

# In vitro response of the striped bass natural resistance-associated macrophage protein, *Nramp*, to LPS and *Mycobacterium marinum* exposure<sup>☆</sup>

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## Abstract

Mycobacteriosis in Chesapeake Bay (USA) striped bass *Morone saxatilis* is an ongoing disease problem with important economic implications for a large commercial and recreational fishery. Additionally, striped bass serve as a reservoir of potential mycobacterial zoonoses. Recently, we described a striped bass gene homolog of the natural resistance-associated macrophage protein family (*MsNramp*), which is responsible for resistance to mycobacterial infections in mice. Striped bass *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 reactive oxygen intermediate (ROI) production in primary cultures of adherent PE after exposure to bacterial lipopolysaccharide (LPS), or live- or heat-killed (HK) *Mycobacterium marinum*. PE expressed significantly higher levels of *MsNramp* at 4 and 24 h post-treatment with live and HK *M. marinum*. *MsNramp* response to LPS was dose-dependent in these cells, with maximum expression at 4 h and 20 µg/ml LPS. Treatment of PE with LPS resulted in increased intracellular superoxide anion levels, whereas treatment with live *M. marinum* caused a significant depression. This study is the first report of induction of a teleost *Nramp* in vitro by mycobacteria, and supports findings of teleost *Nramp* induction by LPS.

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## 1. Introduction

Striped bass (*Morone saxatilis*) is 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., 1999). 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., 2000; M. Rhodes, Virginia Institute of Marine Science, personal communication). In addition, a new species, *Mycobacterium shottsii*, has been identified that is phylogenetically similar by 16 S rRNA to *M. marinum* and *M. ulcerans* (Rhodes et al., 2003). Of particular concern are the human mycobacterial infections attributed to contact with infected fish and/or waters from Chesapeake Bay (Zeligman, 1972; Hoyt et al., 1989).

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

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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). Immunofluorescence and confocal microscopy were used 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 (Gruenheid et al., 1997). 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 (Gruenheid et al., 1997; Blackwell et al., 2001). 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<sup>S</sup>*) and -resistant (*Nramp<sup>R</sup>*) mouse strains and showed a common nonconservative glycine to aspartic acid substitution within the fourth transmembrane domain of *Nramp<sup>S</sup>* 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<sup>-/-</sup>*) 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<sup>+/+</sup>* 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- $\gamma$ -inducible gene expression and NF-IL6 bacterial lipopolysaccharide (LPS) responsiveness. These regulatory sequences suggest that *Nramp1* transcription can be modulated during macro-

phage activation in response to cytokine or bacterial stimuli. Northern blot experiments performed on mouse macrophages demonstrated that *Nramp1* was upregulated by the inflammatory mediators, IFN- $\gamma$  and LPS, and substantially increased by pretreatment with IFN- $\gamma$  followed by LPS exposure (Govoni et al., 1997). IFN- $\gamma$  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* 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<sup>+/+</sup>* 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 Kuhn et al. (1999) report that *M. avium*-infected *Nramp<sup>R</sup>* macrophages accumulate more iron (as  $\text{Fe}^{2+}$ ) than susceptible *Nramp<sup>S</sup>* 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* (GenBank accession no. AY008746) (Burge et al., 2004). Using real-time PCR, we demonstrated a large (approximately 17-fold) induction of *MsNramp* gene expression in peritoneal exudate cells (PE) (Bodammer, 1986) after in vivo exposure to *M. marinum*. 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 *Nramp1<sup>+</sup>* mycobacteria-containing phagosomes has suggested a direct linkage between the expression of *Nramp1*

and the intracellular ROI response (Kuhn et al., 1999, 2001; Zwilling et al., 1999). Iron-treated Nramp-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 *Nramp* 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 *MsNramp* and production of intracellular superoxide anion ( $O_2^-$ ) in primary cultures of striped bass adherent PE exposed to LPS, live *M. marinum*, or heat-killed *M. marinum*. In addition, the short-term bactericidal activity of PE to *M. marinum* was quantified. Possible relationships between *Nramp* and ROI induction and the potential role of *MsNramp* in resistance to mycobacterial infections in fish are discussed.

## 2. Materials and methods

### 2.1. Experimental fish and maintenance

Striped bass, *M. 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 greater than 2 weeks prior to experimental use.

