding region sequences were obtained using primer sets NrampA/B, NrampA/MDNMP4 and MDNMP1F/OmNramp 1463. A total of 1242 bp of the internal coding region was sequenced in this manner. These internal fragments were used to design primers 5RACE1, 3RACE1, 3RACE2 and 3RACE4, for use in 5' and 3' RACE-PCR. 5' RACE, using primers 5RACE1 and the GeneRacer 5' Primer (Invitrogen, Carlsbad, CA) produced a product of 620 bp that included a 183 bp 5' untranslated region (UTR) and the translation start codon at position 184. 3' RACE fragments contained a TAG termination codon at position 1848, and a 3'-UTR of 1682 bp. Compilation and alignment of all the fragments produced by RT-PCR and RACE demonstrated that *MsNramp* is a 3530 bp gene, with a 1665 nt single open reading frame encoding a 554 aa polypeptide (Fig. 5). Three regions of tandem repeats exist in the 3'-UTR at positions 1876-1890 (consensus pattern TTCCTCT), 2050-2071 (AATCAGAA), and 2949-2971 (GTGTGATAAAAT). Two prospective, atypical polyadenylation signals are present at position 3425 (nonamer) and 3436 (hexamer) followed closely by a poly dA tail of at least 32 nt.

Striped bass *MsNramp* structure conserves all the important motifs and regulatory elements of mammalian *Nramp*. The deduced amino acid sequence of striped bass *MsNramp* was a protein with a minimum molecular mass of 61157 M_r containing 12 putative membrane-spanning domains composed of highly hydrophobic amino acids (Fig. 6). Analysis of the protein topology predicted that the amino- and carboxy-termini exist submembrane of the insertion location 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 8. Two potential phosphorylation sites related to protein kinase C, along with two casein kinase phosphorylation signatures, are located in the amino terminus, with a single PKC site and two additional casein kinase motifs in the carboxy terminal end. A single tyrosine kinase site is located between TM 6 and 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 several other families of iron transporters and channels (Dassa and Hofnung, 1985), is located in the intracellular region between TM 8 and 9. Unlike the *Cyprinus carpio* homolog (Saeij et al., 1999) and other *Nramp2* isoforms (Gunshin et al., 1997; Lee et al., 1998), but similar to channel catfish *Nramp* (Chen et al., 2002), no identifiable iron-responsive regulatory-binding-protein site (IRE) consensus sequence (CNNNNNCAGUG) (Casey et al., 1988) was identified in the striped bass 3'-UTR.

Alignment and phylogenetic analysis groups striped bass *MsNramp* with other teleost and mammalian *Nramp2* proteins. Striped bass *MsNramp* aa sequences were

aligned with other vertebrate Nramp1 and 2 homologs (Table 3 and Fig. 6) in order to examine potentially important distinguishing characteristics. Three distinct clades were evident in the phylogenetic analysis of *Nramp* aa sequences. Vertebrate Nramp1 proteins clustered into one subgroup, while all teleost sequences clustered sister to the mammalian Nramp2 proteins. Nramp sequences of the *Cyprinidae* and both paralogs from rainbow trout formed a separate clade within the teleosts. MsNramp was phylogenetically most similar to the *Takifugu rubripes* Nramp β (Fig. 7).

***MsNramp* mRNA expression in striped bass tissues is ubiquitous.** Anterior kidney, brain, heart, gill, female and male gonad, intestine, liver, muscle, PE, peripheral blood leucocytes and spleen were positive for *MsNramp* mRNA transcripts by RT-PCR (Fig. 8) for three primer sets in three male and female striped bass. PCR performed on total RNA prior to cDNA generation confirmed that no genomic DNA contamination was present in the mRNA samples. For each tissue 5 μ g of RNA was analyzed.

Cytology and ultrastructure confirm infection of PE within one day of injection of mycobacteria. To confirm that *Mycobacterium*-exposed striped bass were harboring mycobacteria intracellularly within one day of infection, light and transmission electron microscopy were used to examine PE. All infected and control fish survived to the end of the experiment (15 days), and no outward manifestations of disease were apparent. Fish injected with *M. marinum* demonstrated gross inflammation of visceral fat and mesenteries at 15 days post-infection, whereas sham-injected fish and *M. shottsii*-injected fish displayed no gross inflammation. Wright-Giemsa stained cytospin preparations

showed peritoneal lavages to be composed primarily of macrophages (>50%), with varying numbers of lymphocytes, thrombocytes, and granulocytes. Ziehl-Neelsen staining indicated that both *M. marinum* and *M. shottsii* were readily phagocytosed by PE within one day post injection. Electron microscopy demonstrated mycobacteria within membrane-limited phagosomes of cells phenotypically consistent with activated macrophages (Fig. 9).

Constitutive expression of *MsNramp* in striped bass tissues was highly variable.

Average crossing points for the different tissue types of control striped bass demonstrated large constitutive differences in *MsNramp* expression. Comparison of constitutive expression (Table 4) between tissues revealed that expression of *MsNramp* was highest in spleen (SPL). Expression levels were approximately 28-, 85-, and 240-fold lower in PE, anterior kidney (AK), and white muscle (WM), respectively. ANOVA and Tukey's multiple comparison determined that each tissue type was significantly different from each of the others ($p < 0.01$).

***MsNramp* in *M. marinum*-injected striped bass is highly induced.** A primary objective of this study was to document the induction of *MsNramp* in fish exposed to *Mycobacterium marinum* and *M. shottsii*. Expression in PE of striped bass (Fig. 10D) infected with *Mycobacterium marinum* was 1701.0 ± 14.02 % higher than control PE within one day post-injection. Levels of *MsNramp* in PE continued to be elevated at three and 15 days, with expression of 412.46 ± 10.00 % and 623.47 ± 66.66 % of control, respectively. AK (Fig. 10A) and SPL (Fig. 10B) *MsNramp* expression in *M.*

marinum-exposed fish did not differ significantly from control until 15 days, when AK was measured at 130.31 ± 10.58 %. No significant increases were seen in SPL on any days. Levels of expression in WM (Fig. 10C) were much more variable than in any of the other tissues and did not have significant increases relative to control fish.

***M. shottsii* induces expression of *MsNramp* in striped bass peritoneal exudate cells.**

M. shottsii-injected striped bass PE expressed *MsNramp* at 216.47 ± 9.86 %, 461.34 ± 20.79 %, and 311.89 ± 21.45 % of control levels on days one, three and 15, respectively (Fig. 10D). No significant induction of *MsNramp* was seen in AK (Fig. 10A) at any time post-injection. Expression in SPL (Fig. 10B) was depressed at 3 days with respect to day 3 controls. By 15 days, levels of *MsNramp* in SPL were statistically similar in control and *M. shottsii* fish. Variability in WM (Fig. 10C) was high for *M. shottsii*-injected fish and a depression of *MsNramp* transcription was noted at day one.

DISCUSSION

In this study, we described the *in vivo* expression of the *Morone saxatilis* homolog of the *Nramp* (*MsNramp*) gene during exposure to mycobacteria. It was demonstrated that striped bass *MsNramp* transcription is significantly upregulated (17x) in peritoneal exudate (PE) cells following infection with *Mycobacterium marinum*. *Mycobacterium shottsii* also induced expression, although not to the degree seen for *M. marinum*. Induction of expression was rapid (less than 24 h) and long lasting (greater than 15 d) in PE. The long lasting nature of the induction may be the result of mycobacterial replication, additional infection of previously naïve PE and/or long-lasting induction within individual PE. Stimulation of mouse bone marrow-derived macrophages with LPS and IFN- γ has demonstrated rapid induction of Nramp1, peaking at 12hr (Atkinson et al., 1997), while functional alleles of murine *Nramp1* maintain a bacteriostatic effect on *M. avium*-containing phagosomes for at least 10 days (Frehel et al., 2002). In the murine model, Nramp1 is recruited to the membrane of the phagolysosome during the initial stages of mycobacterial infection (Gruenheid et al., 1997) where it maintains the fusiogenic properties of this compartment (Frehel et al., 2002). It is likely, based on high sequence similarity between mammalian and striped bass homologs and on the pattern of induction seen for mycobacteria-infected PE, that striped bass use the gene product in a similar fashion.

Murine Nramp1 and Nramp2 have a distinctive tissue expression pattern that is not seen in teleost Nramps. Tissue expression of *MsNramp*, as measured by RT-PCR, showed that this gene is expressed ubiquitously, although these results do not take into account the heterogeneous cell types of many tissues. Relatively high constitutive expression was seen in the SPL in the real-time RT-PCR assay, which suggests that *MsNramp* may be playing a dual role as a mycobacteria-inducible resistance gene and as a metabolic divalent metal transporter. Screens of a zebrafish *Danio rerio* mutagenesis library identified a DMT1 (Nramp2 homolog) genotype as responsible for a hypochromic anemia. The gene was shown to be critically important for red blood cell iron uptake in a functional assay (Donovan et al., 2002), suggesting that during certain developmental stages and in erythroid cells, teleost Nramp may play a fundamental role in iron acquisition. These results support the hierarchy of constitutive expression seen in striped bass (see Table 4). Anterior kidney and spleen have relatively high expression, as tissues of hematopoiesis, while naïve peritoneal macrophages express relatively lower levels of *MsNramp* until in contact with mycobacteria.

Peritoneal exudate 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 (Enane et al., 1993). Assuming that the elevated levels of *MsNramp* expression in the peritoneal preparations is of macrophage origin, lower total levels of *MsNramp* in AK and SPL of mycobacteria-injected fish would be expected as the proportion of macrophages in these tissues is significantly lower than that of PE. Trafficking of mycobacteria by infected PE may account for the late increase in *MsNramp* expression observed in AK. Previous work has shown that well developed

granulomas are not present histologically in AK two weeks after intraperitoneal mycobacteria injection (Gauthier et al., 2003). This same study did observe small numbers of acid-fast staining mycobacteria within inflammatory foci of the AK and SPL two weeks after intraperitoneal injection. Longer term analysis of *MsNramp* expression may indicate that as infection occurs within the AK and SPL, differentiation and activation of resident macrophages is accompanied by upregulation of this gene.

The conserved features of *MsNramp* include a topology of 12 transmembrane domains, N- and C- terminal phosphorylation sites, extracytoplasmic glycosylation, and the binding protein-dependent transport systems inner membrane component signature. The transport signature is implicated in ATP-binding related to transport functions (Ames, 1986) and is found in several gene families of iron transporters and channels (Dassa and Hofnung, 1985). Alternative splicing has been identified in several *Nramp2* homologs, including human (Lee et al., 1998), mouse and rat (Tabuchi et al., 2002), macaca (Zhang et al., 2000) and channel catfish (Chen et al., 2002). In the mammalian *Nramp* studies, alternative splicing was shown to correspond to alternate C-terminal amino acids, distinctive subcellular or tissue expression, and presence or absence of an IRE. IREs are stem-loop RNA structures often found in genes that are post-transcriptionally regulated by cellular iron concentrations, as *Nramp2* (DCT1) appears to be in rats (Gunshin et al., 2001). Channel catfish splice variants resulted in a single, non-IRE encoding open reading frame, irregardless 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 aa of channel catfish *NrampC*, identified a single molecular weight 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 better understanding of disease resistance in economically valuable finfish (Fjalestad et al., 1993). Genes responsible for innate resistance to intracellular pathogens are likely candidates for selective breeding efforts in aquaculture (Wiegertjes et al., 1996) and enhance our understanding of the evolution of innate immunity in vertebrates. This study demonstrates that striped bass *Morone saxatilis* possess a highly conserved natural resistance-associated macrophage protein gene, *MsNramp*, of close homology to the mammalian *Nramp2* with all the hallmark features of the *Nramp* gene family described from humans (Kishi, 1994; Kishi and Tabuchi, 1998) and mice (Gruenheid et al., 1995; Vidal et al., 1993). *MsNramp* is rapidly and highly induced in peritoneal exudates cells upon exposure to *Mycobacterium* spp. *in vivo*. This is the first report of induction of an *Nramp* gene *in vivo* from fish exposed to intracellular pathogens.

Table 1
Primers used in tissue-specific RT-PCR, RLM-RACE, and sequencing

Oligo name	Sequence (5' → 3')	Source (citation or accession #)
NrampA	GGAAGCTGTGGGCCTTCAC	consensus mammalian (Chen et al., 2002)
NrampB	CTGCCGATGACTTCCTGCAT	consensus mammalian (Chen et al., 2002)
OmNramp1263	CCCCTCCGCCTATCTTCCACACCAGTCC	<i>Oncorhynchus mykiss</i> (AF054808)
OmNramp1463	CCCGCTCCATCGCCATCTTCCCCAC	<i>Oncorhynchus mykiss</i> (AF054808)
MDNMP1F	GCCCCATAATATCTACCTGCACTC	<i>Oncorhynchus mykiss</i> (Dorschner and Phillips, 1999)
MDNMP4	GGTGCCCGTCATGGTAGAGCTCTG	<i>Oncorhynchus mykiss</i> (Dorschner and Phillips, 1999)
5RACE1	TCAGCCAAAGGATGACCCGAGGAAC	<i>Morone saxatilis</i> (AY008746)
3RACE1	TCTGACCTTCACCAGTCTGACATC	<i>Morone saxatilis</i> (AY008746)
3RACE2	CACTTACTCGGGGCAGTTTGTTATG	<i>Morone saxatilis</i> (AY008746)
3RACE4	GATACCGTCCTCCTACCAACATACTG	<i>Morone saxatilis</i> (AY008746)
T3	ATTAACCCTCACTAAAGGGA	Invitrogen Corp.
T7	TAATACGACTCACTATAGGG	Invitrogen Corp.
GeneRacer 5' Primer	CGACTGGAGCACGAGGACACTGA	Invitrogen Corp.
GeneRacer 3' Primer	GCTGTCAACGATACGCTACCGTAACG	Invitrogen Corp.
GeneRacer 3' Nested	CGCTACGTAACGGCATGACAGTG	Invitrogen Corp.
M13F IRDye 800	CACGACGTTGTAAAACGAC	LI-COR Biosciences
M13R IRDye 700	GGATAACAATTTACACACAGG	LI-COR Biosciences

Table 2

Primers and hybridization probes used in qRT-PCR^a

Oligo role	Oligo name	Sequence (5' → 3')
forward primer	MsNramp736	TTGTCGTAGCGGTCTT
reverse primer	MsNramp1020	GGGACCACCGTAGGTTTA
5' hybridization probe	5'MsNramp942f	GCTGGACAGAGTTCCACCA-f ^b
3' hybridization probe	3'MsNrampRed963p	LCRed640 ^c -ACAGGCACTTACTCGGGG-p ^d

^a developed from *Morone saxatilis* sequence AY008746; ^b3'-fluorescein; ^c5'-LightCycler Red 640 dye; ^d3'-phosphate

Table 3
Nucleotide and amino acid identities of *Nramp* homologs with *Morone saxatilis* *MsNramp* (AY008746)

