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Arginine metabolism in the *Edwardsiella ictaluri*- channel catfish macrophage dynamic

Wes Arend Baumgartner

Louisiana State University and Agricultural and Mechanical College

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ARGININE METABOLISM IN THE *EDWARDSIELLA ICTALURI*- CHANNEL CATFISH
MACROPHAGE DYNAMIC

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
Through the Department of Pathobiological Sciences

by

Wes Arend Baumgartner
B.S., University of Illinois, 1998
D.V.M., University of Illinois, 2002
Dipl. ACVP, 2009
December 2011

DEDICATION

This work is dedicated to:
my wife Denise who makes it all worthwhile,
my infant daughter Applee, who has stolen my heart,
my sweet mother Judith, whom I will ever acutely miss,
and Pan; a more excellent and amiable feline there never was, nor a braver patient

Pin your ear to the wisdom post
Pin your eye to the line
Never let the weeds get higher than the garden
Always keep the sapphire in your mind
Always keep the diamond in your mind

T.A.Waits

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I would like to take this opportunity to recognize some of the people in my life who have contributed to this work and who have bettered my daily walk, both in my time spent in Baton Rouge and time preceding it. First, to my wife Denise and daughter Applee who have given me lasting joy and many Christmases and summer birthdays to look forward to.

I cannot do justice to the memory of my mother Judy. Who can wrap words around a lifetime of love, patience, gentleness, and deep kindness for a son? All of my memories of you are warm and peaceful, for you never gave me any other kind. I have lost you far too soon and I am heartstricken and envious of those who can drive or fly or walk to see their mother. I am more than thankful for the consolation that I have not lost you forever, but only a little while.

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ABSTRACT

Edwardsiella ictaluri encodes a urease operon and an arginine decarboxylase (AdiA) that are required for virulence in head kidney derived macrophages (HKDM). The urease produces ammonia in amounts sufficient to alter environmental pH from acid to neutral. A hypothetical model was proposed, involving arginine metabolism in *E. ictaluri* infected HKDM, focusing on bacterial urease, AdiA, a second arginine decarboxylase (SpeA), and agmatinase (SpeB). Using fluorescence based ratiometric pH determination of *E. ictaluri* in live HKDM, it was shown that *E. ictaluri* modulates HKDM phagosome pH to above six. Urease and AdiA mutants failed to up-regulate vacuole pH, while vacuole pH for the SpeA and SpeB mutants was similar to the wild-type. These mutants could also replicate in HKDM similar to wild type *E. ictaluri*. These data show that urease and AdiA are required for phagosome pH neutralization. To determine the source of urea for *E. ictaluri*'s urease, an arginase inhibitor, L-norvaline, was used to partially block HKDM urea production. In arginase inhibited HKDM, *E. ictaluri* could not neutralize phagosome pH, nor could it replicate. Nitric oxide production in HKDM was not significantly different between controls and experimental groups. This indicates that HKDM have limited capacity to produce NO. Levels of urea produced in infected and control HKDM were at the lowest limit of assay detection and were not significantly different from one another. Together, these data show that *E. ictaluri* uses its urease and AdiA to neutralize phagosome pH, and that it uses urea derived from HKDM arginase to do so.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The genus *Edwardsiella* was so named in 1965 for Philip R. Edwards (a CDC microbiologist), which contained only the type species *E. tarda* until 1980, when *E. hoshinae* and *E. ictaluri* were added. *Edwardsiellae* are members of subgroup 3 of the γ subclass of Gram-negative *Proteobacteria* and are distantly related to other core members of the family Enterobacteriaceae. They are primarily found in aquatic environments and are often isolated from fish, but many species of vertebrate and invertebrate animals carry these organisms. Both *E. tarda* and *E. ictaluri* are known vertebrate pathogens (Abbott and Janda 2006). *Edwardsiella ictaluri* has been one of the most economically significant pathogens of farmed channel catfish, *Ictalurus punctatus* (Rafinesque, 1818) over past 30 years in the United States of America (Hawke 1979; Thune, et al. 2007; USDA 2009a; USDA 2009b). Disease caused by *E. ictaluri*, known as enteric septicemia of catfish (ESC), is typically described as having 2 distinct forms: a fulminant septicemia with high mortality, and a less common chronic presentation most notably characterized by meningoencephalitis with necrotizing extension through the integument of the fontanelles associated with the frontal bones of the cranium (so called “hole in the head” lesion) (Shotts, et al. 1986; Thune, et al. 1993).

Although the channel catfish is most susceptible *E. ictaluri* infection, other ictalurids such as the white catfish (*Ameiurus catus* Linnaeus, 1758), brown bullhead (*Ameiurus nebulosus* Lesueur, 1819), and walking catfish (*Clarias batrachus* Linnaeus, 1758) can be infected (Hawke, et al. 1998). Recently, the laboratory zebrafish, *Danio rerio* (Hamilton, 1822) has been found to be susceptible to *E. ictaluri* infection as well (Petrie-Hanson, et al. 2007). Infected catfish often exhibit erratic swimming, abnormal posture in the water column, and anorexia. Externally fish

have widespread petechiation, most easily seen on the ventrum, as well multifocal, disseminated skin erosions and ulcers (“buckshot” lesions). Internally, fish have abundant yellow to pink ascitic fluid, livers have multiple necrotic foci, and the intestine is hyperemic and distended with loose bloody feces (Hawke, et al. 1998). The head kidney (pronephros) becomes swollen, pale pink, and friable. In populations where an acute outbreak has passed, a small number of fish will develop the hole-in-the-head lesion, which grossly is first seen as a focal depigmentation over the cranial fontanelles. With time, this focus will become hyperemic and ulcerated with progressive recession and necrosis of the integument, in many cases leading to marked exposure of the bones of the skull.

ESC can occur in healthy fish and in wild populations, but is most commonly associated with intensive farming practices at times of increased stress from handling, confinement, poor diet, substandard water quality, and other pond pathogens. The admixture of fingerlings with adults in ponds is particularly important for the persistence and recurrence of ESC, since survivors can occultly carry *E. ictaluri* for up to 200 days. Originally thought to be an obligate fish pathogen, *E. ictaluri* is now known to persist in pond sediment for months (Hawke, et al. 1998). The major route of fish infection is not resolved; the intestine, nares, and gills have been proposed and may all be important routes of infection (Baldwin and Newton 1993; Morrison and Plumb 1994; Nusbaum and Morrison 1996; Thune, et al. 1993). Transmission is commonly fecal-oral during feeding sessions, but cannibalism of infected carcasses is also common. The optimal temperature range for outbreaks in ponds is between 20°C and 28°C (Hawke, et al. 1998).

Histologically, ESC is characterized by multiorgan necrosis and granulomatous disease, often involving the liver, spleen, and intestine (Blazer, et al. 1985; Shotts, et al. 1986). Discrete

granulomas like those seen in mycobacteriosis are not characteristic; instead, sheets of macrophages contain uncommon bacterial rods. *E. ictaluri* can be recovered from pronephros tissue as early as 30 minutes post infection following an immersion challenge (Thune, et al. 2007). Recently it was shown that *E. ictaluri* is able to invade, survive, and replicate in naive pronephros derived macrophages (HKDM) from *I. punctatus* (Booth, et al. 2006). This is of particular interest, since *E. ictaluri* does not survive well within activated macrophages (Shoemaker, et al. 1997). As a pathogen of professional phagocytes, *E. ictaluri* joins the company of such infamous organisms as *Salmonella*, *Legionella*, and *Shigella* (Russell and Gordon 2009). Significant progress in understanding the pathogenesis of ESC stemmed from a signature tagged mutagenesis study (STM) in which numerous potential virulence factors were identified (Thune, et al. 2007), including a type 3 secretion system similar to that found in *Salmonella*, and a urease operon similar to an acid activated urease found in *Yersinia enterocolitica* (Young, et al. 1996). Investigations into the role of bacterial urease in the pathogenesis of *E. ictaluri* found that while it is not important for the invasion of the catfish or catfish macrophage, it is required in order to maintain high virulence and replication in the catfish and its macrophages (Booth, et al. 2009). Although not required for acid tolerance, this urease is upregulated at acid pH and, in the presence of urea, is capable of producing sufficient ammonia to adjust acidic media pH above 7 (Booth 2006). The presence of both an ammonia and a urea transporter in close association with the urease operon, as well as an arginine decarboxylase (AdiA) being identified as a possible virulence factor (Booth, et al. 2009; Thune, et al. 2007), all indicate that arginine (L-arginine) metabolism is of great importance in this macrophage-bacterium dynamic. Consequently, a hypothetical model is proposed that encompasses the major metabolic routes for arginine in this context (Figure 1.1).