### 2.2. Cell culture

All chemicals and cell culture reagents were from Sigma (St. Louis, MO, USA), unless otherwise noted. Primary cell cultures of peritoneal exudate cells (PE) 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, USA) 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 sodium heparin. Lavage fluid was withdrawn via ventral incision after 10 min. 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 intracellular superoxide anion production ( $O_2^-$ ) were seeded at a density of  $8 \times 10^5$  viable cells/well into 96-well tissue culture plates, and cells for RNA extraction were seeded at  $1.5 \times 10^7$  viable cells/well in 10 cm<sup>2</sup> (6-well) tissue culture plates. Cells were allowed to adhere to plates for 4 h, 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 h after adherence to minimize activation due to processing prior to experimental manipulation.

### 2.3. Cell treatments

Cells were stimulated with the following treatments: control untreated, LPS at final concentrations of 1, 5, 10, 20, 50, 100 and 200 µg/ml (*Escherichia coli* 055:A4), heat-killed (HK) *M. marinum*, or live *M. marinum*, both at a multiplicity of infection (MOI) of approximately 1:5. *M. 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  $12,000 \times g$  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 µm 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 Hanks' Balanced Salt Solution (HBSS) to 0.15, and 10-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 h. 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).

### 2.4. Intracellular superoxide production ( $O_2^-$ )

Adherent PE cultures were stimulated for 4 or 24 h prior to measurements of  $O_2^-$ , using a method modified from Secombes (1990). Quadruplicate wells were used for all treatments. Negative controls consisted of unstimulated cells tested in parallel with treated cells at each time point. At the end of the stimulation period, the cells were washed two times in HBSS and 100 µl of 1 mg/ml nitroblue tetrazolium (NBT) in HBSS test solution was overlaid. After 1 h of

development, cells were fixed with repeated washes of methanol, the formazan reduction product was dissolved in 1:1 1 M KOH/DMSO and plates were read on an automated plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 590 nm.

### 2.5. Bactericidal assay

After 24 h in antibiotic-containing medium, cells in 96-well plates were washed twice with L-15 (no additives), then overlaid with 150  $\mu$ l L-15/5% FBS without antibiotics. Fifty microliters of bacterial suspension giving an approximate target/effector ratio of 5:1 was then added to wells. Control wells received 50  $\mu$ l HBSS without mycobacteria. Bacteria were allowed to settle and be phagocytosed, at which point cells were washed twice with L-15 and overlaid with 200  $\mu$ l L-15/5% FBS with no antibiotics. Immediately (Time 0) and after a chase of 4 or 24 h in fresh medium, medium was removed and 50  $\mu$ l sterile 0.1% Tween-20 in distilled water was added to lyse cells. Lysis was allowed to proceed for 10 min, and 150  $\mu$ l MDB was added to each well. The plate was then incubated at 30 °C in a humid chamber for 48 h to allow bacterial outgrowth. After incubation, 10  $\mu$ 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 h. After 4 h, MTT reduction was halted and bacterial cells lysed by addition of 50  $\mu$ 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. Data are presented as Killing Index (Graham et al., 1988), which is mean OD<sub>590</sub> of quadruplicate wells at time  $x$  ( $T_x$ ) divided by mean OD<sub>590</sub> of quadruplicate wells at time 0 ( $T_0$ ), with values above one representing bacterial outgrowth and values below one representing bacterial killing.

### 2.6. RNA extraction

Cells in six-well tissue culture plates were treated as described above (only 10, 20, and 50  $\mu$ g/ml LPS dosages used). After the stimulation period, medium was decanted from treated cells, the monolayers rinsed twice with HBSS, and 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) added to the cells. Samples were immediately extracted or held at –80 °C until processed as per the manufacturer’s instructions. RNA pellets were dissolved in RNA Storage Solution (Ambion, Austin, TX, USA) and stored at –80 °C. Total RNA was assessed for integrity by 1% formaldehyde agarose gel electrophoresis and quantitated by UV spectrophotometry at 260 and 280 nm.