Sequence	Acc #	nucleotide % identity ^a	AA % identity ^b	AA % similarity ^b
<i>Takifugu</i> β	AJ496550	85.23	87	93
<i>Oncorhynchus</i> β	AF048761	81.274	86	90
<i>Oncorhynchus</i> α	AF048760	80.328	86	92
<i>Ictalurus</i>	AF400108	76.285	83	88
<i>Pimephales</i>	AF190773	77.527	82	89
<i>Danio</i>	AF529267	77.707	82	88
<i>Cyprinus</i>	AJ133735	76.717	82	89
<i>Takifugu</i> α	AJ496549	<75	79	89
<i>Homo</i> 2	NP_000608	<75	77	87
<i>Mus</i> 2	AF029758	<75	76	87
<i>Rattus</i> 2	AAC53319	<75	76	87
<i>Macaca</i> 2	AF53279	<75	75	87
<i>Gallus</i> 1	U40598	<75	66	79
<i>Homo</i> 1	L32185	<75	65	78
<i>Mus</i> 1	AAA39838	<75	65	78
<i>Ovis</i> 1	U70255	<75	65	80
<i>Bos</i> 1	U12862	<75	64	80

^aNucleotide % identity calculated using FASTA (Pearson and Lipman, 1988), values <75% generated multiple fragmentary homologies; ^bAmino acid identities and similarities by PSI-BLAST (Altschul et al., 1997)

Table 4

Tissue specific constitutive expression of *MsNramp*

Tissue	Avg Cp Controls ^a ± SEM	PCR _E ^b	Constitutive Expression	Fold Difference with WM ^c
AK	21.01 ± 0.29*	1.72	medium	85
PE	23.54 ± 0.18*	1.80	medium	28
SPL	18.90 ± 0.32*	1.70	high	240
WM	29.20 ± 0.32*	1.72	low	n/a

^aaverage crossing point (Cp) calculated from 9 control fish (3/time point); Cp is the cycle during amplification (of 45 total cycles) at which fluorescence rises above background levels* all tissues types are significantly different from each other (p<0.01); ^bPCR_E is the efficiency of the PCR reaction for each tissue type;

^cfold difference calculated as $\text{PCR}_E^{\Delta\text{Cp}}$

Figure 5. Striped bass *Morone saxatilis* *MsNramp* nucleotide and amino acid sequence. GenBank accession number AY008746. Included are the 183 bp 5'-UTR, the 1665 bp open reading frame, and the 1682 bp 3'-UTR. Numbers to the right of each line refer to nucleotide and aa numbers (inset). Brackets indicate the ATG translation start codon (position 184) and TAG termination codon (position 1848). Single letter aa residues are placed under the second nucleotide of their respective codon. Directional arrows indicate primer binding (see Table 1 and 2 for sequences). Boxed motifs are potential microsatellite tandem repeats, shaded areas represent polyadenylation signals and the double boxed poly dA tail.

Figure 6. CLUSTALX amino acid alignment of selected Nramp homologs.

Abbreviations in the left column refer to each taxon and locus as follows: Ms *Morone saxatilis*, Tr B *Takifugu rubripes* β , Dr *Danio rerio*, Cc *Cyprinus carpio*, Ip *Ictalurus punctatus*, Om B *Oncorhynchus mykiss* β , Hs 2 *Homo sapiens* 2, Mm 2 *Mus musculus* 2. Accession numbers are listed in Materials & Methods. Heavy grid outlined residues represent potential protein kinase C (PKC) phosphorylation sites and boxed areas casein kinase (CK) motifs. Double outlined and shaded polypeptides are putative transmembrane domains (TM) numbered consecutively. The crosshatched area between TM 6 and 7 is a consensus tyrosine kinase (TK) motif and amino-linked glycosylation signatures (N-gly) are broken-line outlined. The consensus 'binding-protein-dependent transport system inner membrane component signature' (transport) is darkened.

Figure 7. Phylogenetic analysis of teleost and mammalian Nramp proteins using neighbor-joining. Numbers at each node are bootstrap values obtained after 10,000 resampling efforts. GenBank accession numbers are given for each taxon and relative genetic distance is indicated by the scale bar and each branch length. Nramp1 and Nramp2 proteins form two distinct clades, and all teleost sequences group with Nramp2.

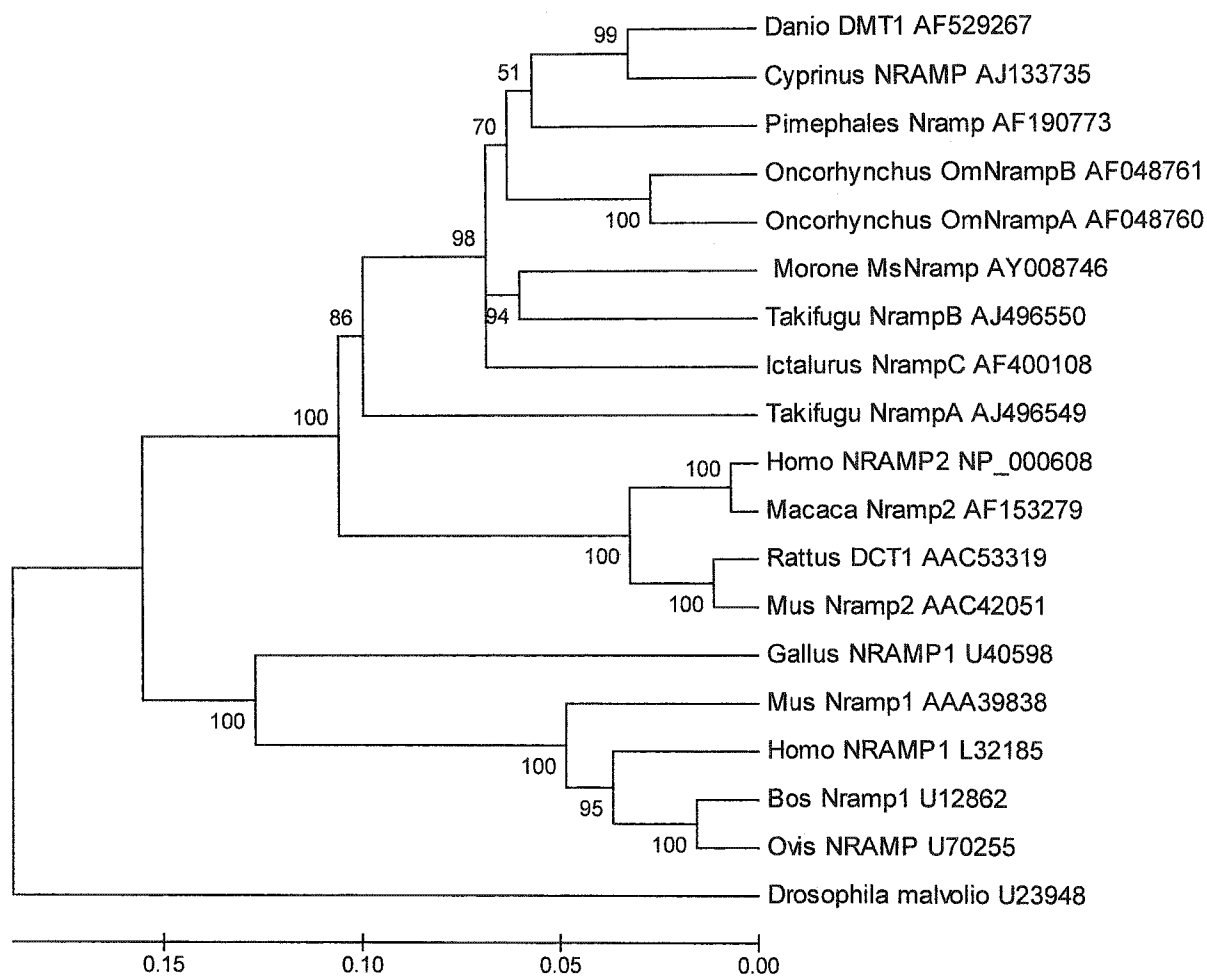


Figure 8. Tissue specific expression of *MsNramp* as measured by RT-PCR. Lanes from left to right are molecular weight marker, anterior kidney, brain, heart, gill, female and male gonad, intestine, liver, muscle, peritoneal exudate cells, peripheral blood leucocytes, spleen, no template and molecular weight marker. PCR products shown were produced utilizing a representative primer set (MsNramp736/ MsNramp1020) of three used in the actual analysis.

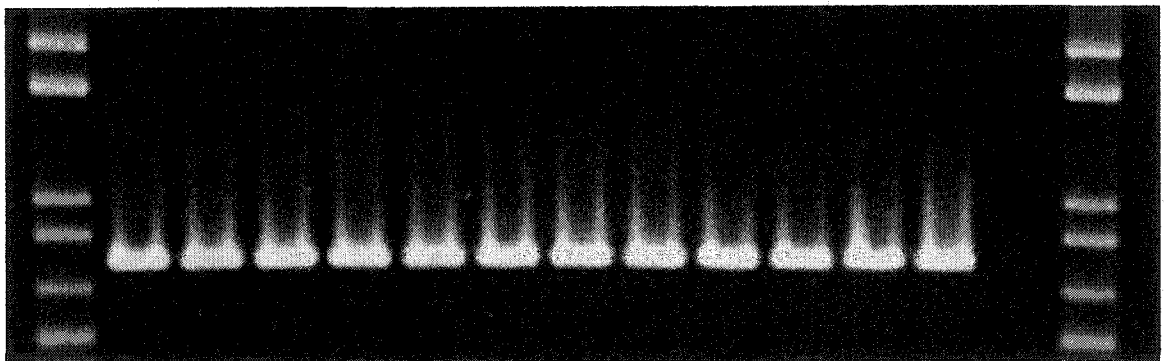


Figure 9. a) *Mycobacterium marinum* (arrow) within macrophage of striped bass. b) *M. shottsii* (arrowheads) within macrophages of striped bass. Mycobacteria are contained within a membrane-limited phagosome and are surrounded by electron-opaque material. 24 hr. post-injection. Bars = 1 μm .

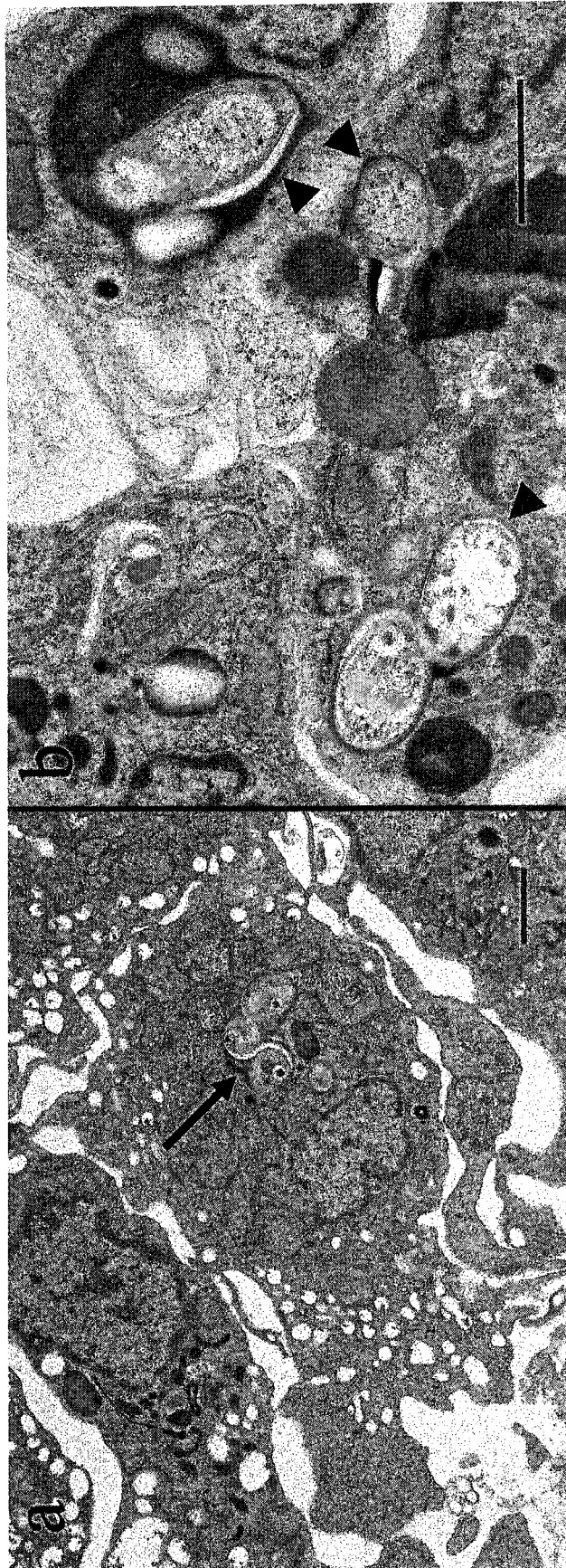
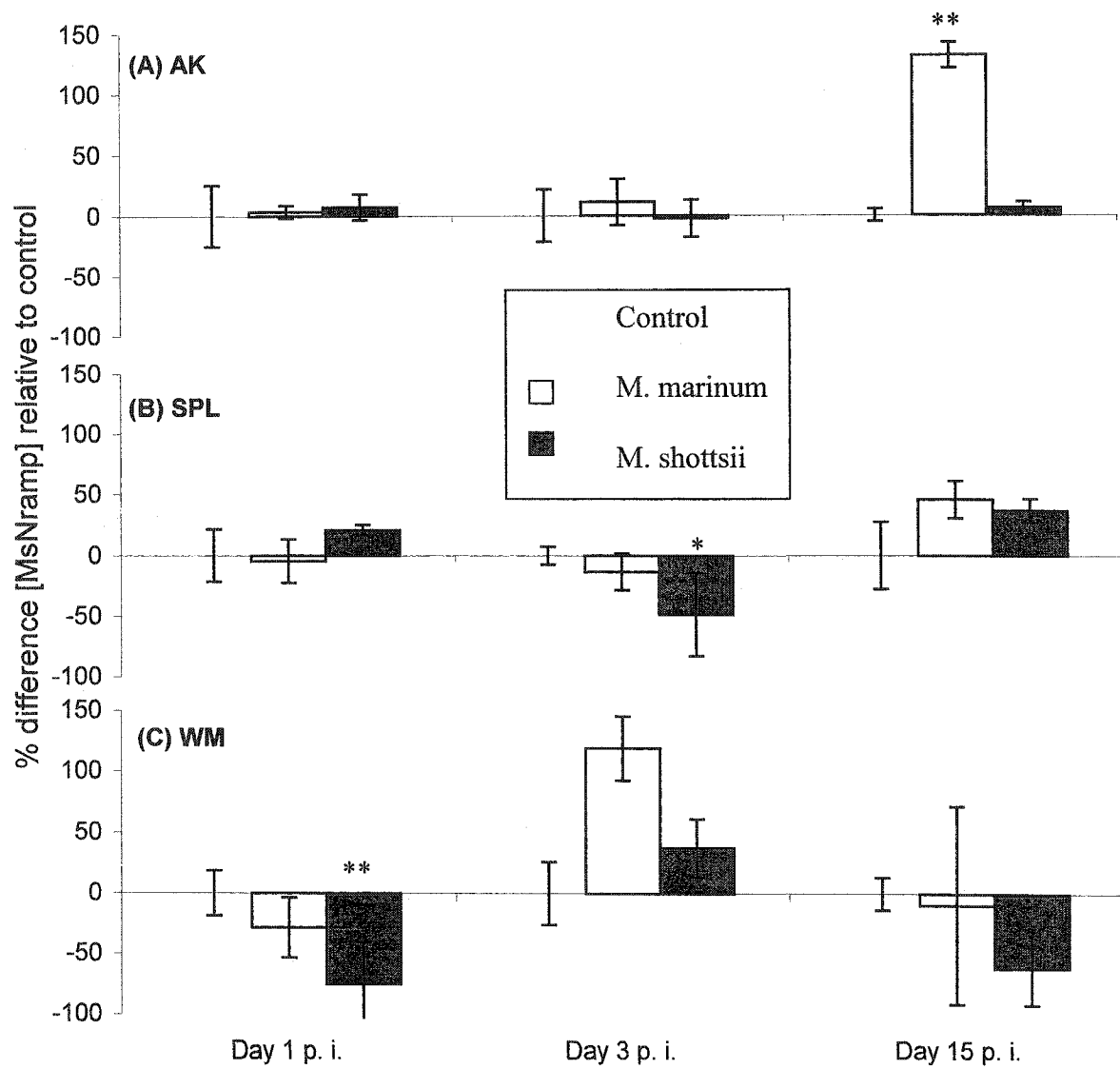
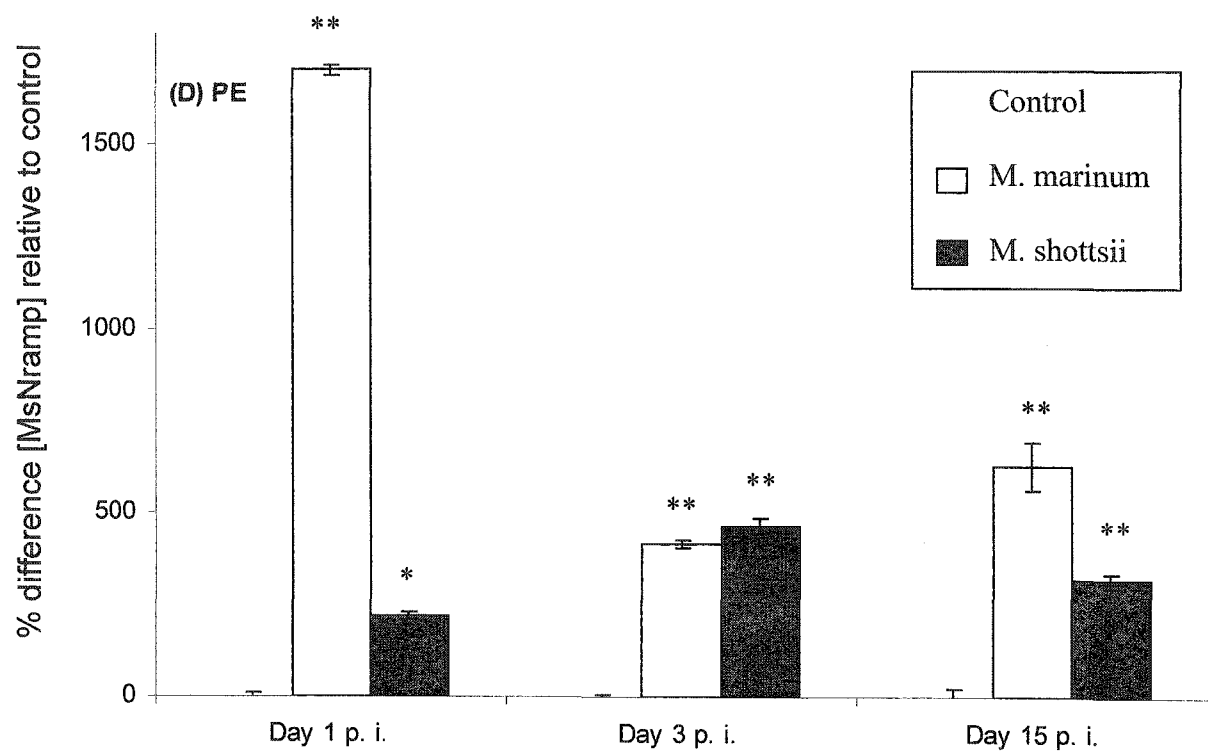