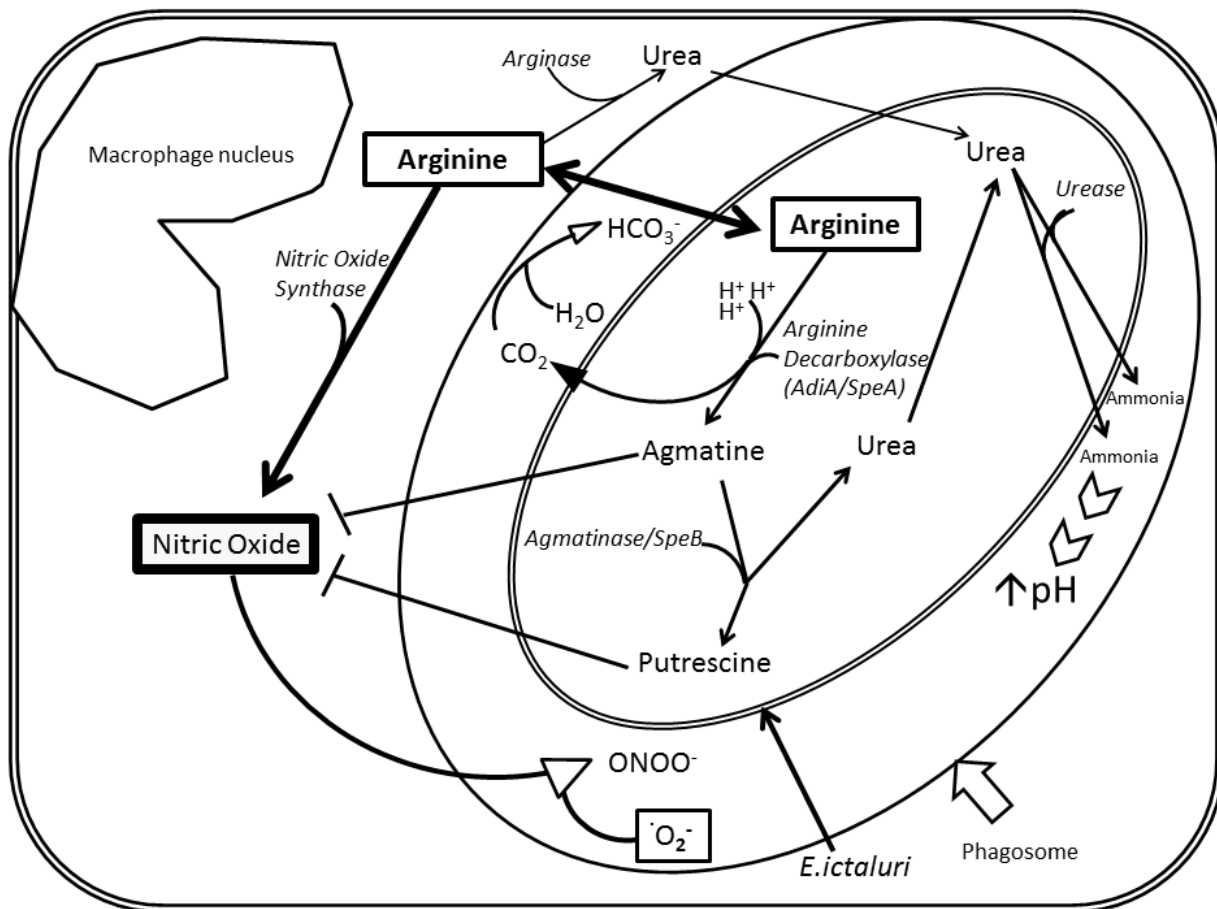


Figure 1.1. Hypothetical model for the interaction of *E. ictaluri* with the catfish macrophage. Arginine may be utilized by nitric oxide synthase, arginase, or arginine decarboxylase (AdiA or SpeA). The metabolites of arginase and arginine decarboxylase may be utilized to promote *E. ictaluri* survival. The nitric oxide produced by nitric oxide synthase has antimicrobial properties.

Briefly, the model, and the discussion, begins with arginine. Arginine is utilized by macrophages for 3 main activities: conversion to nitric oxide (NO) by nitric oxide synthase (NOS), conversion to agmatine by arginine decarboxylase (AdiA or SpeA), or conversion to urea by arginase. If NO is produced, it is used by the macrophage to form reactive nitrogen intermediates (RNI) that combine with oxygen radicals to form potent antimicrobial molecules. If arginine is converted directly to urea by arginase, the *E. ictaluri* urease can use it to produce ammonia (NH₃), either preventing a drop in phagosomal pH or acting to increase pH following acidification. A neutral phagosomal pH is essential for *E. ictaluri* to replicate intracellularly (Booth, et al. 2006). Finally, if arginine is converted to agmatine by the bacterium, agmatine can be converted to either urea or NH₃, which has a similar potential effect on phagosomal pH. Additionally, arginine decarboxylase can function as an acid resistance mechanism. An important secondary benefit for *E. ictaluri* if arginine decarboxylase or arginase is active is the deprivation of arginine to the macrophage, preventing NO production.

MACROPHAGE PHYSIOLOGY AND ARGININE METABOLISM

Macrophages are immunocytes derived from hematopoietic progenitors in the bone marrow of mammals and in the pronephros of fish (Rieger, et al. 2010; Zhang and Mosser 2009). They are of primary importance to immune function in animals in that they respond to various stimuli, both chemotactic and inflammatory, and modulate/direct innate and adaptive immune responses (Vinh and Holland 2009). The range of macrophage physiological function is remarkably wide, involving homeostatic, microbicidal, and inflammatory functions in various tissue environments (Yates, et al. 2009). In order to perform these functions optimally, macrophages must first be *activated*, which is to say that they exhibit enhanced antimicrobial and antineoplastic capacities. Macrophages are highly adaptive and exhibit different activation states,

the best known of which are *classical* and *alternative* activation, which are associated with distinct cytokine and phenotypic profiles (Yeramian, et al. 2006a).

Classically, macrophage activation requires both a priming signal and a triggering signal to achieve full cytolytic activity, which *in vivo* are provided by cytokines and inflammatory stimuli in tissues (Zhang and Mosser 2009). A priming signal enhances the responsiveness of the macrophage to a trigger; the prototypical primer is interferon gamma (IFN- γ). The traditional trigger is lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria. Thus the classical type of activation is often defined as an IFN- γ mediated pro-inflammatory response for enhanced microbicidal activity and cytokine production; however other substances can act as primers and triggers (Vinh and Holland 2009). Certain cytokines are associated with classical activation of macrophages, including interleukin (IL) 12, tumor necrosis factor alpha (TNF- α), and IL-1. Interleukin 12 is important in T cell activation and T helper cell type 1 (Th1) differentiation for cell mediated immunity. Another hallmark of classically activated macrophages is the production of abundant NO and reactive oxygen species (ROS) (Zhang and Mosser 2009). Murine macrophages activated with LPS and IFN- γ exhibit an increase in both arginine uptake and nitrite synthesis (indicative of increased NO production), the latter of which is dependent on extracellular rather than intracellular arginine concentrations (Bogle, et al. 1992; Hammermann, et al. 1998; Hammermann, et al. 1999).