### 2.7. Real-time semiquantitative reverse transcription-PCR

Methods are as previously described in Burge et al. (2004). Briefly, the procedure was performed on a Roche

Molecular Biochemicals LightCycler System and the appropriate primers and hybridization probes developed using LightCycler Probe Design v. 1.0 software (Idaho Technologies). Sequences of primers and probes from 5' to 3' are as follows: forward primer (MsNramp736) TTGTCTAGCGGTCTT, reverse primer (MsNramp1020) GGGACCACCGTAGGTTTA, 5' hybridization probe (5MsNramp942f) GCTGGACAGAGTTCCACCA-fluorescein, 3' hybridization probe (3MsNrampRed963p) LightCycler Red640 dye-ACAGGCACTTACTCGG GG-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  $\mu$ M), primers (0.5  $\mu$ M) and LightCycler RNA Master Hybridization Probes enzyme mixture (1  $\times$ ). To initiate the reaction, 500 ng of PE 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 s. Amplification consisted of 45 cycles of denaturation (95 °C for 1 s), annealing/hybridization (54 °C for 15 s), and elongation (72 °C for 11 s). 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.

### 2.8. qRT-PCR analysis

Six replicates of each of six RNA concentrations (500, 200, 100, 50, 20, and 10 ng RNA) were amplified for PE and a mean efficiency of PCR ( $PCR_E$ ) calculated. The  $PCR_E$  was calculated as:

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

where  $1 \leq PCR_E \leq 2$ .

Measurement of the PE slope was by linear regression of the crossing points of the six replicates against the log<sub>10</sub> 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 (*MsNramp*) in the sample. Percent difference is then calculated as follows (Gentle et al., 2001):

$$\% \text{ difference} = (PCR_E^{\Delta Cp} \times 100) - 100$$

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

*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 100% at both 4 and 24 h.

## 2.9. Statistical analysis

For calculation of crossing points and slope for PCR<sub>E</sub>, 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, Cary, NC, USA) 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 time point in SAS. Controls were excluded from this analysis.

## 3. Results

### 3.1. Intracellular superoxide anion ( $O_2^-$ ) production in adherent peritoneal exudate cells (PE) treated with LPS, HK *M. marinum* or live *M. marinum*

PE  $O_2^-$  production was significantly elevated after stimulation with between 20 and 200  $\mu\text{g/ml}$  LPS at 4 h and between 5 and 200  $\mu\text{g/ml}$  at 24 h (Fig. 1). Significantly

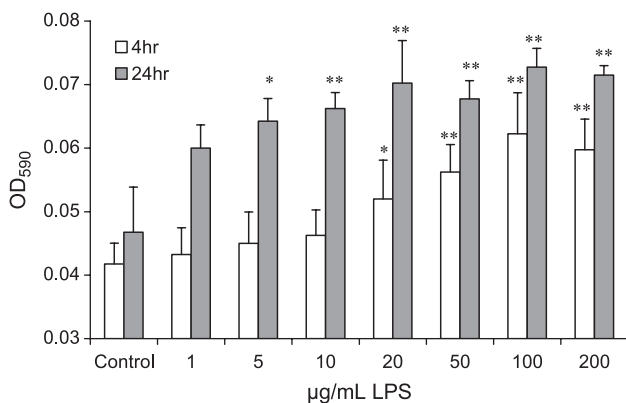


Fig. 1. Intracellular superoxide anion ( $O_2^-$ ) production after treatment with LPS in striped bass (*M. saxatilis*) peritoneal exudate cells (PE) at 4 and 24 h. Bars represent the mean of quadruplicate wells for three fish  $\pm$  S.E.M.; significant differences calculated relative to controls by ANOVA and multiple comparison,  $*p<0.05$ ,  $**p<0.01$ . Time point effects over all doses, excluding controls, were significantly different (*t*-test  $p<0.01$ ) when comparing treatment effects at 4 h vs. 24 h.