Figure 10. *Morone saxatilis* expression of *MsNramp* measured by qRT-PCR, 1, 3 and 15 days post-injection (p. i.) with *Mycobacterium marinum* or *M. shottsii*. (A) results for anterior kidney (AK); (B) spleen (SPL); (C) muscle (WM); (D) peritoneal exudate cells (PE), note the difference in scale from (A-C). Data presented as the mean of duplicate measurements of three fish/tissue/time point \pm SEM. *indicates a significant difference ($p \leq 0.05$) from the corresponding control (uninfected group), ** $p \leq 0.01$. Multiple comparison by the Tukey test or the Kramer modification of Tukey's for unequal sample sizes.





SUPPLEMENTARY DATA

Validation and reproducibility of qRT-PCR data. To determine the validity and reproducibility of *MsNramp* expression in control and experimental samples it is necessary to determine the intra- and inter-assay (total) variation as a statistic (Altman and Bland, 1983). This was accomplished by performing qRT-PCR over a wide range of concentrations (1000, 500, 250, 100, 50 ng starting RNA), with multiple replicates (n=6) at each concentration, comparing the variation within concentration groups, repeating the entire experiment, and then comparing variation of sample concentrations between the experiments. A single 'sample' consisted of the average of three crossing points for each sample dilution, consequently, each experiment consisted of six replicates of each of five concentrations. For example, a 1000 ng RNA sample should contain twice as many *MsNramp* transcripts as 500 ng of the same sample, and 20 times as many as a 50 ng sample. Treating 1000 ng as an arbitrary 'control' would predict the following results for the concentration of *MsNramp*: [500 ng]=1/2[1000 ng], [250 ng]=1/4[1000 ng], [100 ng]=1/10[1000 ng] and [50 ng]=1/20[1000 ng]. ANOVA, linear regression, Student's *t*-test and power analysis were used to calculate coefficients of variation for this experimental system (Gentle et al., 2001). Simply put, did the qRT-PCR method return the same answer each time for the same sample?

Intra-assay variation of qRT-PCR. Validation of the semiquantitative or relative measure of *MsNramp* between control (uninfected) and mycobacteria-infected striped bass was accomplished by comparing the accuracy within replicates of a sample and between replicates from separate assays. Analysis of each of the input RNA concentrations (1000, 500, 250, 125, 50 ng) showed that variance between replicates was low at the 500 ng level while still insuring adequate input RNA for amplification. Intra-assay variation, as measured by the residuals of linear regression, showed that for each of the three replicate experiments there was no systematic error (Table 5).

Inter-assay (total) variation. The comparison of identical experiments conducted on successive days showed that total variation between those duplicated experiments was low enough to accurately assess differences in the abundance of *MsNramp* between uninfected and infected striped bass. Using the series of dilutions revealed a high degree of accuracy and reproducibility for this system (Table 6). The total coefficient of variation (CV) over all RNA concentrations was 66.1%, but for the 500 ng sample CV=9.33% (p=0.96). Therefore, for an RNA sample of 500 ng, using these primers and hybridization probes, this assay could detect a true difference of >10%.

Table 5

Intra-assay variation of LightCycler experiments

	Experiment 1	Experiment 2	Experiment 3
replicate samples, n=	27	29	29
average of residuals	-0.002	-0.082	-0.011
st. dev. of residuals	0.40	0.25	0.25
ANOVA p=	0.73	0.89	0.65

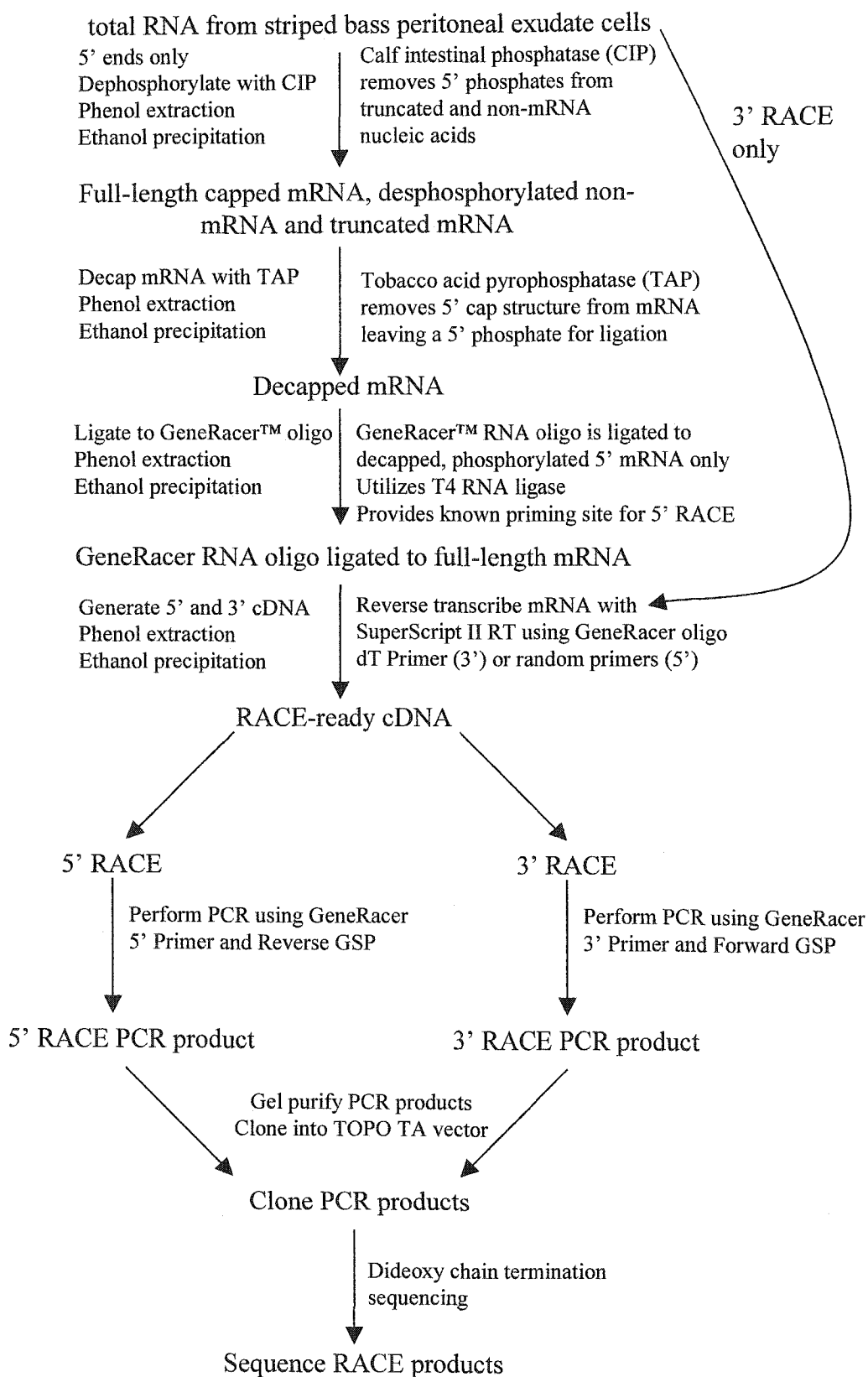
Table 6

Inter-assay (total) variation of repeated LightCycler experiments

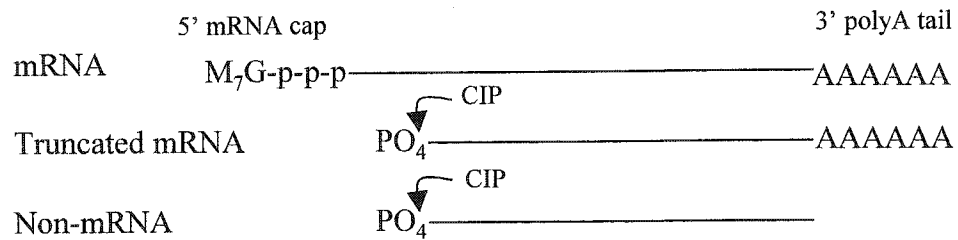
Sample	1/[<i>MsNramp</i>] predicted relative to 1000ng sample	1/[<i>MsNramp</i>] relative to 1000ng sample			coefficient of variation ^b
		Experiment 1	Experiment 2	Experiment 3	
500ng	2	2.28 ^a 2.06	2.07 1.99	1.7 2.11	9.33
250ng	4	3.57 5.97	4.01 4.61	4.16 4.09	19.0
100ng	10	9.14 12.55	8.62 9.91	5.57 7.3	26.8
50ng	20	17.31 19.67	17.5 20.6	15.4 20.1	11.0

^aeach concentration is calculated from the crossing point average of 3 samples of 500, 250, 100, or 50ng; ^bcoefficient of variation (CV)=(standard deviation)/(average)

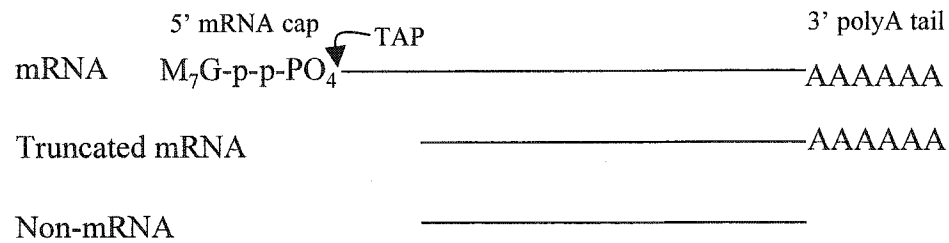
Figure 11. Schematic and graphic diagrams of the GeneRacer™ RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) protocol. This method allows the selective amplification of full-length mRNA for sequencing of the 5' and 3' ends of unknown gene sequences. The protocol is adapted from the GeneRacer™ kit manual from Invitrogen, Inc. (Carlsbad, CA; catalog # L1502-01).