In contradistinction to classical activation, macrophages that are activated by the T helper cell type 2 (Th2) cytokines IL-4 or IL-13 produce large amounts of IL-10, have increased surface expression of innate recognition receptors (macrophage mannose receptor for instance), and are considered to be alternatively activated. Interleukin 10 is a potent immunosuppressive cytokine whose actions include suppression of macrophage pro-inflammatory functions and the

expression of major histocompatibility complex type 2 (MHCII) and costimulatory molecules. The production of high IL-10 levels inhibits IL-12 production in macrophages, leading to Th1 response inhibition and reduced IFN- γ levels, which are vital to immune reactions against intracellular pathogens (Kaiser and O'Garra 2009). Importantly, these macrophages also down-regulate NO production and shift arginine metabolism to the arginase pathway. This type of down-regulation markedly inhibits the macrophage's ability to kill microbes and is most commonly associated with parasite infections and allergic responses (Zhang and Mosser 2009).

As professional phagocytes, macrophages are recruited to sites of infection where they bind and engulf a tremendous variety and amount of biological matter including microbes in a process referred to as phagocytosis. Phagocytosis is a critical function of the immune system and is defined as a receptor mediated engulfment of particles over 0.5 μm wide (Steinberg, et al. 2007). This process is conceptually split into phagosome formation and phagosome maturation. Formation involves the progressive wrapping of cytoplasmic extensions around extracellular particles, initiated and promoted by cell receptor-ligand interactions. Once a new intracellular organelle (phagosome) is formed by this process, maturation begins within minutes (Swanson 2009). Phagosome maturation is a progressive remodeling of the phagosome membranes and content to form a microbicidal organelle (Vinh and Holland 2009). Functionally, this consists of a cadre of hydrolytic enzymes, cationic peptides, ROS, and RNS present in a highly acidified lumen that provide an optimally hostile environment for ingested microbes (Steinberg, et al. 2007).

The acidification process in the macrophage phagosome is of primary importance to its function and heavily influences phagosome-endosome and phagosome-lysosome interactions throughout maturation. Acidification is achieved mainly by vacuolar-type ATPases that pump

protons unidirectionally without regard to electro-neutrality, eventually generating a steady state whereby proton entry matches proton leakage (Steinberg, et al. 2007). This pH change occurs incrementally, beginning with sorting endosomes at pH 6 and ending with phagolysosomes at less than pH 5. Along the way, minor changes in pH (as little as 0.5 pH units) cause various endosome ligands to release from receptors and traffic to later endosomes and other cellular recycling compartments. In addition acidification activates the degradative enzymes in lysosomes, which serves 2 purposes; first, their lytic activity aids in killing ingested microbes and secondly, it enables enzymatic degradation of material, removal of waste, recycling of metabolites, and generation of antigens for immune functions (Mukherjee and Maxfield 2009). The rate of acidification of ingested material varies from 5-30 minutes, and phagolysosomal maturity can occur in 40-90 minutes (Blanchette, et al. 2009; Fairn, et al. 2009; Yates, et al. 2009).

Despite the obvious utility of acidification as an aid to microbe destruction, acidification does not always lead to killing. *Salmonella enterica*, for example requires phagosomal acidification to survive in macrophages, in part through the acid mediated upregulation of virulence factors (Thompson and Holden 2009). In contrast, for pathogens such as *Legionella pneumophila* and *Mycobacterium tuberculosis*, the prevention of phagosome acidification is necessary for bacterial survival (Clemens and Horwitz 1995; Sturgill-Koszycki and Swanson 2000).

Within the phagosomal maturation/acidification process of the macrophage, arginine utilization is an important and highly regulated function. Arginine is a dibasic semi-essential amino acid formed from glutamate or proline and is best known for its role in the urea cycle in most mammals. It is also indirectly involved in the regeneration of adenosine triphosphate

(ATP), cell proliferation, neurotransmission, vasodilation, calcium release, and ultimately in immunity (Nieves Jr. and Langkamp-Henken 2002). The potential beneficial effects of arginine on immune stimulation and cardiovascular function even led to its use in disease management (Nieves Jr. and Langkamp-Henken 2002; Tapiero, et al. 2002). In the context of immunity, inflammation, and immunopathology, arginine has a role as a precursor of both NO and polyamines (putrescine, spermidine, spermine, cadaverine), making it an amino acid of considerable research interest (Peranzoni, et al. 2008). Arginine and NO metabolism play vital roles in macrophage phenotypic change, cell activation, and progression to apoptosis or necrosis in the context of a bacterial infection (Peranzoni, et al. 2008). As a component of the urea cycle and multiple cellular activities, arginine concentrations are highly regulated at the level of cell membrane transport and in catabolism, the latter most notably by arginase and NOS.

Macrophages require arginine for protein synthesis and for NO and polyamine elaboration. These cells are highly sensitive to extracellular arginine concentrations, which can up or down regulate macrophage functions depending on their activation state (Albina, et al. 1989). Surprisingly, arginine deprivation or low (6 μ M) concentrations do not seem to inhibit macrophage activation or immunobiological function, but may actually enhance it (Albina, et al. 1989; Choi, et al. 2009). Macrophages cultured in arginine-free media showed no change in phagocytic capacity, expression of activation markers (CD69,CD40,CD80,CD86,CD206, MHCII,TNF- α), production of cytokines (IL-6, IL-10, IL-12p70), NOS protein expression in classically activated cells, or arginase protein expression in alternatively activated cells (Choi, et al. 2009). However, the lack of arginine substrate for NOS and arginase in macrophages does inhibit production of NO and ornithine/urea, respectively (Yeramian, et al. 2006b).

Arginine can cross cell membranes through several transport mechanisms, the most relevant of which is the cationic amino acid transporter (CAT) system γ^+ , an inducible sodium and pH independent, high affinity transporter family for arginine, lysine, and ornithine (Peranzoni, et al. 2008; Verrey, et al. 2004). These transporters are comprised of 14 glycosylated transmembrane domains, a conserved and essential Glu¹⁰⁷ residue in domain 3 and an 80 amino acid region in domain ten responsible for substrate affinity (Verrey, et al. 2004). Cationic amino acid transporters are part of the SLC7 family of amino acid transporters, which is divided into 2 groups: SLC7A1-4 (CATs) and SLC7A5-11, which are heterodimeric amino acid transporters. SLC7A1 and SLC7A2 correspond to CAT1 and CAT2, which are important transporters in activated macrophages. Interestingly, expression of CAT2 is upregulated by IFN- γ and LPS or IL-4 and IL-10, which are activating cytokines for classically and alternatively activated macrophages, respectively (Peteroy-Kelly, et al. 2001; Yeramian, et al. 2006b). Differentiated or activated murine macrophages have 2 intracellular pools of arginine, one of which is freely exchangeable with extracellular arginine and is accessible to NOS. This exchangeable pool is regulated by both CAT1 and CAT2, but only CAT2 has a role in NOS substrate supply (Closs, et al. 2000; Verrey, et al. 2004). The relationship between NOS expression and arginine transport is not fully understood, partly due to variations in macrophage populations used by various studies (Baydoun, et al. 1993; Hammermann, et al. 2001; Venketaraman, et al. 2003). In murine bone marrow derived macrophages for instance, NOS or arginase blocking did not alter CAT2 expression or arginine transport in classically or alternatively activated cells (Yeramian, et al. 2006b). However, in murine activated alveolar macrophages, NOS inhibition prevented LPS induced arginine uptake (Hammernann, et al. 2001). Under basal conditions, non-activated macrophages can use CAT1, or a secondary transporter from the γ^+L HAT family to maintain arginine levels (Baydoun, et al. 2006; Martín, et al. 2006; Yeramian, et al. 2006b).