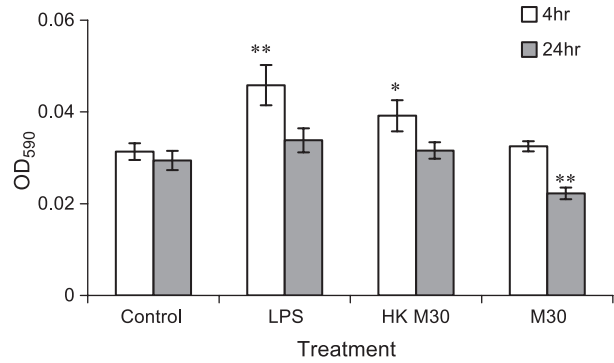


Fig. 2. Intracellular superoxide anion ( $O_2^-$ ) production after treatment with mycobacteria or LPS in adherent peritoneal exudate cells (PE) from striped bass (*M. saxatilis*) at 4 and 24 h. Control—untreated cells, LPS—20  $\mu\text{g/ml}$ , HK M30—heat-killed *M. marinum* MOI 1:5, M30—live *M. marinum* MOI 1:5. Bars represent the mean of quadruplicate wells for three fish  $\pm$  S.E.M.; significant difference relative to control by ANOVA and multiple comparison,  $*p<0.05$ ,  $**p<0.01$ . Time point effects over all treatments, excluding controls, were not significantly different (*t*-test  $p=0.1148$ ) when comparing treatment effects at 4 h vs. 24 h.

higher responses ( $p<0.01$ ) averaged over all LPS concentrations for PE were seen at 24 h. Superoxide production was significantly depressed by live mycobacteria at 24 h (Fig. 2) and PE were generally unresponsive to heat-killed mycobacteria, with the exception of a slight but significant increase ( $p<0.05$ ) in  $O_2^-$  at 4 h. In a separate experiment, PE were treated with conditioned cell culture supernatants (1:8 w/v) from mitogen-stimulated (10  $\mu\text{g/ml}$  concanavalin A and 5 ng/ml phorbol myristate acetate, PMA) cultures of striped bass adherent anterior kidney (Graham and Secombes, 1988), 0.5  $\mu\text{g/ml}$  PMA, or live *M. marinum*, and significant ROI increases were seen for the supernatant and PMA treated cells, but a lack of effect or depression was noted for live mycobacteria at both 4 and 24 h (Fig. 3).

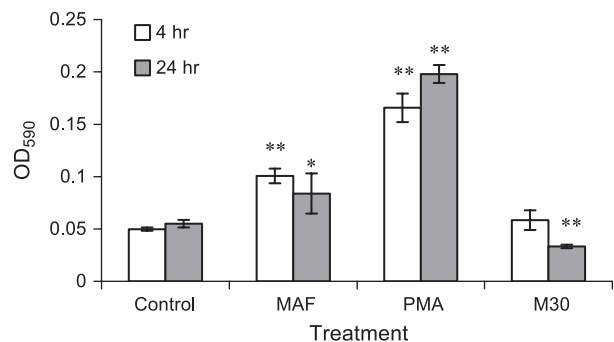


Fig. 3. Intracellular superoxide anion ( $O_2^-$ ) production after treatment with stimulants or mycobacteria in striped bass (*M. saxatilis*) peritoneal exudate cells (PE) after 4 and 24 h. Control—untreated cells, MAF—cell conditioned supernatants (1:8 w/v, 10  $\mu\text{g/ml}$  concanavalin A, 5 ng/ml PMA for 60 h on anterior kidney cells), PMA—0.5  $\mu\text{g/ml}$  phorbol myristate acetate, M30—live *M. marinum* MOI 1:5. Bars represent the mean of quadruplicate wells for three fish  $\pm$  S.E.M.; significant difference relative to control by ANOVA and multiple comparison,  $*p<0.05$ ,  $**p<0.01$ . Time point effects over all treatments, excluding controls, were not significantly different (*t*-test  $p=0.1788$ ) when comparing treatment effects at 4 h vs. 24 h.

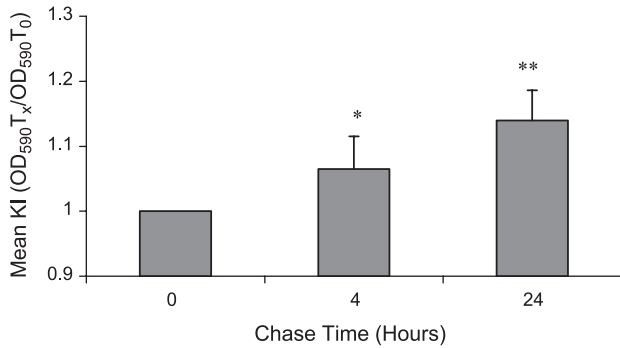


Fig. 4. Persistence of *M. marinum* in striped bass (*M. saxatilis*) peritoneal exudate cells (PE). Chase times represent time elapsed after removal of nonadherent extracellular bacteria and addition of fresh medium. Killing Index (KI) is calculated by dividing OD<sub>590</sub> of quadruplicate wells at time  $x$  ( $T_x$ ) by mean OD<sub>590</sub> of quadruplicate wells at time 0 ( $T_0$ ). Mean KI is the average of KI from three representative fish. Values >1.0 represent bacterial outgrowth. Significant differences relative to  $T_0$  were calculated from untransformed OD<sub>590</sub> data by ANOVA with Tukey's multiple comparison (\* $p$ <0.05, \*\* $p$ <0.01).