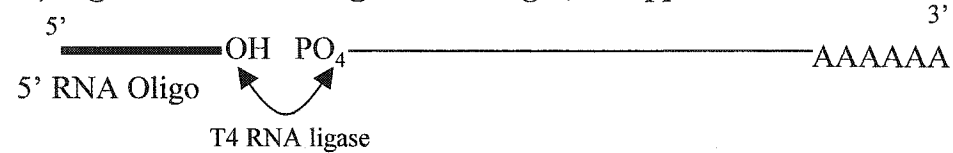
A) Dephosphorylate non-mRNA and truncated mRNA



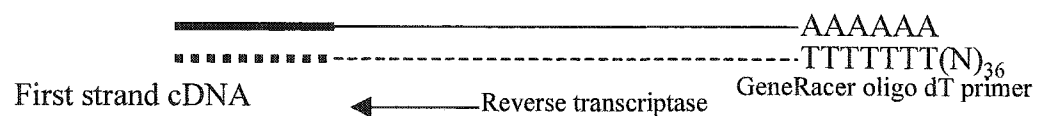
B) Phosphorylate full length mRNA and remove 5' cap structure



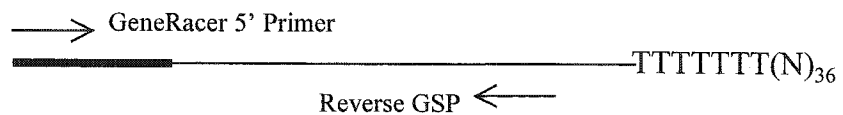
C) Ligate the 5' RNA oligo to full-length, decapped mRNA



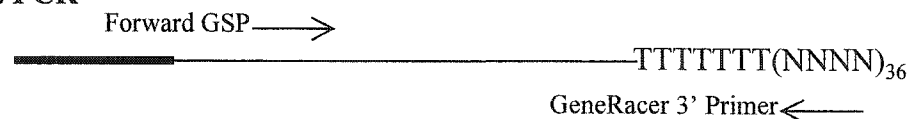
D) Reverse transcribe mRNA to first strand cDNA



E) 5' RACE PCR



F) 3' RACE PCR



CHAPTER TWO. *IN VITRO* RESPONSE OF THE STRIPED BASS NATURAL
RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN, *NRAMP*, TO LPS AND
MYCOBACTERIUM MARINUM EXPOSURE

ABSTRACT

Mycobacteriosis in Chesapeake Bay (USA) striped bass *Morone saxatilis* is an emerging disease problem with potentially important economic implications for a large commercial and recreational fishery. Recently, we have described a striped bass gene homolog of the natural resistance-associated macrophage protein (*Nramp*), which is responsible for innate resistance to mycobacterial infections in mice. The striped bass homolog, *MsNramp*, is strongly induced in peritoneal exudate cells (PE) *in vivo* after intraperitoneal injection with *Mycobacterium* spp. The purpose of the present study was to investigate short-term *in vitro* *MsNramp* expression and ROI production in primary cultures of adherent anterior kidney cells (AK) and PE after exposure to bacterial lipopolysaccharide (LPS), and live- or heat-killed (HK) *Mycobacterium marinum*. Relative quantification of *MsNramp* expression was accomplished using a real-time RT-PCR technique. PE expressed significantly higher levels of *MsNramp* at 4 and 24 hours post-treatment with live and HK *M. marinum*. *MsNramp* response to LPS was dose-dependent in these cells, with maximum expression at 4 hr and 20 µg/ml LPS. Treatment of PE with LPS caused an increase in intracellular superoxide anion (O_2^-) levels, whereas treatment with live *M. marinum* caused a significant depression. AK responded to LPS with increased ROI and *MsNramp* production, but were uninduced or suppressed relative to controls by mycobacteria. This study is the first report of induction of a teleost *Nramp* *in vitro* by mycobacteria, and supports reports of teleost *Nramp* induction by LPS.

INTRODUCTION

Striped bass (*Morone saxatilis*) are an economically and ecologically important finfish species on the east coast of North America. An epizootic of mycobacteriosis in striped bass is currently occurring in the Chesapeake Bay, with reported disease prevalence as high as 62.7% in wild fish (Cardinal, 2001). Mycobacteriosis in striped bass is characterized by widespread visceral granulomatous inflammation, and infrequently, ulcerative skin lesions (Vogelbein et al., 2000). This disease has been associated with severely emaciated fish, but mortality rates due to mycobacteriosis in the wild are currently unknown. Numerous mycobacterial species have been cultured from diseased bass, including *Mycobacterium marinum*, and isolates similar to *M. avium*, *M. gordonae*, *M. peregrinum*, *M. scrofulaceum*, and *M. terrae* complex (Kaattari et al., 2001; Rhodes et al., 2000). In addition, a new species, *M. shottsii*, has been identified that is phylogenetically similar by 16S rRNA to *M. marinum* and *M. ulcerans* (Rhodes et al., 2003b).

Pathogenic mycobacteria are intracellular parasites of macrophages whose persistence within the host is dependent on circumventing or resisting phagolysosomal fusion, free-radical-based killing mechanisms, and genetically-based resistance mechanisms (Flynn and Chan, 2001). The formation of the phagolysosome is a dynamic process that involves fusion with endocytic vacuoles containing a variety of degradative enzymes capable of digesting macromolecules and/or microorganisms and recruitment of

the natural resistance-associated macrophage protein 1, Nramp1 (Vidal et al., 1993) into the membrane (Gruenheid et al., 1997). Gruenheid *et al.* (1997) used immunofluorescence and confocal microscopy to localize Nramp1 protein in late endocytic compartments (late endosome/lysosome) of mouse macrophages. Double immunofluorescence of phagosomes indicated that Nramp1 is recruited to the phagosomal membrane during maturation of the microbial phagosome. The presence of 5' and 3' endocytic targeting signals in *Nramp1* transcripts (Atkinson et al., 1997) is consistent with protein recruitment directly from the Golgi apparatus to the late endosome/lysosome of the macrophage (Blackwell et al., 2001; Gruenheid et al., 1997). In a mycobacterial infection, the pathogen becomes localized to this compartment. In the absence of effective host defense, the bacteria multiply within the phagosome.

Nramp1 belongs to a small family of highly conserved proteins that includes two known mammalian genes, *Nramp1* (Vidal et al., 1993) and the ubiquitously expressed *Nramp2* (Gruenheid et al., 1995), as well as related sequences in many other taxa (Cellier et al., 1996). Besides mice, *Nramp* gene sequences have been isolated from humans (Cellier et al., 1994; Kishi, 1994), rats (Gunshin et al., 1997), birds (Hu et al., 1995), fish (Dorschner and Phillips, 1999), insects (Rodrigues et al., 1995), nematodes (The C. elegans Sequencing Consortium, 1998), plants (Belouchi et al., 1995), yeast (Portnoy et al., 2000), and bacteria (Makui et al., 2000). Vertebrate homologs share a 12 transmembrane domain topology, glycosylation, extensive phosphorylation, and a highly conserved binding protein-dependent transport system inner membrane component signature. Malo et al. (1994) and Vidal et al. (1995a) sequenced *Nramp* cDNA clones from 27 mycobacteria-susceptible (*Nramp^S*) and -resistant (*Nramp^R*) mouse strains and

showed a common nonconservative glycine to aspartic acid substitution within the fourth transmembrane domain of *Nramp*^S strains, confirming a role for *Nramp1* in mycobacterial resistance in the mouse. Gene knockout experiments in mice revealed that *Nramp1* plays an important role in the early stages of parasite-macrophage interactions (Govoni et al., 1996). In that study, a null allele (*Nramp1*^{-/-}) transfectant mouse line was developed that lacked the normal resistance of its parent strain to *Mycobacterium* spp. while maintaining normal appearance and longevity. Reintroduction of the wild-type *Nramp1*^{+/+} onto that genetic background completely restored resistance to intracellular parasites .

Govoni *et al.* (1995) analyzed the regulatory sequences found within the promoter region of *Nramp1* and found nucleotide binding motifs that were characteristic of macrophage-specific transcription factor PU.1, IFN- γ -inducible gene expression and bacterial lipopolysaccharide (LPS) responsiveness. These regulatory sequences suggest that *Nramp1* transcription can be modulated during macrophage activation in response to cytokine or bacterial stimuli. Northern blotting experiments performed on mouse macrophages demonstrated that *Nramp1* was upregulated by the inflammatory mediators, IFN- γ and LPS, and substantially increased by pretreatment with IFN- γ followed by LPS exposure (Govoni et al., 1997). IFN- γ and LPS also regulate expression of mouse *Nramp2* transcripts, and are believed to influence macrophage Fe metabolism during anemia by modifying *Nramp2* transcription (Wardrop and Richardson, 2000).

Research conducted with rat *DCT1*, an *Nramp2* homolog, indicated that members of the Nramp protein family function to transport divalent cations (Gunshin et al., 1997). Jabado et al. (2000) reported that Nramp1 functions as a proton-gradient coupled divalent

cation symporter that removes redox-active metals from the phagosome by monitoring divalent cation flux within macrophage phagosomes *in situ* and in real time using fluorescent-tagged zymosan particles. It was shown that *Nramp1*^{+/+} macrophages accumulated less Mn than null allele cells. These differences were abrogated when proton flux across the membrane was inhibited. Another study found that overexpression of Nramp1 in a macrophage cell line also increased the efflux of radiolabelled iron (Atkinson and Barton, 1998). In contrast to these results, Zwilling et al. (1999) and Khun et al. (1999) report that *M. avium*-infected *Nramp*^R macrophages accumulate more iron (as Fe²⁺) than susceptible *Nramp*^S phagosomes and control mycobacterial growth more effectively.

We have previously reported nucleotide and amino acid sequences for a striped bass homolog of the natural resistance-associated macrophage protein, *MsNramp*. Using real-time PCR, we have demonstrated a large (approximately 17-fold) induction of *MsNramp* gene expression in peritoneal exudate cells (PE) after *in vivo* exposure to *M. marinum* (Burge et al., 2004). These results are suggestive of similarities between the roles for *MsNramp* and mammalian *Nramp1* in resistance to intracellular parasite infection, although the involvement of *MsNramp* (or any teleost *Nramp*) in resistance has not been demonstrated.

One of the primary effector functions of macrophages in the innate immune response is the generation of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) produced during the macrophage 'respiratory burst'. These are potent bactericidal and bacteriostatic compounds (Goldsby et al., 2000; Nathan and Shiloh, 2000). The complementary roles of RNI, ROI and Nramp1-mediated resistance

to pathogens are most fully understood in the mouse model, but all three antimicrobial systems have also been found in teleost fish, and current research in the mouse is centered on investigating the redundancy, synergy, and regulation of these responses (Karupiah et al., 2000). Evidence demonstrating the accumulation of iron within Nrp1⁺ mycobacterial phagosomes has suggested a direct linkage between the expression of Nrp1 and the intracellular ROI response (Kuhn et al., 1999; Kuhn et al., 2001; Zwilling et al., 1999). Iron-treated Nrp1-expressing phagosomes more effectively control growth of *M. avium* (Zwilling et al., 1999), and have increased hydroxyl radical formation (Kuhn et al., 1999). Iron cations are essential metal cofactors in the Fenton reaction, producing hydroxyl ions from superoxide anion, and may contribute to resistance using this upregulation of microbial killing. Measurements of both ROI production and *Nrp1* expression would, therefore, serve as useful markers of macrophage activation and anti-mycobacterial activity.

In the current study, we measured the *in vitro* expression of *MsNrp1* and production of intracellular superoxide (O₂⁻) in primary cultures of adherent AK and PE exposed to LPS, live *M. marinum*, or heat-killed *M. marinum*. In addition, the short-term bactericidal activity of AK and PE to *M. marinum* was quantified. Possible relationships between *Nrp1* and ROI induction and the potential role of *MsNrp1* in resistance to mycobacterial infections in fish are discussed.

MATERIALS AND METHODS

Experimental fish and maintenance. Striped bass *Morone saxatilis* (500-2000 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 fish were maintained in 3000 l tanks with flow-through, sand-filtered York River water at ambient temperature and salinity. At the time of these experiments, temperature was approximately 15°C and salinity 17 ppt. Tanks were illuminated with fluorescent lighting adjusted to local photoperiod. Fish were fed daily to satiation with wild-caught small fish and crabs and acclimatized to captivity for at least two weeks prior to experimental use.

Cell Culture. All chemicals and cell culture reagents were from Sigma Chemical (St. Louis, MO), unless otherwise noted. Primary cell cultures of peritoneal exudate cells (PE) and anterior kidney cells (AK) were isolated and cultured with modifications of the methods of Secombes (1990). PE were elicited by intraperitoneal injection of 750 µl Freund's Incomplete Adjuvant 7-10 days prior to harvesting. Fish were anesthetized with an overdose of tricaine methanesulfate (Argent Chemical, Redmond, WA) and injected with 10-15 ml ice-cold Leibowitz L-15 medium containing 2% fetal bovine serum (FBS), 100 U/ml penicillin + 0.1 mg/ml streptomycin (P/S) and 100 U/ml heparin. Lavage fluid was withdrawn via ventral incision after 10 minutes. AK tissue was harvested aseptically

after lavage, and a single cell suspension was made by pressing tissue through a 100 μm stainless steel screen. AK leukocytes were purified on a 38% discontinuous Percoll gradient by centrifuging at 4°C for 20 minutes at 800 x g. AK and PE were washed twice in L-15/2% FBS/P-S/10 U/ml heparin, and counted on a hemacytometer. Trypan blue exclusion staining showed viability of cells to be greater than 95% in all cases. After counting, cells were resuspended to appropriate concentrations in L-15/0.1% FBS/P-S. Cells for respiratory burst assays (ROI) were seeded at a density of 8×10^5 viable cells/well into 96 well tissue culture plates, and cells for RNA extraction were seeded at 1.5×10^7 viable cells/well in 10cm^2 (6 well) tissue culture plates. Cells were allowed to adhere to plates for four hours, at which point the medium was replaced with L-15/5% FBS. Cells were kept at 4°C until adherence, after which they were gradually warmed to 18°C, where they were maintained throughout the remainder of the assays. Cell monolayers were rested 24 hours after adherence to minimize activation due to processing.

Cell treatments. Cells were stimulated with the following treatments: control untreated, LPS at final concentrations of 1, 5, 10, 20, 50, 100 and 200 $\mu\text{g/ml}$ (*E. coli* 055:A4), heat-killed (HK) *M. marinum*, or live *M. marinum*, both at a multiplicity of infection (MOI) of approximately 1:5. *Mycobacterium marinum* (VIMS isolate M30, fish-passaged) was grown and prepared as described previously (Gauthier et al., 2003). Briefly, turbid cultures were grown in Middlebrook 7H9 medium with OADC enrichment and 0.05% polyoxyethylenesorbitan monooleate (Tween 80)(MDB) at 30°C. Bacteria were pelleted by centrifugation at 12,000 x g for 20 min and washed once in Butterfield's phosphate

buffer with 0.05% Tween 80 (PB) Washed cultures were resuspended in 1-2 ml PB, vortexed vigorously with glass beads (500 μ m diameter) for 2 min and filtered through Whatman No. 1 paper to remove clumps of bacteria and obtain a homogeneous suspension. Absorbance at 590 nm was adjusted with Hank's Balanced Salt Solution (HBSS) to 0.15, and ten-fold dilutions plated on Middlebrook 7H10 agar with OADC enrichment and 0.5% glycerol (MDA) for subsequent enumeration. HK *M. marinum* were prepared by heating aliquots of the bacterial suspension to 70°C in a water bath for 2 hr. Sterility was confirmed by plating on MDA. Based on turbidimetric measurements for this strain of *M. marinum*, M30 growth in Middlebrook 7H9 broth with ADC enrichment and containing 0.05% Tween 80, doubling times are approximately 2-3 days at 23°C and 1 day at 28°C (M. Rhodes, personal communication).