The potential for a bacterial pathogen to alter arginine metabolism in the macrophage is a phenomenon of which we know little. A *M. bovis* Bacillus Calmette-Guérin (BCG) arginine transporter mutant strain (AS1) was found to have severely limited arginine transport capabilities in culture, but intracellularly could increase arginine uptake 4 fold in unstimulated macrophages due to the upregulation of CAT1 and CAT2. In this model, infected macrophage NO production was comparable to controls, but urea production and bacterial intracellular survival were increased significantly over controls. Although arginase was assumed to be the source of the urea, inhibition of arginase led to significant improvement of AS1 survival in macrophages. The authors concluded that arginine was more important for AS1 survival than urea and ornithine (Talaue, et al. 2006). However, they did not investigate other possible metabolic pathways for arginine which may produce urea or ammonia. Such metabolic pathways may be upregulated to account for increased arginine utilization and urea production. Similarly, *S. enterica* infection of unstimulated murine bone marrow macrophages leads to increased arginine uptake by cells and increased CAT1 and CAT2 expression (Das, et al. 2010b). When a *S. enterica* arginine transporter (ArgT) is knocked out, bacterial survival in macrophages and *in vivo* was significantly decreased and NO production was significantly increased. Interestingly, the macrophage CAT1 is colocalized to the *Salmonella* containing vacuole (SCV) in 75% of bacteria by 12 hours, indicating that host arginine transporters are actively recruited to the SCV by *S. enterica* (Das, et al. 2010b). In *L. pneumophila*, intra-amoebic replication and growth in *Legionella* containing vacuoles (LCV) is in part regulated by LCV arginine concentrations through *argR*. ArgR is an arginine biosynthesis regulon repressor that functions as an arginine sensor and in the case of *L. pneumophila* may be involved in the regulation and expression of a type IVB secretion system vital to the prevention of phagosome acidification (Hovel-Miner, et al. 2010).

Within cells, arginine can be hydrolyzed to urea and ornithine by arginase, a trimeric manganese metalloenzyme with 2 recognized vertebrate isoforms, type I and type II. These isoforms share 60% amino acid sequence identity but exhibit distinct isoform and species specific cellular and tissue localization, subcellular regulation, and immunologic activity depending on the vertebrate model under investigation (Peranzoni, et al. 2008). Type I arginase (ARG1, liver type) is highly expressed in macrophages and in the liver as a component of the urea cycle. Type II arginase (ARG2) is a mitochondrial enzyme also expressed in monocytes and macrophages, among other tissues. Depending on the species and type of macrophage (histiocytes, Kupffer cells, microglia, osteoclasts, etc.), ARG1 or ARG2 may be the predominantly induced isoform in inflammation (Bogdan 2009; Joerink, et al. 2006; Lewis, et al. 2011). In mammalian macrophages, ARG1 is induced by Th2 cytokines, LPS, and pathogen associated molecular patterns to modulate various aspects of inflammation, resulting in tissue regeneration, cell proliferation, and the reduction of inflammation (Grillo and Colombatto 2004; Munder 2009). It is the production of the polyamines spermine, spermidine, and putrescine (from ornithine) from ARG1 expressing myeloid cells that has such direct and potent effects for the outcome and severity of infection (Das, et al. 2010a; Munder 2009). Polyamines, as discussed later, are cationic molecules that function in a wide variety of fundamental cell processes (Munder 2009). Cellular arginases compete with NOS for arginine in infected macrophages (Figure 1.2), enabling arginase to regulate levels of, or even counteract the effects of NO (Chang, et al. 1998; Gobert, et al. 2001). Macrophage activation leads to increased arginine consumption, which results in extracellular arginine depletion in sites of inflammation. This has been shown to have important immunoregulatory effects, particularly in the down regulation of activated T cell receptor zeta chains (CD3 ζ), which leads to T cell hyporesponsiveness and immunosuppression (Munder 2009; Peranzoni, et al. 2008).

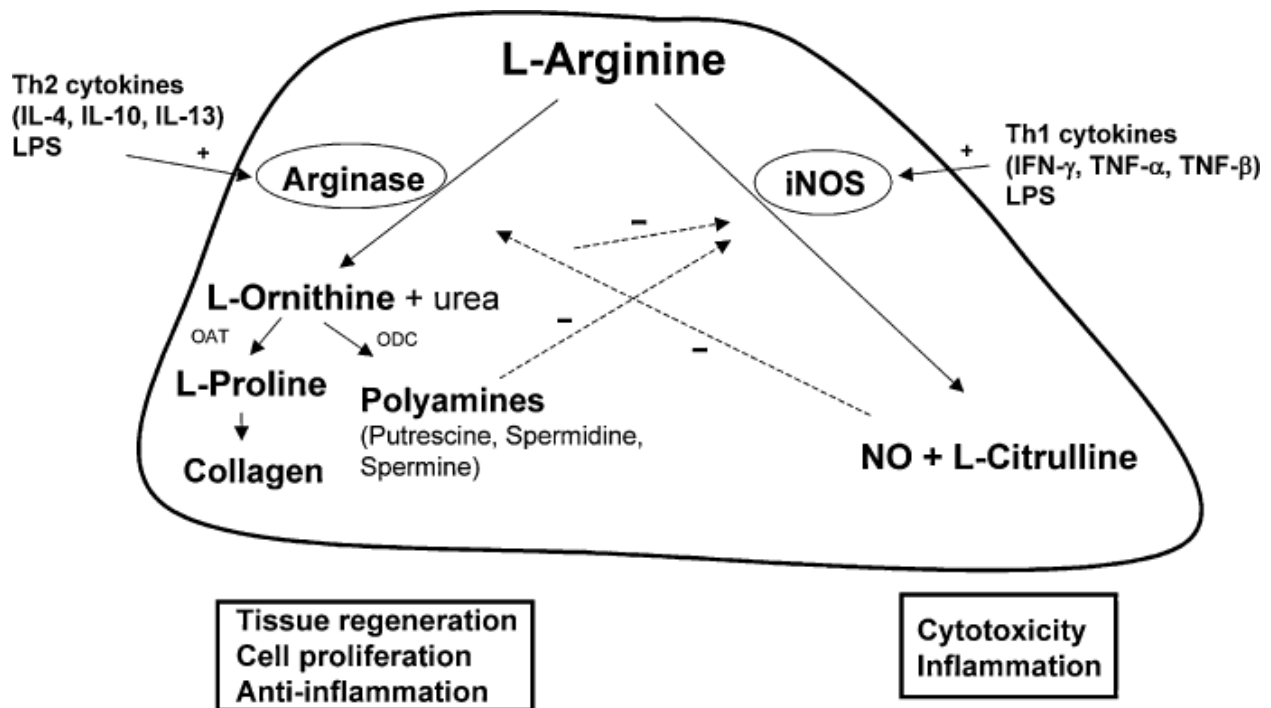


Figure 1.2. Reciprocal regulation of arginase and inducible nitric oxide synthase (iNOS) in murine myeloid cells. Downstream metabolic products of arginase and their association with components of inflammatory responses. OAT, ornithine aminotransferase; ODC, ornithine decarboxylase. (Munder, 2009).

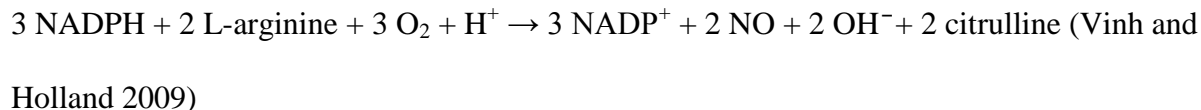
Bacterial arginases have been characterized in relatively few species compared to their mammalian counterparts. The best known arginase pathways are from *Helicobacter pylori*, *Bacillus subtilis*, and *Agrobacterium tumefaciens* (Lu 2006). Arginase is the first enzyme in the arginine utilization pathway, which ultimately produces 2-ketoglutarate and urea, both of which can be used as nitrogen sources. It is a thermostable binuclear metalloenzyme that has optimal activity with manganese, typically in a pH range above nine (McGee, et al. 2004). In *B. subtilis*, arginase (RocF) is encoded by the *rocDEF* operon, which is complemented by a *rocABC* operon. Expression of the *rocABC* operon is induced by arginine through RocR, a positive regulator. Overall this genetic organization is not conserved amongst bacilli nor is it similar to *A. tumefaciens*, a plant pathogen (Calogero, et al. 1994; Gardan, et al. 1995; Lu 2006). The *H. pylori* arginase, RocF, has some unique qualities; optimal activity occurs with cobalt instead of manganese and enzyme activity is best at pH 6.1, possibly to aid in gastric survival (McGee, et al. 2004). At the post-translational level, RocF is modulated by thioredoxin 1, a chaperone with the capacity to protect against oxidative and nitrosative stress and damage (McGee, et al. 2006). At the gene level, *H. pylori rocF* exhibits extensive variation that is strain specific, possibly due to hypervariability in the upstream sequence including the Shine-Dalgarno site. Phenotypic activity was also highly varied (>100 fold), including variation due to *in vitro* passage and when *rocF* was cloned into *Escherichia coli*, giving evidence for trans-acting elements in arginase regulation (Hovey, et al. 2007). Investigations in our lab for an *E. ictaluri* arginase in the genome database found no promising candidates.