Histology and electron microscopy confirmed phagocytosis of both live and dead bacteria at both time points (data not shown), and cell counts of mycobacteria-exposed wells confirmed that depression of O<sub>2</sub><sup>-</sup> was not due to loss of cells from culture.

### 3.2. Mycobactericidal activity of adherent PE

Mycobacteria were persistent within PE throughout the 24 h observation (Fig. 4) and were unable to kill intracellular *M. marinum* within the first 24 h of infection. A slight but significant increase in bacterial numbers was noted at both 4 and 24 h. Generation time for this strain (VIMS M30) of *M. marinum* is approximately 4 days at 18 °C.

### 3.3. Calculation of PCR efficiency (PCR<sub>E</sub>) for PE

The most important parameter associated with accurate relative quantification of real-time PCR results involves the

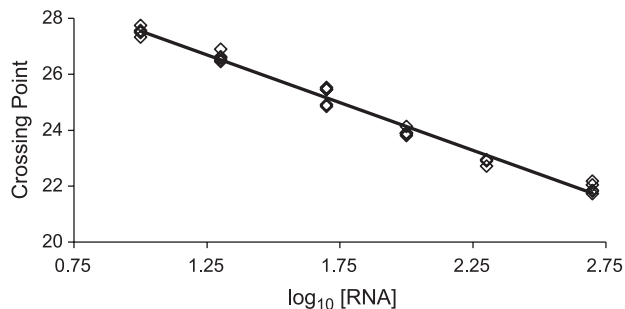


Fig. 5. Calculation of PCR efficiency (PCR<sub>E</sub>) for peritoneal exudate cells (PE) by linear regression ( $y = -3.4147x + 30.961$ ). Diamonds represent measurements of replicate samples of 500, 200, 100, 50, 20 and 10 ng of template RNA ( $n = 6$ /concentration). PE PCR<sub>E</sub> = 1.86 ( $p$ <0.01,  $R^2 = 0.992$ ).

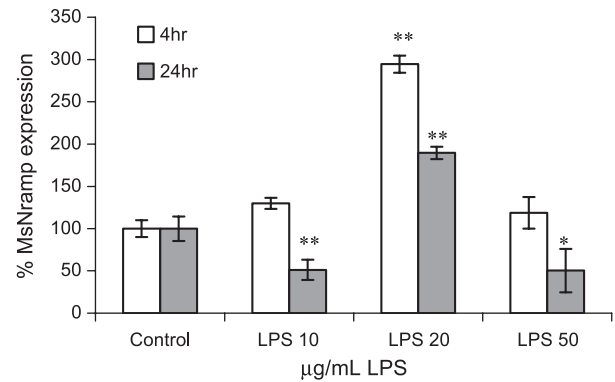


Fig. 6. *MsNramp* expression after LPS treatment in adherent peritoneal exudate cells (PE) from striped bass (*M. saxatilis*) 4 and 24 h post-treatment. Bars represent the mean expression for six fish  $\pm$  S.E.M.; significant difference relative to control by ANOVA and multiple comparison, \* $p$ <0.05, \*\* $p$ <0.01. Time point effects over all doses, excluding controls, were significantly different ( $t$ -test  $p$ <0.01) when comparing treatment effects at 4 h vs. 24 h.

calculation of the overall efficiency of the reaction. PCR<sub>E</sub> was calculated for PE using linear regression of crossing points against log<sub>10</sub> transformed RNA concentrations (Fig. 5). PCR<sub>E</sub> was estimated at 1.86 for PE.