Intracellular superoxide production (O_2^-). Adherent AK and PE cultures were stimulated for 4 or 24 hours prior to measurements of O_2^- . Quadruplicate wells were measured for all treatments. Negative controls consisted of unstimulated cells tested in parallel with treated cells at each timepoint. At the end of the stimulation period, the cells were washed 2x in HBSS and 100 μ l of 1 mg/ml nitroblue tetrazolium (NBT) test solution was overlaid. After 1 hour of development, cells were fixed with repeated washes of methanol, and the formazan reduction product was dissolved in 1:1 KOH/DMSO and the plates were read on an automated plate reader (Molecular Devices, Sunnyvale, CA) at 590 nm.

Bactericidal assay. After 24 hr in antibiotic-containing medium, cells in 96-well plates were washed twice with L-15 (no additives), then overlaid with 150 μ l L-15/5% FBS without antibiotics. Fifty μ l of bacterial suspension giving an approximate target:effector ratio of 5:1 was then added to wells. Control wells received 50 μ l HBSS without mycobacteria. Bacteria were allowed to settle and be phagocytosed for 90 min, at which point cells were washed twice with L-15 and overlaid with 200 μ l L-15/5% FBS with no antibiotics. Immediately (Time 0) and after a chase of 4 or 24 hrs in fresh medium, medium was removed and 50 μ l sterile 0.1% Tween-20 in distilled water was added to lyse cells. Lysis was allowed to proceed for 10 minutes, and 150 μ l MDB was added to each well. The plate was then incubated at 30°C in a humid chamber for 48 hours to allow bacterial outgrowth. After incubation, 10 μ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and color was allowed to develop for 4 hr. After 4 hr., MTT reduction was halted and bacterial cells lysed by addition of 50 μ l 20% SDS to each well. Plates were placed on a “belly dancer” mechanical rocker overnight to dissolve the purple MTT reduction product and scanned at 590 nm with an automated plate reader. Data are presented as Killing Index (Graham (nee Chung) et al., 1988), which is the mean OD₅₉₀ of quadruplicate wells at time x (T_x) divided by mean OD₅₉₀ of quadruplicate wells at time 0 (T₀), with values above one representing bacterial outgrowth and values below one representing bacterial killing.

RNA extraction. Cells in six-well tissue culture plates were treated as described above (only 10, 20, and 50 μ g/ml LPS dosages used). After the stimulation period, medium was decanted from treated cells, the monolayers rinsed twice with HBSS, and one ml TRIzol

(Invitrogen, Carlsbad, CA) added to the cells. Samples were immediately extracted as per the manufacturer's instructions or held at -80°C until processed. RNA pellets were dissolved in RNA Storage Solution (Ambion, Austin, TX) and stored at -80°C . Total RNA was assessed for integrity by agarose gel electrophoresis and quantitated by UV spectrophotometry.

Real-time semiquantitative reverse transcription-PCR. This procedure was performed on the Roche Molecular Biochemicals LightCycler System and the appropriate primers and hybridization probes developed using LightCycler Probe Design v. 1.0 software (Idaho Technologies, Inc, Salt Lake City, UT). Sequences of primers and probes from 5' to 3' are as follows: forward primer (MsNramp736) TTGTCGTAGCGGTCTT, reverse primer (MsNramp1020) GGGACCACCGTAGGTTTA, 5' hybridization probe (5MsNramp942f) GCTGGACAGAGTTCCACCA-fluorescein, 3' hybridization probe (3MsNrampRed963p) LightCycler Red640 dye-ACAGGCACTTACTCGGGG-phosphate. All reagents were prepared at 4°C in low light to minimize nonspecific amplification and fluorophore degradation.

The PCR reaction consisted of a master mix of water, manganese acetate (final concentration 4.25 mM), hybridization probes (0.2 μM), primers (0.5 μM) and LightCycler RNA Master Hybridization Probes enzyme mixture (1x). To initiate the reaction, 500 ng of sample RNA was added to each capillary and LightCycler cycling immediately begun. RNA samples were quantified immediately before use by spectrophotometric detection at 260 and 280 nm, and corrected for protein concentration

at 320 nm. RNA sample concentrations calculated by spectrophotometry were reproducible within 5%.

Reverse transcription was performed at 61°C for 20 min, followed by primary denaturation of the RNA:cDNA hybrid at 95°C for 30 sec. Amplification consisted of 45 cycles of denaturation (95°C for 1 sec), annealing/hybridization (54°C for 15 sec), and elongation (72°C for 11 sec). Each cycle was followed by fluorescence monitoring by the LightCycler at 640 nm. Four amplification reactions were performed for each RNA sample. Data collection and preliminary analyses were conducted using LightCycler Data Analysis software v. 3.3.

qRT-PCR analysis. Six replicates of each of five RNA concentrations (500, 200, 100, 20, and 10 ng RNA) were amplified for each cell type and a mean efficiency of PCR (PCR_E) calculated. The PCR_E was calculated as:

$$PCR_E = 10^{-1/\text{slope}}$$

$$\text{where } 1 \leq PCR_E \leq 2$$

Measurement of the slopes for AK and PE was accomplished by linear regression of the crossing points of the six replicates against the \log_{10} RNA concentration. The crossing point (Cp) of the qRT-PCR is the point during amplification at which fluorescence of a sample rises above the background. That point on the amplification curve is proportional to the amount of starting mRNA template (*MsNramp*) in the sample. Percent difference is then calculated as follows:

$$\% \text{ difference} = (\text{PCR}_E^{\Delta\text{Cp}} \times 100) - 100 \text{ (Gentle et al., 2001)}$$

where ΔCp = (control sample crossing point - experimental sample crossing point)

MsNramp expression was quantified by calculation of percent increase or decrease in treated cells compared to untreated controls. This served to normalize treatment effects to control (constitutive) expression. Crossing points for each fish and treatment were averaged, the standard error of the mean calculated for the group, and the formula above used to calculate a percentage difference between treated and untreated groups. Control *MsNramp* expression was normalized to one hundred per cent at both 4 and 24 hr.

Statistical analysis. For calculation of crossing points and slope for PCR_E , linear regression was performed by LightCycler software v. 3.3. Treatment effects associated with *MsNramp* expression and intracellular superoxide anion production were analyzed by single-factor ANOVA ($\alpha=0.05$), and multiple comparisons performed using Tukey's Studentized Range Test ($\alpha=0.05$ and 0.01) in SAS v. 8.0 (SAS Institute Inc., Cary, NC) with Kramer's modification for unequal samples sizes where appropriate. Analysis of time post-treatment differences was performed with paired two sample for means *t*-test ($\alpha=0.05$), which tested the aggregate difference at each timepoint in SAS.

RESULTS

Intracellular superoxide anion (O_2^-) production in adherent AK and PE cells treated with LPS. AK responded to LPS in a dose-dependent manner at 4 hr post-treatment, with peak O_2^- production for the treatment with 20 $\mu\text{g/ml}$. Intracellular superoxide production declined but remained significantly higher than controls after 24 hr stimulation. No optimal LPS concentration was seen at 24 hr (Fig. 11A). Intracellular superoxide production by AK at 4 hr was significantly different from production at 24 hr. PE O_2^- production was significantly elevated after stimulation with between 20-200 $\mu\text{g/ml}$ LPS at 4 hr and between 5-200 $\mu\text{g/ml}$ at 24 hr (Fig. 11B). Maximal responses for PE were seen at 24 hr, in contrast to results for AK. In the intracellular superoxide anion assay, control AK values were higher than PE, and in general, had higher inherent variance.

Intracellular superoxide anion (O_2^-) production in adherent AK and PE cells treated with mycobacteria. Differential responses in O_2^- production were seen between AK and PE treated with either HK or live *Mycobacterium marinum*. Histology and electron microscopy confirmed phagocytosis of both live and dead bacteria at both timepoints (data not shown), but O_2^- production relative to control cells was not significantly elevated at either timepoint. Both cell types were generally unresponsive to HK mycobacteria, with the exception of a slight but significant increase in O_2^- at 4 hr in PE.

Superoxide production was significantly depressed by live mycobacteria at 24 hr in both PE and AK (Fig. 12). Cell counts of mycobacteria-exposed wells confirmed that depression of O_2^- was not due to loss of cells from culture. In a separate experiment, PE were treated with conditioned cell culture supernatants (1:8 w/v) derived from mitogen-stimulated (10 μ g/ml concanavalin A and 5 ng/ml PMA) cultures of AK, 0.5 μ g/ml phorbol myristate acetate, or live *M. marinum*, and significant ROI increases were seen for the supernatant and PMA treated cells, but a lack of effect or depression noted for live mycobacteria at both 4 and 24 hours (Fig. 13).

Mycobactericidal activity of AK and PE cells. Mycobacteria were persistent within both AK and PE of striped bass throughout the 24 hr observation (Fig. 14). No significant increases in bacterial numbers were observed for AK over 24 hr. A slight but significant increase in bacterial numbers was noted at both 4 and 24 hr in PE. Generation time for this strain (VIMS M30) of *M. marinum* is approximately 4 days at 18°C.

Calculation of PCR efficiency (PCR_E) for AK and PE cells. The most important parameter associated with accurate relative quantification of real-time PCR results involves the calculation of the overall efficiency of the reaction. PCR_E was calculated for AK and PE using linear regression of crossing points against \log_{10} transformed RNA concentrations (Fig. 15). PCR_E was estimated at 1.87 for AK and 1.86 for PE. PE constitutively express approximately 10 times (9.71 ± 1.09 ; $p < 0.01$) the *MsNramp* measured in adherent AK.

***MsNramp* gene expression after LPS treatment in AK and PE cells.** No significant change in *MsNramp* gene expression was observed in AK after treatment with the LPS concentration optimal for superoxide production (20 µg/ml). LPS concentrations of 10 and 50 µg/ml resulted in significant depression in *MsNramp* in AK at both 4 and 24 hr (Fig. 16A). Treatment with 20 µg/ml LPS caused a significant 2-3 fold elevation of *MsNramp* levels at both 4 and 24 hr in PE (Fig. 16B). Higher or lower LPS concentrations did not induce *MsNramp* at 4 hr, and caused a depression in *MsNramp* at 24 hr.

***MsNramp* expression after live and HK *M. marinum* exposure in AK and PE .** No significant differences in *MsNramp* expression were seen after treatment of AK with HK mycobacteria, but high fish to fish variability may have masked any true differences. Expression was unchanged 4 hr after treatment with live mycobacteria, but was significantly depressed after 24 hr (Fig. 17A). In PE (Fig. 17B), 4 hr treatments of live and HK mycobacteria significantly increased expression of *MsNramp*. A nearly two-fold induction was noted for cells treated with HK *M. marinum* at 4 and 24 hr. Live mycobacteria stimulated a significant, approximately 50% increase in *MsNramp* transcript abundance during both timepoints.

DISCUSSION

Nramp1 protein in mice is critically important for resistance to intracellular pathogens, including *Mycobacterium* spp. (Vidal et al., 1993). Mouse *Nramp1* is induced *in vitro* and *in vivo* by LPS administration (Govoni et al., 1997) and exposure to mycobacteria (Vidal et al., 1995b; Zhong et al., 2001) or *Salmonella* (Govoni et al., 1999). We have cloned and sequenced a homolog of the *Nramp* gene family from striped bass *Morone saxatilis* and showed that it is highly induced *in vivo* in PE after exposure to *Mycobacterium marinum* (Burge et al., 2004). Previous work with fish *Nramp* homologs demonstrated that LPS upregulated expression *in vivo* in channel catfish (Chen et al., 2002; Dorschner, 1998). Channel catfish treated intraperitoneally for 5 hr with 30 mg/kg LPS were shown to upregulate *Nramp* transcription in AK and spleen, but not in peripheral blood leukocytes or intestinal tissue. Additionally, the channel catfish monocyte cell line 42TA responded to a 48 hr cocktail treatment (mitogen-activated cell supernatants, 25 µg/ml *Salmonella* LPS, and 1 µg/ml extracellular protein from *Edwardsiella ictaluri* cultures) with an approximately five-fold increase in *Nramp* transcripts (Chen et al., 2002). Several fish species have been shown to respond to LPS by upregulating expression of genes associated with the innate immune system (Engelsma et al., 2001; Neumann et al., 1995). For example, rainbow trout RTS11, a macrophage-like cell line of the spleen, increased expression of IL-1 β and COX-2, both

of which are involved in innate immune regulation and host defense after treatment with 5 µg/ml LPS (Brubacher et al., 2000).

In this study, we measured the expression of the striped bass *MsNramp* gene *in vitro* after exposure of AK or PE to LPS, HK- or live-*Mycobacterium marinum*. Striped bass PE responded to LPS stimulation *in vitro* with increasing mRNA transcription (3-fold induction) of *MsNramp* within 4 hr of exposure. At 24 hr, post-treatment levels of *MsNramp* were still elevated by 20 µg/ml LPS, but depressed by greater and lesser doses. These results support the contention that *MsNramp* transcription in striped bass PE responds to LPS in a similar way to mouse *Nramp1*. Transcription factors associated with LPS stimulation are present in the mouse *Nramp1* promoter (Govoni et al., 1995) and these results indicate that similar regulatory sequences may operate within *MsNramp*. Govoni et al. (1997) demonstrated rapid induction of *Nramp1* by LPS within 2 hr, with a lessening of expression noted by 24 hr post-treatment in mouse RAW264.7 cells.

MsNramp appears to require considerably higher LPS concentration for upregulation, however. Mouse *Nramp1* was stimulated by LPS doses as low as 0.1 ng/ml, with a maximal response noted for 250 ng/ml, whereas striped bass demonstrated maximal response at 20 µg/ml. Research into lower vertebrate innate immunity has previously noted a lack of effect of LPS dosages that are stimulatory to mammalian cells (Berczi, 1998). Whereas PE had a robust *MsNramp* response to LPS treatment, AK were refractory to LPS stimulation, although it cannot be concluded that a subset of the total adherent cell population was not responding by increasing transcription. Relative to AK, which contain large numbers of lymphocytes and immature leucocytes, adherent PE populations are highly enriched in activated, mature macrophages (Bodammer, 1986;

Press and Evensen, 1999). Mature macrophages are the primary mediator of LPS-induced transcription of *Nramp1* in mice (Govoni et al., 1997). Therefore, it may be that AK *MsNramp* response to LPS could have been masked by the diluting presence of other, refractory cell types, or by relatively nonreactive immature macrophages.