For intracellular pathogens, either the microbial or macrophage arginase can be used for a survival advantage. The *H. pylori* arginase RocF is constitutively expressed and can effectively consume extracellular arginine, preventing NO production in co-cultured macrophages, allowing

bacterial survival (Gobert, et al. 2001). Furthermore, RocF has a role in acid resistance and in reducing the expression of CD3 ζ in T cells (McGee, et al. 1999; McGee, et al. 2004). In a *H. pylori* infection model, macrophage ARG2 was upregulated by *H. pylori* via nuclear factor kappa beta (NF- κ B), resulting in the inhibition of NOS translation and the induction of macrophage apoptosis through polyamine production (Gobert, et al. 2002). In an ARG2 knockout mouse model, infection with *H. pylori* led to increased gastric inflammation, with a lower bacterial load, decreased macrophage apoptosis, increased NO production in tissues, and enhanced Th1 cytokine levels versus wild type (Lewis, et al. 2011). *Salmonella* serovar Typhimurium can also upregulate host macrophage arginase for survival, antagonism of NOS, and reduction in NO production which can be reversed by arginase blocking. Furthermore, treatment of infected mice with nor-NOHA (an arginase inhibitor) led to significantly reduced bacterial burdens in the spleen, liver, and mesenteric lymph nodes. This therapy also delayed, but did not prevent, progressive disease (Lahiri, et al. 2008). These findings demonstrate that the induction of host or bacterial arginase can be used by a pathogen to enhance virulence at the cellular and animal level. Remarkably, the intermediates and products of the ARG-ornithine decarboxylase pathways (agmatine, polyamines) are NOS inhibitors, and NO itself is an inhibitor of ornithine decarboxylase (ODC), further solidifying the concept that ARG and NOS function in an antagonistic and reciprocal fashion (Grillo and Colombatto 2004; Peranzoni, et al. 2008; Satriano 2004).

As part of the classically activated macrophage arsenal, the production of RNS starts with the conversion of arginine to NO, which is a free radical gas and antimicrobial agent synthesized in all mammalian cells from arginine by NOS (Tapiero, et al. 2002). NOS is homodimer with an

incorporated heme that is complexed to (6R)-tetrahydrobiopterin. Multiple cofactors and substrates are involved in an oxidoreductase reaction according to the following formula:



Three NOS isoenzymes are known: NOS1 (neuronal NOS, nNOS), NOS2 (inducible NOS, iNOS), and NOS3 (endothelial NOS, eNOS), which differ in their distribution, regulation, and degree of NO production (Bogdan 2001; Peranzoni, et al. 2008). While nNOS and eNOS are constitutively expressed at low levels in many cells types, iNOS (referred to throughout as NOS) is a high output isoform in macrophages, hepatocytes, and endothelium activated by endotoxin and Th1 cytokines (Peranzoni, et al. 2008; Tapiero, et al. 2002). In nonstimulated macrophages, NOS mRNA and protein are not easily detectable, but expression is quick and marked due to promoter activation in one of 3 ways: IFN- γ activation, Toll-like receptor (TLR) activation via myeloid differentiation primary-response protein 88 (MyD88), and TLR activation independent of MyD88 (Bogdan 2009). A variety of substances are known inducers of NOS, including IFN- γ , LPS, flagellin, bacterial DNA, and TNF (Bogdan 2004). NO production is mainly regulated by NOS expression at the transcriptional and post-transcriptional level. As mentioned previously, NOS activity depends on arginine concentration, arginine transport into cells, and consumption of arginine by other metabolic pathways. Studies in human and murine models show marked cell-specific and species-specific variation in signal transduction pathways for NOS expression. However, NF- κ B seems to be a central target for activators and repressors of NOS expression. Nitric oxide synthase mRNA stability and translation are influenced by the cytokine milieu; IFN- γ stabilizes mRNA while transforming growth factor beta (TGF- β) destabilizes it (Pautz, et al. 2010). Additionally, NOS protein stability in intracellular pathogen models can be decreased

due to IL-4 or TGF- β (Bogdan 2004). In most cases, NOS protein produces NO until the enzyme is degraded. In nonstimulated macrophages NOS is diffusely present, but will localize to phagosomal membranes in activated cells. This recruitment requires a functional actin cytoskeleton with complementary proteins, which can be exploited for the benefit of phagocyte pathogens such as the Mycobacteriae (Bogdan 2009).

In terms of infectious disease, NO exhibits a wide range of activity and has been implicated in antimicrobial, proinflammatory, anti-inflammatory, cytotoxic, and cytoprotective effects (Bogdan 2001). Nitric oxide may also exert antimicrobial effects through various means. Perhaps the best known is that of directly toxic effects to DNA, structural proteins, enzymes and membrane lipids, along with the formation of the RNS peroxynitrite (ONOO⁻). Peroxynitrite can form peroxynitrous acid (ONOOH) that spontaneously decomposes at physiological pH to the hydroxyl radical (OH[•]) and nitrogen dioxide ([•]NO₂) (Bogdan 2004). In addition, NOS is able to deplete cellular arginine levels which can impede intracellular pathogen growth and can produce N^ω-hydroxy-L-arginine which can block arginase function, leading to a lack of microbe-beneficial polyamines (Bogdan 2009). Furthermore, NO has been reported to overcome phagosome maturation inhibition by *Leishmania donovani*, inhibit the escape of *Listeria monocytogenes* from phagolysosomes, and inhibit type III secretion system gene expression in *Salmonella* serovar Typhimurium (McCollister, et al. 2005; Myers, et al. 2003; Winberg, et al. 2007).

In fish as in mammals, macrophages function as professional phagocytes and share many of the same characteristics, including classical and alternative activation, ROS production, and RNS production (Chadzinska, et al. 2008; Chettri, et al. 2010; Joerink, et al. 2006; Plouffe, et al. 2005; Rieger, et al. 2010). Such studies involving macrophage activation use well known

activators and triggers, like IFN- γ and LPS in addition to various other substances such as squalene (peritoneal irritant) and macrophage activating factor (MAF) of goldfish to induce activation (Joerink, et al. 2006; Rieger, et al. 2010). Results of recent experiments into the differential aspects of goldfish (*Carassius auratus auratus*, Linnaeus 1758) pronephros macrophage cultures have provided some important insights for our model in channel catfish. The pronephros contains myeloid progenitors, monocytes, and mature macrophages, all of which have distinct morphologies, gene expression profiles, and functional capabilities (Belosevic, et al. 2006). From pronephros derived *in vitro* primary cultures, these 3 myeloid cell types remain viable for at least nine days. While progenitor cells exhibit minimal phagocytic and respiratory burst capabilities, monocytes and mature macrophages show increased phagocytic, respiratory burst, and phagolysosomal fusion capability when activated with LPS and MAF. Interestingly, unactivated macrophages had high phagocytic capacity but low ROI production and phagolysosome formation compared to unstimulated monocytes. The authors speculate that in unstimulated fish monocytes and macrophages, phagocytosis is uncoupled from degradative or killing responses. In cases where pathogens can rapidly infect unactivated macrophages in the pronephros, the advantage is to the microbe (Rieger, et al. 2010). Only until recently have cytokine measurements in fish cells become available in order to better characterize the molecular details of activation (Chadzinska, et al. 2008).