### 3.4. MsNramp expression after LPS treatment in PE

Treatment with 20 µg/ml LPS caused a significant two- to three-fold elevation of *MsNramp* levels at both 4 and 24 h (Fig. 6). LPS concentrations of 10 and 50 µg/ml were not stimulatory to PE at 4 h, but resulted in significant depression in *MsNramp* transcripts after 24 h. Comparison of 4 and 24 h stimulation periods revealed a significantly higher response ( $p$ <0.01) at 4 h post-treatment.

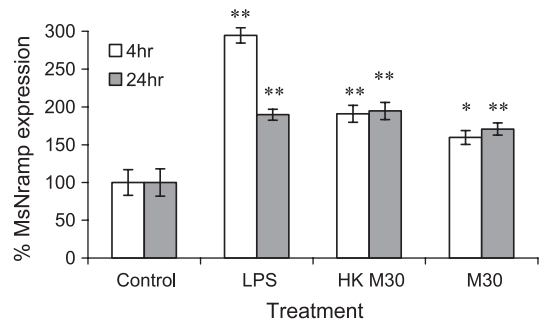


Fig. 7. *MsNramp* expression after LPS, live or dead *M. marinum* treatment in striped bass (*M. saxatilis*) peritoneal exudate cells (PE) 4 and 24 h after addition of bacteria. Control—untreated cells, LPS—20 µg/ml, HK M30—heat-killed *M. marinum* MOI 1:5, M30—live *M. marinum* MOI 1:5. Bars represent the mean expression for six fish  $\pm$  S.E.M.; significant difference relative to control by ANOVA and multiple comparison, \* $p$ <0.05, \*\* $p$ <0.01. Time point effects over all treatments, excluding controls, were not significantly different ( $t$ -test  $p = 0.7842$ ) when comparing treatment effects at 4 h vs. 24 h.

### 3.5. *MsNramp* expression after live and HK *M. marinum* treatment in PE

In PE, 4 and 24 h treatments of live and HK mycobacteria significantly increased expression of *MsNramp* (Fig. 7). A nearly two-fold induction was noted for cells treated with heat-killed *M. marinum* at 4 and 24 h. Live mycobacteria stimulated approximately a 50% increase in *MsNramp* transcript abundance during both time points.

## 4. Discussion

*Nramp1* 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 similarly upregulated after exposure to mycobacteria (Vidal et al., 1995b; Zhong et al., 2001) and *Salmonella* (Govoni et al., 1999). Additionally, channel catfish (*Ictalurus punctatus*) *NrampC* expression is upregulated by LPS treatment (Chen et al., 2002), and the authors suggest that this is evidence of functional similarity between fish and the mouse. *Nramp1* expression in mice is also regulated by proinflammatory cytokines like TNF- $\alpha$  and IFN- $\gamma$  that have a demonstrated efficacy during mycobacterial infection (Brown et al., 1997; Ables et al., 2001).

In this study, we measured the expression of striped bass *MsNramp* in vitro after exposure to LPS, HK-, or live-mycobacteria. PE responded to LPS stimulation in vitro by increasing mRNA transcription (three-fold induction) of *MsNramp* within 4 h of exposure. At 24 h, post-treatment levels of *MsNramp* were still significantly elevated by 20  $\mu$ g/ml LPS, but depressed by greater and lesser doses. This apparent narrow range of *MsNramp*-responsiveness to LPS stimulation will require further study, but nevertheless, these results support the hypothesis that *MsNramp* transcription in striped bass is responsive to LPS, as has been demonstrated in vitro for mouse *Nramp1* (Govoni et al., 1997) and in vivo for channel catfish (Chen et al., 2002). NF-IL6 transcription factor motifs associated with LPS stimulation are present in the mouse *Nramp1* promoter (Govoni et al., 1995) suggesting that similar regulatory sequences may operate within *MsNramp*. Govoni et al. (1997) demonstrated rapid induction of *Nramp1* by LPS within 2 h, with a lessening of expression noted by 24 h 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 at 250 ng/ml, whereas striped bass demonstrated maximal response at 20  $\mu$ g/ml. Research into fish innate immunity has previously noted a lack of effect of LPS dosages that are stimulatory, or even toxic, to mammalian cells (Berczi et al., 1966).