AK had no significant *MsNramp* response to either live- or HK-mycobacterial stimulation, with the exception of a slight but significant inhibition at 24 hr. In contrast, PE showed a clear and significant induction of *MsNramp* in response to both live- and HK-mycobacteria at both time 4 and 24 hr post-exposure. These results, coupled with findings on LPS-stimulated AK, suggest that AK may be less optimal than PE for *in vitro* expression assays of *MsNramp* modulation.

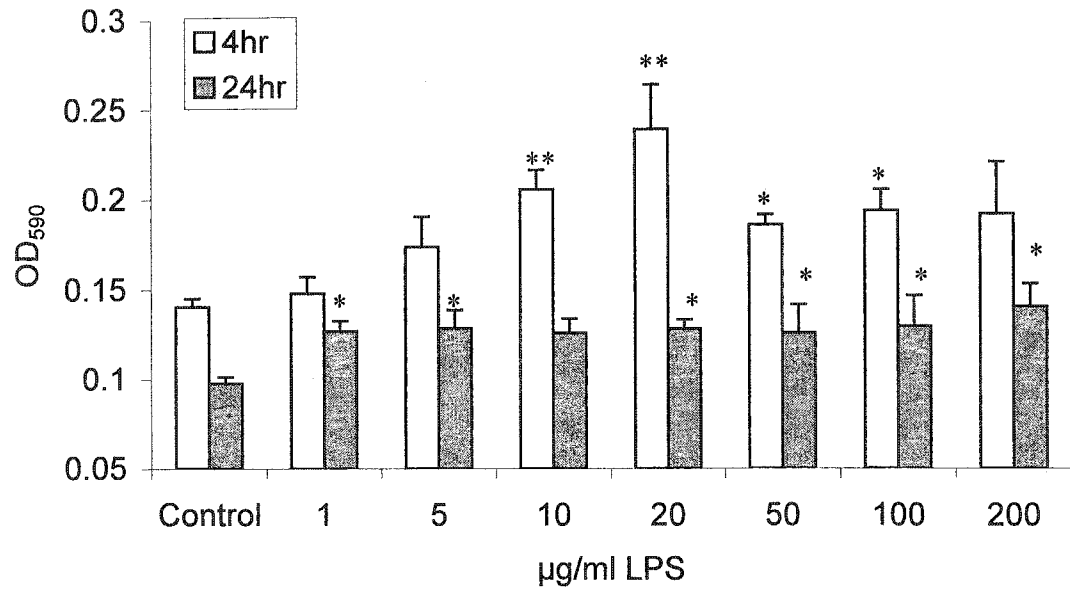
Measurements of ROI production are useful for measuring the overall activation state of macrophages, and in the present study, were applied to striped bass primary cell cultures derived from anterior kidney and peritoneal exudates in response to LPS and mycobacteria. Striped bass AK and PE responded to LPS were dose-dependent. Temporal patterning for the two cell types was different, with a maximal response noted for AK at 4 hr post-stimulation, and 24 hr for PE. In PE, ROI production and *MsNramp* expression were stimulated by both HK- and live-mycobacteria at 4 hr, while ROI after 24 hr treatments were equal to or significantly depressed from control cells. These results suggest that live mycobacteria may have a specific inhibitory effect on ROI production in striped bass while *MsNramp* mRNA expression levels remained high at 24 hr in PE. Treatment with macrophage-activating cell supernatants or PMA confirmed that striped bass PE could respond with increased O_2^- , while also being suppressed by live mycobacteria (Fig. 13).

Mycobactericidal assays of cultured AK and PE demonstrated that these cells were unable to control the growth of *M. marinum* effectively *in vitro*. Significant outgrowth of mycobacteria occurred by 24 hr post-exposure. These results are supported by the findings of Wolf and Smith (1999) and Gauthier et al. (2003). The former showed striped bass to be a more susceptible species to mycobacteriosis, compared to tilapia and demonstrated more destructive pathology associated with infection. The latter work established the persistence of live mycobacteria within AK tissue of striped bass.

In summary, these studies present evidence of the *in vitro* induction of striped bass *MsNramp* in response to LPS and mycobacterial treatments. This represents the first report of *in vitro* induction of a teleost *Nramp* gene by mycobacteria, and supports the findings of Chen (2002) and Dorschner (1998), that teleost *Nramps* can be induced by LPS treatment.

Figure 12. (A) Intracellular superoxide anion (O_2^-) dose response to LPS in anterior kidney (AK) cells at 4 hr and 24 hr. The elevated OD_{590} readings for AK in this assay are the result of a cell seeding density of 1.6×10^6 cells/well. AK seeding densities for all subsequent experiments were as described in Materials and Methods. Results were significantly different ($p < 0.01$) at 4 and 24 hr using paired two sample for means t -test. (B) O_2^- dose response to LPS in peritoneal exudate (PE) cells. Bars represent the mean of quadruplicate wells for three fish + SEM; significant difference relative to control by ANOVA and multiple comparison, * $p < 0.05$, ** $p < 0.01$.

A) AK. 4 hr vs. 24 hr (t -test $p < 0.01$)



B) PE. 4 hr vs. 24 hr (t -test $p < 0.01$)

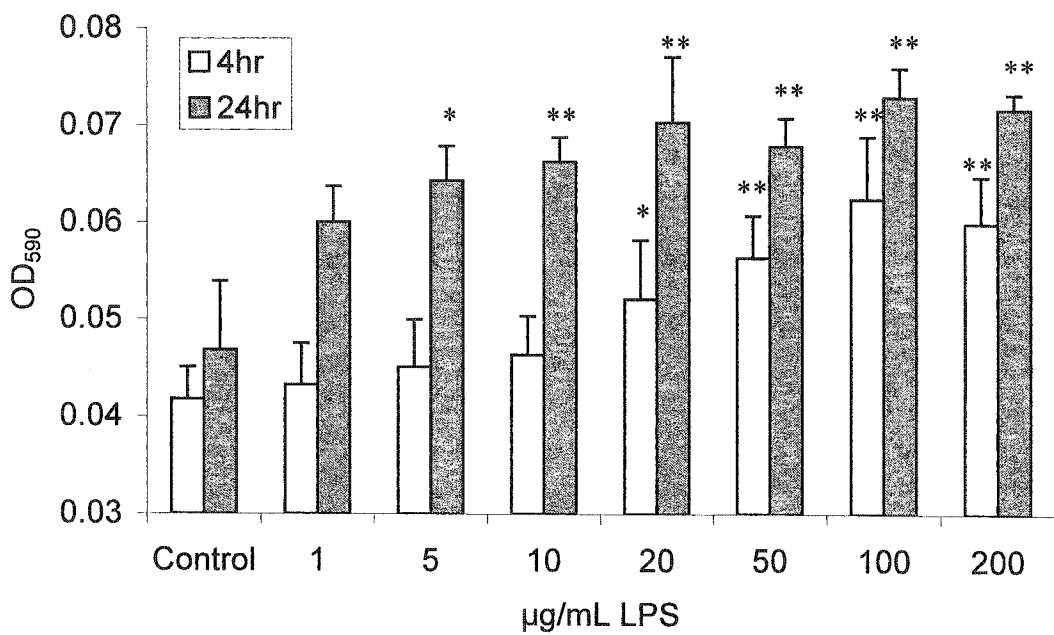
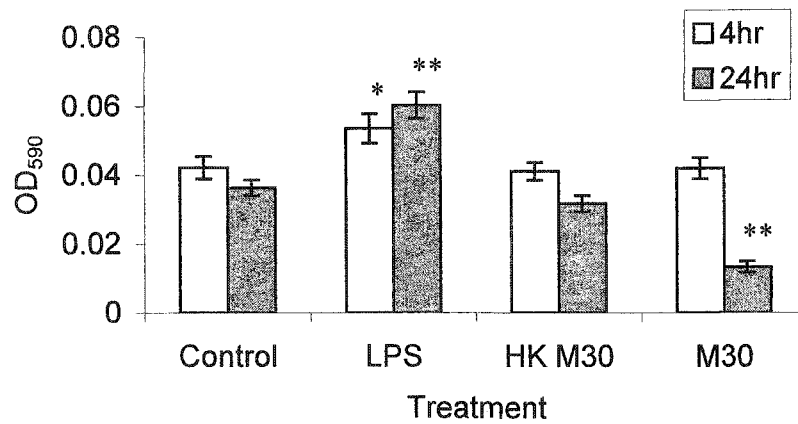


Fig. 13. Intracellular superoxide anion (O_2^-) production in adherent AK (A) and PE (B) cells treated with mycobacteria or LPS at 4 hr and 24 hr. Control- untreated cells, LPS- 20 μ g/ml, HK M30- heat-killed *M. marinum* MOI 1:5, M30- live *M. marinum* MOI 1:5. Bars represent the mean of quadruplicate wells for three fish \pm SEM; significant difference relative to control by ANOVA and multiple comparison, * $p < 0.05$, ** $p < 0.01$.

A) AK. 4 hr vs. 24 hr (t -test $p=0.0197$)



B) PE. 4 hr vs. 24 hr (t -test $p=0.1148$)

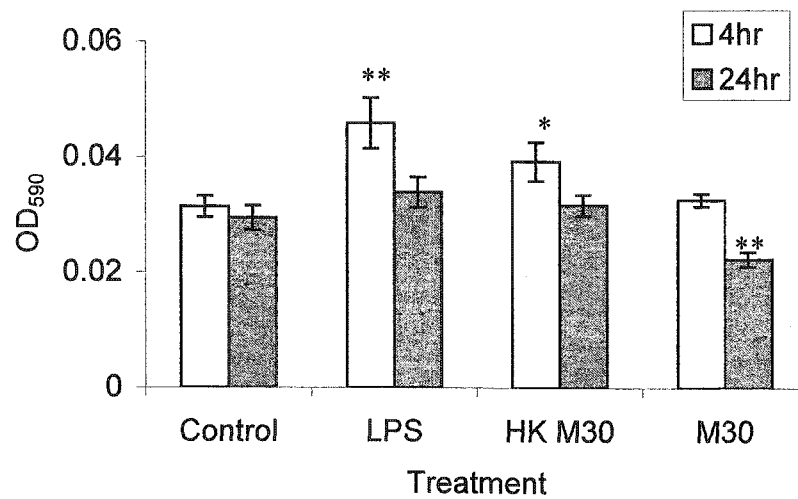


Fig. 14. Effect of stimulants on ROI response in PE. Control – untreated cells, MAF – cell conditioned supernatants (1:8 w/v, 10 µg/ml Concanavalin A, 5 ng/ml PMA for 60 hr on AK cells), PMA – 0.5 µg/ml phorbol myristate acetate, M30 – live *M. marinum* MOI 1:5. Bars represent the mean of quadruplicate wells for three fish \pm SEM; significant difference relative to control by ANOVA and multiple comparison, * $p < 0.05$, ** $p < 0.01$.

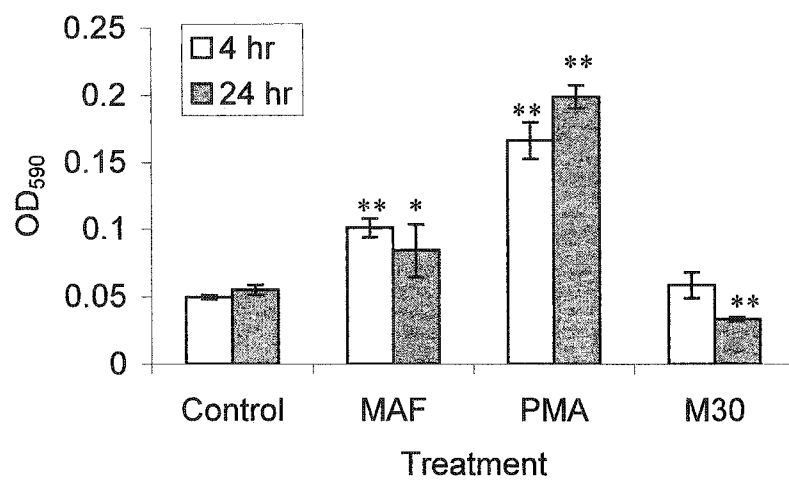


Figure 15. Persistence of *M. marinum* in striped bass AK and PE cells. Chase times represent time elapsed after removal of nonadherent extracellular bacteria and addition of fresh medium. Killing Index (KI) is calculated by dividing OD₅₉₀ of quadruplicate wells at time x (T_x) by mean OD₅₉₀ of quadruplicate wells at time 0 (T₀). Mean KI is the average of KI from three representative fish. Significant differences relative to T₀ were calculated from untransformed OD₅₉₀ data by ANOVA with Tukey's multiple comparison (* p<0.05, ** p<0.01).

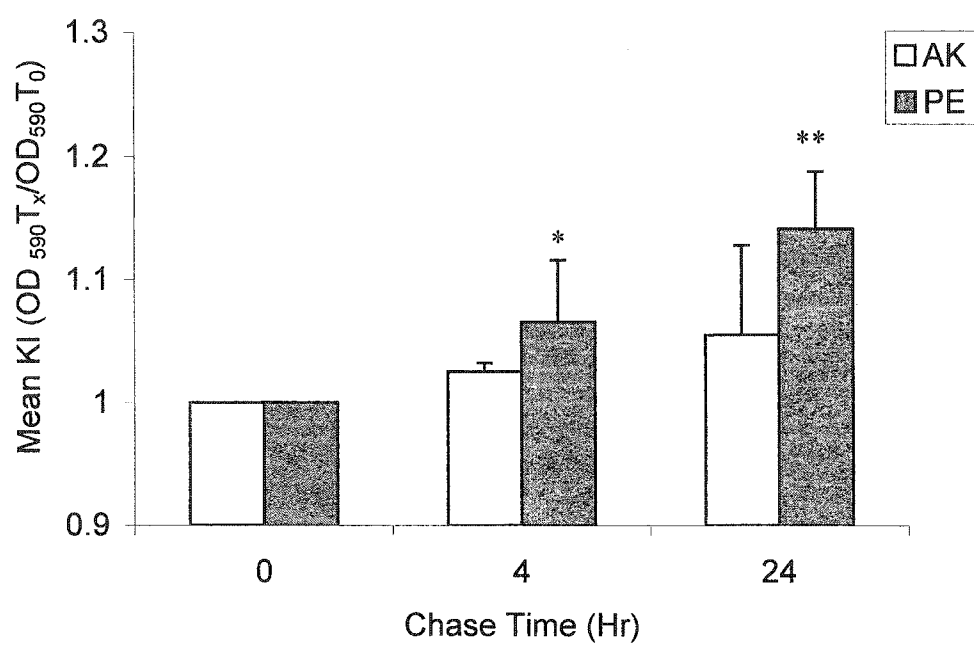
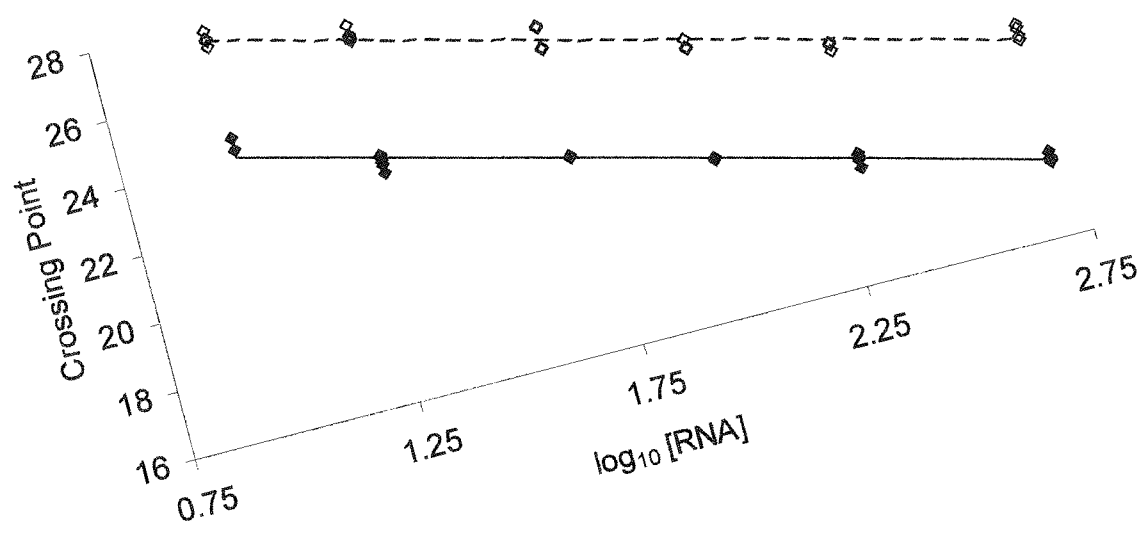