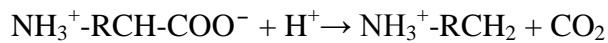
Another important consideration when using a fish macrophage model is the difference in elicited responses from different macrophage cell populations. Pronephros derived macrophages in the barramundi (*Lates calcarifer* Bloch 1790) have markedly different functional capabilities (ROI and RNI production) compared to peritoneal derived, activated macrophages (Tumbol, et al. 2009). Relatively little is currently known about the molecular characteristics of channel

catfish macrophage activation and functional physiology. In one study, activated (LPS injection) peritoneal lavage macrophages from *I. punctatus* were pooled and activated *in vitro* with LPS to measure NO activity via nitrite and nitrate assays. Nitrite production was measured after 96 hours and was found to be greatest when cells were incubated with 0.5 mM arginine and glutamine but was inhibited when N^G -monomethyl-L-arginine was used (competitive inhibitor for NOS). Thus, there is an arginine dependent NO production pathway in catfish macrophages that can be stimulated with LPS (Buentello and Gatlin III 1999). Finally, it is important to bear in mind that there are marked differences in macrophage NO production and functional capacity in domestic mammal species; it is safe to say that with regards to fish, such differences also exist (Schneemann, et al. 2002).

BACTERIAL ARGININE METABOLISM

There are at least 5 arginine utilization pathways in bacteria: arginine decarboxylase, arginase, arginine deiminase, arginine transaminase/oxidase/dehydrogenase and arginine succinyltransferase. Arginine is important for microorganisms as it is a source of carbon, nitrogen, and energy; it is synthesized and catabolized by many species (Lu 2006). In addition, arginine has a well known role in acid resistance in Enterobacteriaceae through arginine decarboxylase (Kieboom and Abee 2006). Biosynthetic arginine decarboxylases that function at neutral pH are to be distinguished from those induced by acid pH (AdiA), which are inducible or biodegradative (Viala, et al. 2011). In the acid induced Enterobacteriaceae system, arginine is converted to agmatine by AdiA, which is subsequently either converted to urea and putrescine by agmatinase, or to putrescine and ammonia by agmatine deiminase (Liu, et al. 2009; Rhee, et al. 2007). In *E. coli*, 5 acid resistance (AR) pathways are known, 3 of which are amino acid decarboxylases (glutamate, arginine, lysine). The AdiA system (AR3) consists of AdiA and the

arginine-agmatine exchange transporter AdiC, along with an AraC-like regulator AdiY. This system functions to consume arginine and protons, which can increase intracellular pH in extreme acid stress; in the case of *E. coli*, from that of internal pH 3.5 to 4.5. The diamine or triamine product of this reaction can function as an environmental buffer. Inducible amino acid decarboxylases consume protons according to the following formula:



Amongst enteropathogenic gammaproteobacteria, only *Salmonella* serovar Typhimurium has an AR3 similar to *E. coli*; however, this is induced only in anoxic conditions (Iyer, et al. 2003; Richard and Foster 2004; Wortham, et al. 2007; Zhao and Houry 2010). *S. enterica* also has 2 other acid inducible decarboxylases for lysine (CadA) and ornithine (SpeF), which function in a similar manner to AR3, albeit with a different substrate. All 3 were able to improve survival and growth at pH 2.3, although AdiA was the most efficient at this pH. At moderate pH (4.5), CadA and SpeF were able to improve *in vitro* growth and increase media pH. In the murine macrophage SCV, CadA and SpeF activity could delay SCV acidification; AdiA activity in this assay was not examined. Importantly, individual gene knockouts for all 3 decarboxylases were not significantly attenuated *in vivo* (Viala, et al. 2011). Recently, *E. ictaluri* has been shown to have an AdiA that is involved in virulence. Two AdiA knockout mutants were found to have low *in vivo* competitive indices compared to wild type *E. ictaluri*, indicating that AdiA has an important pathogenic role (Thune, et al. 2007). Similar to *E. coli* AR3, *E. ictaluri* has a putative AdiY, but also an arginine/agmatine transporter associated with AdiA. Additionally, a second arginine decarboxylase, SpeA, is associated with agmatinase in *E. ictaluri*, which is a potential source of urea. With reference to the model, urea produced by the AdiA/SpeA/agmatinase pathway could be shuttled to the bacterial urease for ammonia production; such a situation seems

both elegant and efficient (Booth, et al. 2009). Despite these findings, arginine decarboxylase is widely considered to be of minor importance in the arginine regulation of infectious disease (Bogdan 2009).

In addition to the functions of arginine in the preceding discussion, it is also a major source of polyamines. Arginine is first converted to ornithine and urea by arginase in eukaryotes, which is then converted by ODC to putrescine. Plants and bacteria however, synthesize putrescine by an alternate mechanism, where AdiA converts arginine to agmatine, which is converted by agmatinase to urea and putrescine (agmatine urohydrolase) (Rhee, et al. 2007). Alternatively there is another bacterial pathway, seen in *Yersinia pestis*, *H. pylori*, and *Pseudomonas aeruginosa*, where agmatine is converted to putrescine and ammonia by agmatine deiminase (ADI) and N-carbamoylputrescine amidohydrolase (Jones, et al. 2010; Nakada, et al. 2001; Wortham, et al. 2007).

Agmatine, a metabolite of arginine via arginine decarboxylase, is considered to merely be a precursor of polyamines in bacteria, but has important regulatory effects in mammals in regard to polyamine and NO suppression (Satriano 2003). It is present in almost all organs of the rat, including plasma, and has an equally wide range of biological activity (Aricioglu and Regunathan 2005). At physiologic pH agmatine carries a positive charge and probably doesn't cross cell membranes by diffusion, but agmatine transporters and nicotinic acetylcholine receptor channels facilitate entry into cells (Berkels, et al. 2004; Molderings, et al. 1999). Agmatine can inhibit all isoforms of NOS due to the formation of the active aldehyde, guanidinobutyraldehyde, which can aid in the transition from the acute inflammatory phase to the reparative/proliferative tissue phase (Sastre, et al. 1998; Satriano 2004). In a murine macrophage cell line, LPS stimulation leads to increased agmatinase and NOS function while decreasing AdiA function.

Thus, when classically activated by LPS, macrophages can upregulate arginine uptake and NO production while inhibiting other arginine degradation pathways and stimulating the hydrolysis of NOS inhibitors (agmatine). Conversely, IL-10 and TGF- β inhibit agmatinase and NOS (Sastre, et al. 1998).

Agmatine is also able to suppress eukaryotic ODC mediated polyamine biosynthesis. Normally, polyamines are able to autoregulate their intracellular concentrations by the upregulation of antizyme, which is able to block ODC and polyamine transport (Berkels, et al. 2004). However, agmatine, in a dose dependent fashion, can directly induce antizyme by a +1 translational frameshift in the ribosome, leading to cellular polyamine depletion (Satriano, et al. 1998). The agmatine regulated reduction in polyamines has several interesting and pertinent effects in eukaryotic cells. In leukemia cell lines, agmatine has a dose dependent antiproliferative effect that may be due to ODC regulation at the translational level, rather than due to antizyme production (Haenisch, et al. 2011). Furthermore, agmatine exhibits a protective, anti-apoptotic effect, evidenced by mitochondrial membrane stabilization and decreased Bcl-2 and caspase 3 expression in mouse fibroblasts (Arndt, et al. 2009). In the context of pathogenic microbes, agmatine can be considered a virulence factor in some cases; *H. pylori* is able to secrete sufficient agmatine to cause increased gastric acid secretion *in vivo*, leading to gastroduodenal ulcers in humans (Molderings, et al. 1999). The vital function of AdiA in ESC pathogenesis indicates that agmatine is produced in physiologically relevant amounts, which could directly affect infected catfish at the cell, tissue, and organism levels. If agmatine is produced by *E. ictaluri* intraphagosomally, it could then disrupt macrophage NOS and polyamine balance, leading to reduction in microbicidal NO and cell protective polyamines. In addition, *E. ictaluri*

derived agmatine could confer antiproliferative and anti-apoptotic effects in the pronephros, a site of lymphocyte and macrophage immunoregulation and immunostimulation.