Peritoneal exudate cells (PE) showed a clear and significant induction of *MsNramp* in response to both live and heat-killed mycobacteria at both 4 and 24 h post-exposure. Mature macrophages are the primary mediator of LPS-induced transcription of *Nramp1* in mice (Govoni et al., 1997) and adherent PE from fish are highly enriched in activated, mature macrophages (Bodammer, 1986; Press and Evensen, 1999). Previous work with fish *Nramp* homologs has demonstrated that LPS upregulated expression in vivo in channel catfish (Chen et al., 2002). Channel catfish treated intraperitoneally for 5 h with 30 mg/kg LPS upregulated *Nramp* transcription in anterior kidney and spleen, but not in peripheral blood leukocytes or intestinal tissue. Additionally, the channel catfish monocyte cell line 42TA responded to a 48 h cocktail treatment (mitogen-activated cell supernatants, 25  $\mu$ g/ml *Salmonella* LPS, and 1  $\mu$ g/ml extracellular protein from *Edwardsiella ictaluri* cultures) with an approximately five-fold increase in *Nramp* transcripts. Several fish species have been shown to respond to LPS by upregulating expression of genes associated with the innate immune system (Neumann et al., 1995; Brubacher et al., 2000; Engelsma et al., 2001).

Measurements of ROI production are useful for measuring the overall activation state of macrophages. Striped bass PE responded with significant increases in superoxide production to LPS concentrations above 5  $\mu$ g/ml, the protein kinase C activator, PMA, and cell-conditioned supernatants, demonstrating the ability of these cells to produce ROI, but live *M. marinum* appeared to have an inhibitory effect on this response. Specific enzymes, such as catalase, superoxide dismutase, alkylhydroperoxide reductase, and mechanisms for the detoxification and inhibition of ROI, including antioxidant cell wall components, have been described from *M. tuberculosis* (Sherman et al., 1995; Ehrt et al., 1997), and it is likely that similar ROI resistance-mechanisms are shared within the *M. tuberculosis* clade that includes *M. marinum* (Harmsen et al., 2003).

*MsNramp* levels were significantly stimulated by both heat-killed and live mycobacteria at 4 h, and remained high at 24 h in PE. Mycobactericidal assays of cultured PE demonstrated that these cells were unable to control the growth of *M. marinum* effectively in vitro. Significant outgrowth of mycobacteria occurred by 24 h 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 tissues of striped bass. The current results and our previous work (Burge et al., 2004) suggest that *MsNramp* expression alone is unable to control the growth of *M. marinum* in striped bass cells, but we are unable to conclude whether a complete abrogation of gene expression would not result in more rapid outgrowth of mycobacteria and severity of disease. Sequencing efforts to investigate *Nramp*

in several fish species demonstrate that, at least in two cases (Dorschner and Phillips, 1999; Sibthorpe et al., 2004), two *Nramp* loci are present in fish, and have distinct subcellular or tissue distribution patterns. These studies are suggestive of the functional specialization of independent loci well established for *Nramp1* and *Nramp2* from mammals (Blackwell et al., 2000). *Nramp2* from mammals appears to retain a more ancestral function of divalent cation transport related to metal homeostasis, with *Nramp1* displaying a more evolutionarily derived role in disease resistance. Phylogenetic reconstructions firmly place all published teleost *Nramp* sequences with mammalian *Nramp2* (Burge et al., 2004; Sibthorpe et al., 2004), but the results of this study together with the phylogenetic relationship between mammalian *Nramp2* and *MsNramp* suggest that *MsNramp* may play an intermediate role combining disease resistance and metabolic divalent cation transport functions.

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 in vitro report of induction of a teleost *Nramp* gene by mycobacteria, and supports the finding of Chen et al. (2002), that teleost *Nramp* can be induced by LPS treatment. Currently, the exact role, if any, of *MsNramp*-mediated resistance to intracellular infection by mycobacteria is unknown. Unlike the murine system where direct action of *Nramp1* on antimycobactericidal activity can be shown (Vidal et al., 1995a), no 'resistant' or 'susceptible' phenotypes are available in striped bass. Direct confirmation or refutation of the role of *MsNramp* in resistance to intracellular pathogens awaits further experimentation. A potential experiment to address this question would likely involve the transfection of *MsNramp* into *Nramp1*<sup>-/-</sup> mice to test for functional homology subsequent to challenge with pathogenic mycobacteria. Alternatively, selective blockage of *MsNramp* expression, perhaps by dsRNA interference (Hammond et al., 2001), may better elucidate the putative disease resistance role of this gene in fish.

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