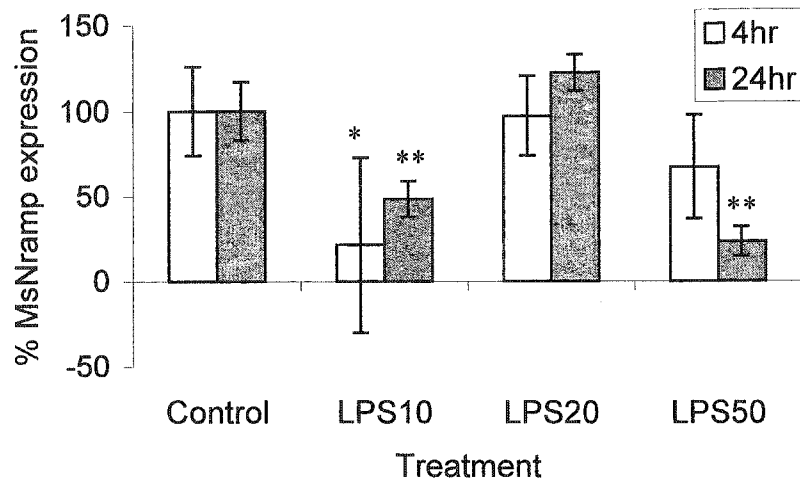
Figure 16. Calculation of PCR efficiency (PCR_E) for AK (open symbols) and PE (closed symbols) cells by linear regression. AK $PCR_E = 1.87$ ($p < 0.01$, $R^2 = 0.987$), PE $PCR_E = 1.86$ ($p < 0.01$, $R^2 = 0.992$).



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Figure 17. *MsNramp* expression after LPS treatment in AK (A) and PE (B) cells. Bars represent the mean expression for six fish \pm SEM; significant difference relative to control by ANOVA and multiple comparison, * $p < 0.05$, ** $p < 0.01$.

A) AK. 4 hr vs. 24 hr (t -test $p < 0.01$)



B) PE. 4 hr vs. 24 hr (t -test $p < 0.01$)

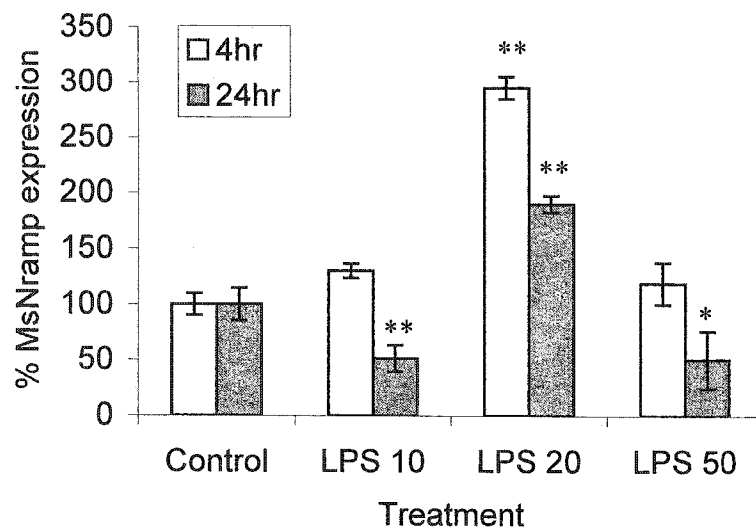
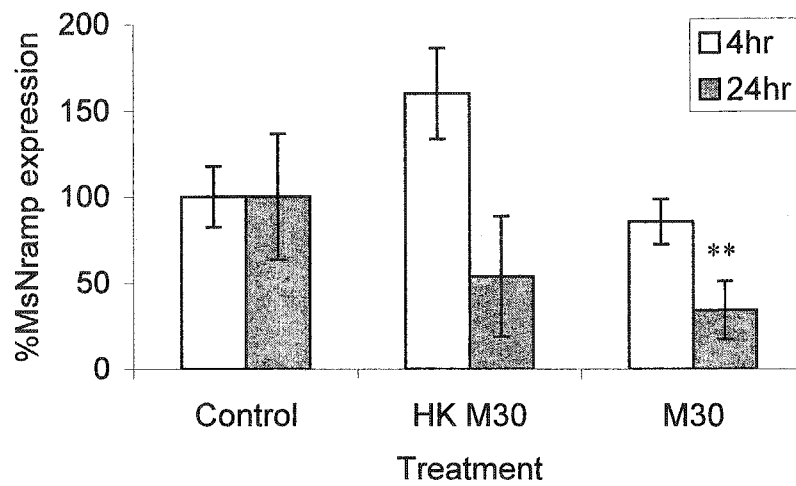
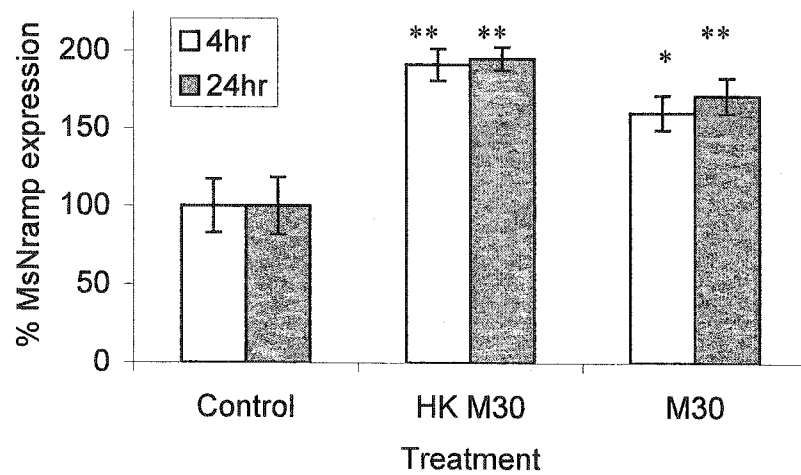


Figure 18. *MsNramp* expression after live and dead *M. marinum* treatment in (A) AK (ANOVA 4 hr $p=0.0624$) and (B) PE cells. Bars represent the mean expression for six fish \pm SEM; significant difference relative to control by ANOVA and multiple comparison, * $p<0.05$, ** $p<0.01$.

A) AK. 4 hr vs. 24 hr (t -test $p < 0.01$)



B) PE. 4 hr vs. 24 hr (t -test $p = 0.7842$)



CHAPTER THREE. COMPARISON OF THE EXPRESSION OF *MSNRAMP*
BETWEEN STRIPED BASS AND WHITE PERCH (*MORONE AMERICANA*)

INTRODUCTION

Striped bass (*Morone saxatilis*) and white perch (*M. americana*) are congeneric anadromous fishes that co-occur over portions of their range and life history. Larvae, juveniles and young adults are especially likely to interact in the upper tributaries of Chesapeake Bay (Petrocci, 1995). Hybridization, and subsequent introgression of white perch genetic characters into striped bass, has been documented in the wild (Harrell et al., 1993; Waldman and Bailey, 1992). Molecular and morphological phylogeny of the species within the genus *Morone*, the striped bass *M. saxatilis*, white bass *M. chrysops*, white perch *M. americana*, and yellow bass *M. mississippiensis*, has suggested that *M. saxatilis* and *M. chrysops* are a sister taxon to *M. americana* and *M. mississippiensis* (Leclerc et al., 1999). Comparative studies of the larval development (Limburg et al., 1997; North and Houde, 2001; Waldman et al., 1999), physiology (Kellogg and Gift, 1983; Neumann et al., 1981; Noga et al., 1994), and competitive interactions (Limburg et al., 1997; Monteleone and Houde, 1992; Rudershausen and Loesch, 2000) have documented significant overlap in these characters. Adverse impacts on the native striped bass population have been noted in the Cape Fear River estuary, North Carolina, and attributed to competition with stocked hybrids (*M. saxatilis* x *M. americana*) of white perch and striped bass (Patrick and Moser, 2001).

Habitat selection, feeding and reproduction are limited by temperatures above approximately 25°C for adult striped bass, with a preferred temperature range of 10 to

20°C (Coutant, 1985), while white perch have an optimum feeding temperature range of 27 to 30°C (Luo et al., 1994). Chesapeake Bay tributaries where white perch and striped bass often co-occur are routinely greater than 28°C during summer and early fall periods (Murdy et al., 1997), and these areas are often associated with low dissolved oxygen levels. Striped bass are susceptible to temperature and dissolved oxygen stress (Coutant and Benson, 1990; Zale et al., 1990) that would be very likely to decrease their disease resistance, while white perch in the same areas are less likely to be under stressful conditions because of their adaptations to higher environmental temperatures.

Currently, an epizootic of mycobacteriosis is occurring in Chesapeake Bay striped bass at high prevalence (Cardinal, 2001; Rhodes et al., 2001b; Rhodes et al., 2003b; Vogelbein et al., 1998), but preliminary attempts to find mycobacterial infections in wild white perch have been unsuccessful (D. Gauthier, E. Burge, C. Bonzek, and R. Latour, unpublished data)(M. Rhodes, personal communication). The same diseases and parasites commonly infect both species (Snieszko et al., 1964; Wolke et al., 1970). The present study investigated the expression of the striped bass homolog of the natural resistance-associated macrophage protein, *MsNramp*, in striped bass and white perch collected from mycobacteriosis-endemic areas of northern Chesapeake Bay. It was hypothesized that high mycobacterial infection rates in striped bass would be correlated to low expression of *MsNramp*, a known *Mycobacterium*-resistance gene in mammals (Vidal et al., 1993), while higher *MsNramp* expression in white perch would be associated with a lack of mycobacterial infection.

MATERIALS AND METHODS

Experimental fish. Striped bass (*Morone saxatilis*) and white perch (*M. americana*) were collected from two sites in the northern portion of Chesapeake Bay, Maryland, in May 2003, by bottom trawl at predetermined sampling locations as part of the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAAP). Stations were chosen from a grid that randomly and proportionally sampled areal and depth parameters of the mainstem of Chesapeake Bay. The trawl net is a 45 foot four seam balloon otter trawl with six inch stretch mesh in the wings and body, and three inch stretch mesh on the cod end with no liner. A 'tickler chain' was attached to both trawl doors to ensure sampling of demersal fishes. The nets were deployed from the R/V *Bay Eagle*, Virginia Institute of Marine Science, a 65' aluminum hull, twin diesel-engine vessel capable of housing crew and scientists for multi-day deployments, and rigged to be an efficient trawler by constructing and installing a removable modular platform, trawling winch, A-frame, and net reel. Each tow was for 20 minutes at a speed of 3.5 knots with the current. Net spread and depth were monitored by acoustic sounders attached to the net.

Tissue sampling. Fish were weighed and measured immediately after capture on board the R/V *Bay Eagle*, before tissue sampling. Spleen and liver samples were preserved in formalin for histological evaluation of mycobacteriosis (n=20 per species). Samples of

spleen and white muscle (approximately 100 mg) from both species were removed and placed in RNAlater™ (Ambion, Austin, TX) for later RNA extraction. Tissue samples were stored at 4°C while at sea and transferred to -20°C after return. A total of nine striped bass and nine white perch were sampled for RNA.

RNA extraction. Total RNA was isolated with TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Integrity of total RNA was assessed by electrophoresis in 1% denaturing formaldehyde-agarose gels. RNA quality and concentration was determined by UV spectrophotometry at 260/280 nm, with background correction for protein contamination at 320 nm. Total RNA was resuspended in RNA Storage Solution (Ambion, Austin, TX) and stored at -80°C until use.

Real-time semiquantitative reverse transcription-PCR. Two gene-specific primers and two gene-specific hybridization probes were used to measure PCR product formation in real time (Table 2) (Wittwer et al., 1997). This procedure was performed on the Roche Molecular Biochemicals LightCycler System and the appropriate primers and hybridization probes developed based on striped bass sequence for *MsNramp* (see Chapter 1) using LightCycler Probe Design v. 1.0 software (Idaho Technologies, Inc, Salt Lake City, UT). All reagents were prepared at 4°C in low light to minimize nonspecific amplification and fluorophore degradation.

The PCR reaction was as previously described. To initiate the reaction, 500 ng of sample RNA was added to each capillary and LightCycler cycling immediately begun. RNA samples were quantified immediately before use by spectrophotometric detection at

260 and 280 nm, and corrected for protein concentration at 320 nm. RNA sample concentrations calculated by spectrophotometry were reproducible within 5%.

Reverse transcription and amplification were as previously described. Two amplification reactions were performed for each RNA sample. Data collection and preliminary analyses were conducted using LightCycler Data Analysis software v. 3.3.

qRT-PCR analysis. *MsNramp* expression was quantified by calculation of percent increase or decrease in transcript number in striped bass and white perch spleen and muscle. PCR efficiencies were previously calculated from striped bass spleen and muscle, and found to be 1.70 and 1.72, respectively. Crossing points were determined by the LightCycler software from replicates of each sample. The crossing point (Cp) of the qRT-PCR is the point during amplification at which fluorescence of a sample rises above the background. That point on the amplification curve is proportional to the amount of starting template mRNA (*MsNramp*) in the sample. Percent difference is then calculated as follows:

$$\% \text{ difference} = (\text{PCR}_E^{\Delta\text{Cp}} \times 100) \text{ (Gentle et al., 2001)}$$

where ΔCp = (white perch average crossing point – striped bass average crossing point)

RESULTS

Mycobacteriosis in white perch and striped bass. For the stations where white perch and striped bass were both collected, the prevalence of granulomatous inflammation indicative of histological mycobacteriosis in striped bass was 40% (8 of 20). No white perch (0 of 20) from these stations were found to have granulomatous inflammation characteristic of mycobacteriosis (D. Gauthier, personal communication).