Agmatine metabolism in bacteria also occurs through ADI operons, where putrescine, ATP, ammonia, and carbon dioxide are produced (Griswold, et al. 2006; Jones, et al. 2010; Nakada, et al. 2001). In *Streptococcus mutans*, the etiological agent of dental caries in humans, an *aguBDAC* ADI operon contains an agmatine-putrescine antiporter (AguD), agmatine deiminase, putrescine carbamoyltransferase, and a carbamate kinase which are upregulated by the transcriptional activator AguR in the presence of agmatine and acidic pH (5.5) (Liu, et al. 2009). With 4 ammonia molecules produced for every agmatine consumed, there is potential for ADI to contribute to acid resistance and disease pathogenesis (Griswold, et al. 2006; Jones, et al. 2010). Interestingly, in *S. mutans* a proposed role for ADI in virulence associated with acidic oral biofilms has been proposed. First, acidic conditions upregulate AdiA in biofilm mixed-bacterial populations to produce agmatine, which is inhibitory to *S. mutans* growth. The *S. mutans* ADI is then upregulated to remove agmatine and produce putrescine, ammonia (which consumes protons), and ATP (to power proton pumps), which function to increase environmental pH, allowing survival (Griswold, et al. 2006). Investigations for an *E. ictaluri* ADI in the genome database have found no promising candidates.

Polyamines themselves are important for prokaryotic and eukaryotic cell growth, cell signaling, and homeostasis, but are also involved in immunomodulation (Rhee, et al. 2007; Satriano 2004). They are small aliphatic hydrocarbons which promote efficient DNA replication, transcription, and translation and have a net positive charge at physiological pH. Bacterial regulation of polyamine uptake, production, and catabolism is highly regulated (Shah and Swiatlo 2008). Polyamine uptake systems are best characterized in the *E. coli* ATP binding

cassette (ABC) transporters *potABCD* (spermidine) and *ptFGHI* (putrescine). Both transporters consist of an ATPase, a periplasmic binding protein, and 2 proteins to form the cell membrane channel. PotE acts as a putrescine-ornithine antiporter and is present in an acid-inducible operon with ODC (Wortham, et al. 2007). The biosynthetic pathways for polyamines typically convert amino acids via decarboxylation first to putrescine, and then to spermidine, which is often the predominant polyamine in bacteria. The 2 best known pathways are: (i) arginine decarboxylation to agmatine by *speA/adiA*, with subsequent conversion to putrescine and urea by agmatinase (*speB*), and (ii) decarboxylation of ornithine directly to putrescine by ODC (*speC*). Ornithine decarboxylase is the key enzyme for putrescine production in *E. coli* and, along with arginine decarboxylase, is inhibited by increased putrescine or spermidine. Spermidine synthesis genes *speE* and *speD* are in a separate operon from *speABC* (Shah and Swiatlo 2008; Tabor and Tabor 1984). Polyamines can activate multiple stress regulons in response to acid, osmolarity change, ultraviolet light exposure, ROS, and heat in many organisms. They may also function as free radical scavengers and are important mediators for acid resistance in bacteria. In *E. coli* polyamines can bind and stabilize bent DNA, and 90% of spermidine is bound to RNA, giving polyamines a potential role in gene transcription and translation regulation (Wortham, et al. 2007). Polyamines in *E. coli* also alter outer membrane function by blocking porins, contributing to acid resistance (Shah and Swiatlo 2008; Yohannes, et al. 2005).

In the context of infectious disease, polyamines secreted by bacteria can negatively affect host macrophages. *H. pylori* infection leads to an ineffective macrophage response in part through its ability to upregulate macrophage arginase and ODC, leading to increased spermine. High spermine levels inhibit macrophage arginine uptake, NOS, and NO production (Bussière, et al. 2005; Chaturvedi, et al. 2010). In addition, *H. pylori* upregulates expression of polyamine

oxidase in macrophages, causing increased spermine oxidation and the subsequent formation of hydrogen peroxide which leads to mitochondrial damage and apoptosis. The upregulation of polyamines has been shown to upregulate virulence factors in *Salmonella* spp., *Shigella flexneri*, and *Streptococcus pneumonia* (Shah, et al. 2008; Shah and Swiatlo 2008).

BACTERIAL UREASE

Urea is an important nitrogenous waste compound produced in abundance by most terrestrial vertebrates and some species of fish. The majority of teleosts are ammoniotelic however, meaning they primarily excrete ammonia and relatively little urea due in part to ammonia's lower metabolic cost and high water solubility (Saha and Ratha 2007). Urea is a small, polar, relatively lipid-insoluble compound that is produced by fish through arginine breakdown, purine degradation, or synthesis by an ornithine urea cycle (OU-C). The OU-C is lacking in most adult fish, including channel catfish, which are ammoniotelic (McDonald, et al. 2006; Walsh 1998). Despite lacking an OU-C as adults, ammonioteles do have a functional OU-C during early life stages that is eventually lost, but urea transporters are retained and are functional. For many species, the gill and kidney tubules are the primary sites for urea transport and excretion (McDonald, et al. 2006). Channel catfish have recently been shown to excrete approximately 18% of their nitrogenous waste as urea when housed in freshwater (Altinok and Grizzle 2004).

The regulation of urea transporter expression in fish is not well characterized, but they likely share much similarity to mammalian urea transport mechanisms (Bagnasco 2005). Although there are virtually no data indicating the presence of urea transporters in macrophages, urea can be transported nonspecifically by aquaporin nine, found in leukocytes and in monocyte lineage cells (Aharon and Bar-Shavit 2006; Litman, et al. 2009). Interestingly, aquaporin one

(AQP1) has been shown to modulate the integrity of pathogen containing vacuoles in mouse fibroblast cell lines and HeLa cells infected with *S. enterica*. Aquaporin one was found to be colocalized with pathogen containing vacuoles and when overexpressed in cells, *Salmonella* could proliferate significantly (Radtke and O'Riordan 2008).

Urea itself can enter cells slowly by diffusion when in high concentrations, but active urea transporters function at times of low availability or increased need. In bacteria there are 3 urea uptake systems in neutralophiles, all of which contribute to acid tolerance; an ABC type transporter which requires ATP, and 2 energy independent transporters (Yut and UreI) which form membrane channels (Sachs, et al. 2006). The *E. ictaluri* UreI, present in the urease operon, has 53.6% identity to Yut at the protein level, and shares the same tandem sequence signature repeats that characterize Yut (Booth 2006). The Yut permease is a 35 kD protein with ten predicted transmembrane segments and no homology to other bacterial permeases, but has 21% homology to human urea transporter one (Sands, et al. 1997). It is not pH regulated and functions as a channel, allowing unrestricted urea entry. In order to prevent excessive and lethal ammonia and carbon dioxide production by urea hydrolysis, *Yersinia*, as opposed to *H. pylori*, has a urease with an acidic pH optimum like that in *E. ictaluri* (Booth, et al. 2009). Conversely, the UreI of *H. pylori* is an acid activated permease that functions as a proton gated urea channel that is directly regulated by the periplasmic pH. (Sachs, et al. 2006).

For many species of medically relevant bacteria (*H. pylori*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Yersinia* spp.) urea is important as a substrate for the enzyme urease (urea amidohydrolase), which is a known virulence factor. Bacterial ureases are multiprotein complexes encoded by operons. For *E. ictaluri* the urease operon is organized comparably to *E. coli* and *H. pylori*, but is most similar to *Y. enterocolitica*, being arranged as *ureABCEFGD*,

with separately regulated downstream urea and ammonia transporters (Booth 2006). In *Klebsiella aerogenes* and *E. coli*, the functional urease apoprotein UreABC assembles into a trimer, then complexes with 3 UreDFG accessory protein complexes to enable UreE to deliver nickel ions (Ni^{2+}) to the enzymatic metallocenter; dissociation of accessory proteins produces the active cytoplasmic enzyme (Boer, et al. 2010; Booth 2006; Burne and Chen 2000; Mobley, et al. 1995). Once activated, urease hydrolyzes urea into ammonia and carbamate; the latter then spontaneously decomposes to produce ammonia, bicarbonate, hydroxide ions, and carbon dioxide in a process known as the urease reaction (Burne and Chen 2000; Huynh and Grinstein 2007; Mobley, et al. 1995). Urease in many bacterial species is constitutively expressed, while in others it is synthesized in response to environmental conditions, including nitrogen starvation, the presence of urea, or environmental acidity (De Koning-Ward and Robins-Browne 1995; Mobley, et al. 1995).