Expression of *MsNramp* in striped bass and white perch spleen. Striped bass *MsNramp* as measured by real-time RT-PCR in the spleen had an average crossing point of 22.59 ± 0.28 (n=9 fish). White perch *MsNramp* expression average crossing point was 27.38 ± 0.20 (n=9 fish). Raw fluorescence values and averages for each species are in Figure 18. Figure 19 graphically represents the relative relationships of the expression of *MsNramp* in white perch, arbitrarily set to 100% expression, and striped bass, calculated relative to white perch. Striped bass expressed $1665.95 \pm 18.0\%$ of the gene, relative to white perch ($100 \pm 12.38\%$). Two sample *t*-test analysis assuming equal variance showed that the white perch and striped bass crossing points were significantly different ($p < 0.000$).

Expression of *MsNramp* in striped bass and white perch muscle. White perch and striped bass *MsNramp* values were calculated for muscle tissue from both species. It had

been previously shown that levels of expression of *MsNramp* in striped bass muscle were constitutively much lower (1-2 orders of magnitude) than splenic tissue. Muscle was used to serve as a control for differences in amplification efficiency between white perch and striped bass due to potential unknown sequence differences, and also as a control for mycobacterial infection, as mycobacteria are not generally found within muscle tissue. Striped bass and white perch muscle were not significantly different in expression of *MsNramp* among the nine fish of each species sampled ($p=0.1737$).

DISCUSSION

Striped bass (*Morone saxatilis*) and white perch (*M. americana*) are closely related congeners that can have extensive interactions during their juvenile stages. In the present study, a preliminary survey of mycobacterial prevalence and expression of a potential mycobacteria-resistance gene, *MsNramp*, was observed in fish from Chesapeake Bay. Striped bass collected from a mycobacteriosis-endemic area of the northern Bay expressed much higher levels of *MsNramp* than did white perch collected at the same locations. Additionally, white perch (n=20) were found to be free of the splenic granulomatous inflammation, characteristic of mycobacteriosis in striped bass. While it is possible that differences in *MsNramp* expression are due to minor sequence differences between white perch and striped bass, it is unlikely for the following reasons. First, *Nramp* genes are very highly conserved in all vertebrates (see Chapter 1) and the regions that the primers and probes (Table 2) were designed from in the striped bass sequence are even more highly conserved than average nucleotide positions. Second, striped bass and white perch are readily hybridized artificially (Harrell, 1998), and have been known to do so in the wild (Harrell et al., 1993; Waldman and Bailey, 1992), indicating a high degree of compatibility within their genomes. Finally, while spleen tissue expression of *MsNramp* was highly divergent for the two species, no significant differences existed for muscle tissue. This indicates that there are not appreciable differences in sequence, and therefore, amplification efficiency.

Striped bass are widely recognized as a particularly susceptible species to mycobacterial infection (Harms et al., 2003; Lansdell et al., 1993; Rhodes et al., 2001b; Sakanari et al., 1983; Wolf and Smith, 1999), and this study was conducted on the hypothesis that white perch, as congeners that share many life history traits, physiological preferences, diet sources and infectious organisms, would also be susceptible. Lack of infection in white perch suggests that a predisposing susceptibility is present in striped bass, but not white perch. This seems less likely than a physiological or genetic difference, however, when so many life history parameters overlap between the species.

Potential experiments needed to address this apparent difference in *MsNramp* expression in the two species would include a large-scale infection trial under controlled conditions. Observations of the degree of pathology associated with infection with *M. marinum* (Gauthier et al., 2003; Wolf and Smith, 1999) and measurement of *MsNramp* in both species would provide insight into the pathogenesis and functional conservation of *MsNramp* during mycobacterial infection. In addition to infection trials, it would be necessary to confirm that the differences between the species in gene expression are not due to subtle sequence differences between the striped bass and white perch homologs.

Figure 18. Comparison of LightCycler RT-PCR curves for *MsNramp* in striped bass (*Morone saxatilis*) and white perch (*M. americana*) spleens. Fluorescence values from real-time RT-PCR are graphed against cycle number for striped bass (diamond, n=9) and white perch (square, n=9). Fish were obtained from a mycobacteriosis-endemic area of northern Chesapeake Bay. Histological prevalence of granulomas was 40% for striped bass (n=20) and 0% for white perch (n=20). Crossing points for the two species were significantly different ($p<0.000$).

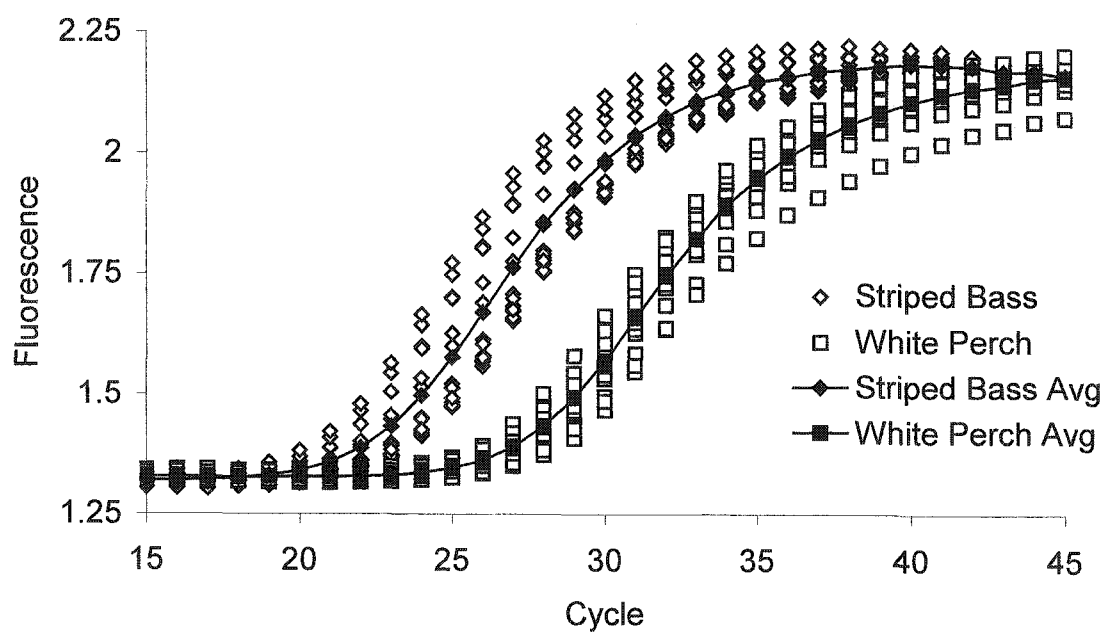
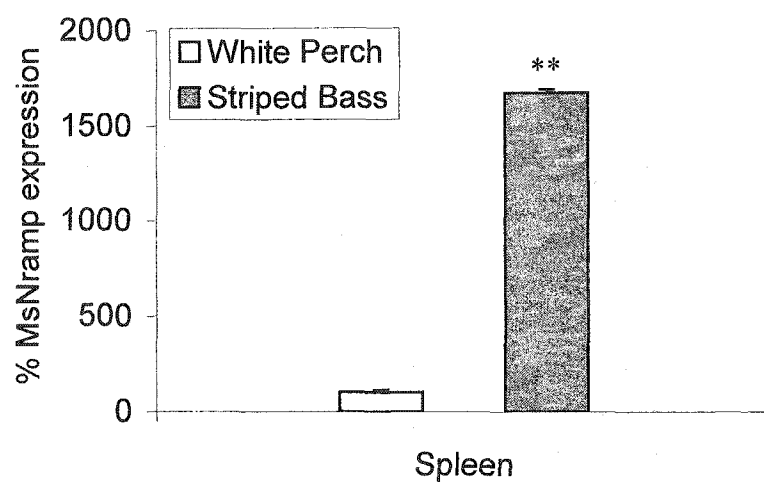
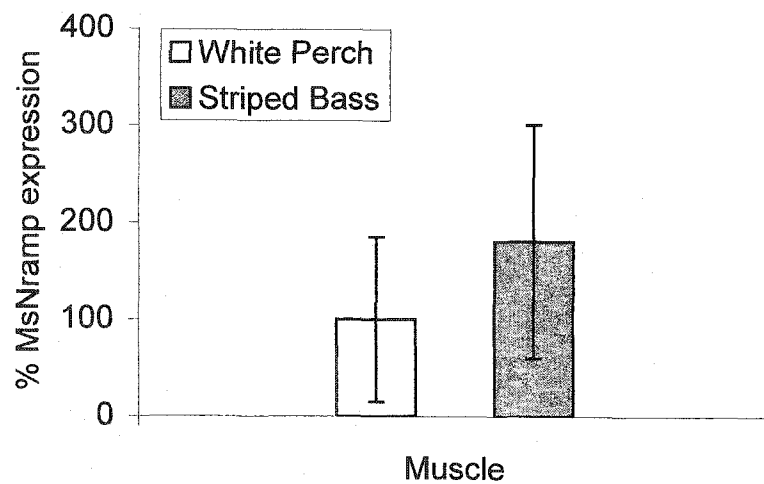


Figure 19. Relative expression of *MsNramp* in striped bass and white perch spleens (A) and muscle tissue (B). White perch values for *MsNramp* were normalized to 100% and striped bass compared relative to that value. Error bars represent the SEM of nine fish per species. Significant differences were determined by *t*-test for means (spleen $p < 0.000$; muscle $p = 0.1737$).

(A) *MsNramp* expression in spleen



(B) *MsNramp* expression in muscle



GENERAL CONCLUSIONS

Significance of this Dissertation

This dissertation demonstrates for the first time the inducible nature of a teleost natural resistance-associated macrophage protein gene in response to *Mycobacterium* spp.

exposure *in vivo* and *in vitro*. These results suggest that striped bass *MsNramp* may be functionally equivalent to the disease resistance role of mammalian Nramp1.

Additionally, this work supports the findings of Chen et al. (2002) and Dorschner (1998), that fish *Nramp* genes are responsive to LPS, as has been shown for mice (Govoni et al., 1995).

Conclusions

1. The striped bass (*Morone saxatilis*) genome contained a natural resistance-associated macrophage protein gene, *MsNramp*, that encoded a 3530 base pair gene and a 554 amino acid protein.
2. The *MsNramp* cDNA indicated that the gene encoded an approximately 60 kDa protein containing 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 amino-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.

3. *MsNramp* was highly similar to mammalian *Nramp2* sequences, and phylogenetically most closely related to other teleost *Nramp* genes. Neighbor-joining using the pairwise-distance model showed the highest degree of relatedness between striped bass *MsNramp* and pufferfish *Takifugu rubripes Nrampβ*.

4. The gene was expressed in all tissues tested, as measured by RT-PCR. Significant constitutive differences were apparent by tissue type. These results were consistent with the expression pattern of other teleost *Nramp* genes.

5. *In vivo*, *MsNramp* was highly induced approximately 20-fold by intraperitoneal injection of *Mycobacterium marinum* and to a lesser degree by *M. shottsii*. The response occurred within 24 hours and continued to be elevated at least 15 days post-injection.

6. Striped bass adherent anterior kidney (AK) and peritoneal exudate (PE) cells responded *in vitro* to bacterial lipopolysaccharide (LPS) with increases in the production of superoxide anion (O_2^-) in a dose and time-dependent manner.

7. AK cells did not respond to heat-killed or live *Mycobacterium marinum* with increased O_2^- , but had a significant decline when treated with live mycobacteria by 24

hours post-treatment. PE cells were moderately stimulated by heat-killed mycobacteria at 4 hours, but suppressed by live cells at 24 hours.

8. AK cells did not significantly upregulate transcription of *MsNramp* in response to optimal concentrations of LPS. PE cells showed a significant increase in transcription to the same dose of LPS.

9. Live *M. marinum* significantly depressed AK *MsNramp* transcription by 24 hours post-treatment. PE cells expressed significantly higher amounts of mRNA for *MsNramp* after 4 and 24 hours of stimulation with heat-killed and live *Mycobacterium*.

10. Interspecific comparisons of the expression of *Nramp* between white perch (*Morone americana*) and striped bass collected from the same locations in Chesapeake Bay demonstrated that striped bass spleen tissue contained approximately 17-fold higher levels of *MsNramp* mRNA than did white perch spleen.

Future Research

Several important questions remain in regards to the function and role of *Nramp* genes in fish. In humans and mice, it is apparent that the two *Nramp* loci fulfill different roles. *Nramp1* is involved in resistance to intracellular parasites (Vidal et al., 1995b), while *Nramp2* appears to play a metabolic role in dietary iron acquisition (Han et al., 1999). Two *Nramp* loci have been reported from rainbow trout (*Oncorhynchus mykiss*) (Dorschner and Phillips, 1999) and pufferfish (*Takifugu rubripes*) (Sibthorpe, 2002), but

carp (*Cyprinus carpio*) (Saeij et al., 1999), channel catfish (*Ictalurus punctatus*) (Chen et al., 2002), zebrafish (*Danio rerio*) (Donovan et al., 2002), and striped bass appear to only possess one *Nramp* locus. Additional sequencing efforts are necessary to determine whether or not diverse fish species contain one or multiple *Nramp* loci.

Major questions remain about the function and role of teleost *Nramp* genes and their potential role in disease resistance. It has yet to be conclusively demonstrated that expression of fish *Nramp* genes is critical to mycobacterial resistance, as is the case for the mouse (Vidal et al., 1995b). The following experimental approaches may be able to answer the question: Is expression of *Nramp* in fish critical for mycobacterial resistance? It is technically feasible to transfect *MsNramp* into *Nramp*^{-/-} mice, challenge them with mycobacteria and assess survival due to *MsNramp* function. Transfection of *MsNramp* into mice and survival after mycobacterial challenge would demonstrate a functional homology between mammalian *Nramp1* and *MsNramp*. Cation transport assays that measure ion flux would be useful for examining the similarities and differences between the transport functions of mammalian and fish genes. Controversy exists as to the directionality of metal ion transport and the subsequent effect that this has on resistance to mycobacteria. Functional overlap in substrate specificity and transport orientation would support the hypothesis that *MsNramp* is an *Nramp1* homolog. A promising new avenue of approach may include the utilization of zebrafish morpholinos that have been selectively blocked for *Nramp* transcription and then assessed for mycobacterial resistance. This approach may more directly assess the role of *MsNramp* in resistance to mycobacteria as induction cues are more likely to be shared between zebrafish and striped bass, because of their closer evolutionary relationship. A further investigation of

the differences in *Nramp* expression between striped bass and white perch will contribute to our understanding of the susceptible nature of striped bass to this disease and may suggest ecological or environmental variables that determine disease in wild populations of fish.

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