Urease expression may be tightly regulated in various ways; positive transcriptional regulators, two-component signal transduction, and sigma 70 type promoters are described in different species (Burne and Chen 2000). In *H. pylori*, where this is best understood, acid pH is sensed by the histidine kinase ArsS, which phosphorylates the response regulator ArsR, an OmpR family protein. ArsR binds to the promoters of both transcriptional units of the urease operon, *ureAB* and *ureIEFGH* (Pflock, et al. 2006). A second sensor kinase, HP0244, responds to cytoplasmic acidity by precipitating inner membrane assembly of the active urease. HP0244 functions in this manner without need of its response regulator HP0703, and without the regulation of transcription (Scott, et al. 2010). Additionally, transcription of *ureAB* is regulated by NikR in response to increased Ni^{2+} and acid pH (Pflock, et al. 2006). In *Y. pseudotuberculosis*, OmpR binds to the promoters of the 3 urease transcriptional units, *ureABC*,

ureEF, and *ureGD*, positively regulates gene expression, and controls urease in a pH dependent fashion (Hu, et al. 2009). The expression regulation of urease in *E. ictaluri* is largely unknown; no NikR has been found and there is limited identity to an AraC-type regulator found in *E. coli*. Furthermore, it is still unclear how many transcriptional units are present in the operon (Booth 2006).

The contribution of urease to bacterial acid resistance and virulence is potentially broad, in that bacteria often find themselves in acidic and hazardous conditions both extracellularly and intracellularly, where pH regulation is necessary for survival. Firstly, the ammonia that is produced sequesters protons by forming ammonium in biological systems, functioning as a powerful acid neutralizer. Secondly, carbon dioxide produced by urease can be converted to bicarbonate (HCO_3^-), which is an important cytoplasmic and periplasmic buffer (Huynh and Grinstein 2007). Thus, within phagosomes, alteration of pH (prevention of acidification) by the bacterial urease products can prevent optimal microbicidal activity, phagosome maturation, and ultimately may prevent phagolysosome formation (Huynh and Grinstein 2007).

In *H. pylori* a model (Figure 1.3) has been proposed to explain its acid acclimation ability, so called because *H. pylori* can maintain periplasmic pH at 6.1 and cytoplasmic pH near neutral, enabling not only survival, but growth in pH 2.5 stomach acid. Acid tolerance or resistance mechanisms in other neutralophiles (AR systems) are only able to maintain cytoplasmic pH between 4 and 5 and cannot alter periplasmic pH (Marcus, et al. 2005; Scott, et al. 2010). In this model, urea diffuses through an outer membrane porin and through the inner membrane channel, UreI, in acidic periplasmic conditions ($\text{pH} < 6.2$). Urease is assembled by HP0244 on the cytoplasmic side of the inner membrane during cytoplasmic acidity, and converts urea to ammonia and H_2CO_3 (carbonic acid). The former combines with protons in the cytoplasm

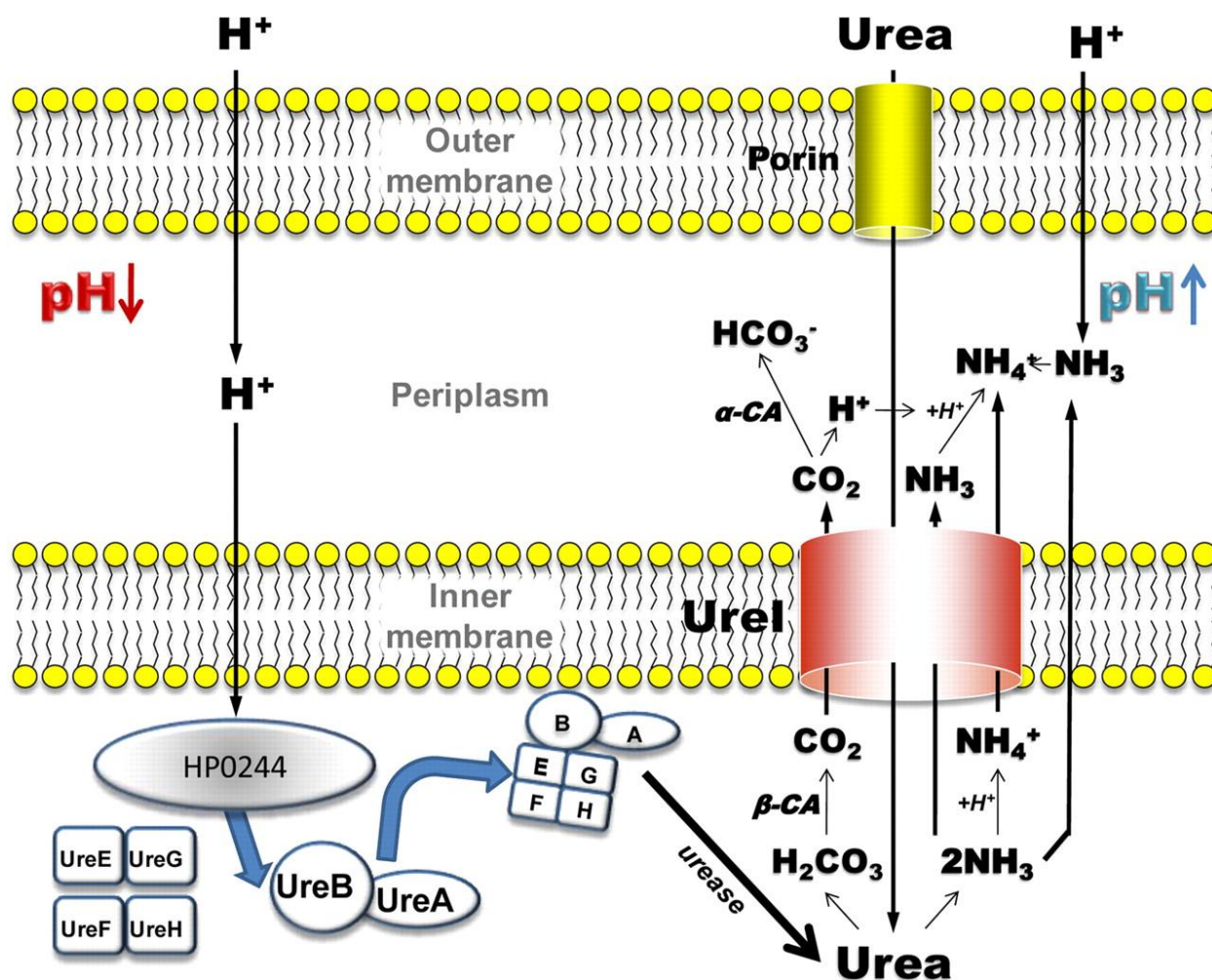


Figure 1.3. Model of the mechanism of action of UreI and HP0244 in *H. pylori*. UreI transports urea at acidic periplasmic pH (pH < 6.2) and increasing urease activity, forming H₂CO₃ and 2 ammonia. Carbonic acid is converted to CO₂ in the cytoplasm by β-carbonic anhydrase and enters the periplasm, where it is converted by α-carbonic anhydrase to HCO₃⁻, enabling maintenance of periplasmic pH at ~6.1. Ammonia exits via the bilayer and UreI, and NH₄⁺ is formed from the H⁺ generated by α-carbonic anhydrase and from protons entering from the medium. The NH₄⁺ generated in the cytoplasm exits via UreI, or perhaps via NH₃ + H⁺ exit. Acidification of the cytoplasm activates HP0244, which in turn allows assembly of the apoenzyme UreA/UreB with the nickel insertion pairs, UreE/UreG and UreF/UreH, activating urease at the membrane, providing local generation of carbonic acid and ammonia. (Scott, et al. 2010).

to form NH_4^+ (ammonium), and the latter is converted to CO_2 by β -carbonic anhydrase. Ammonium, carbon dioxide, and ammonia can then exit through UreI to the periplasm. In the periplasm, carbon dioxide is converted to bicarbonate by α -carbonic anhydrase where it functions as a periplasmic buffer (Scott, et al. 2010).

Such a situation may occur in murine macrophages infected with *H. pylori*, which evades killing by altering phagosome maturation through megasome formation, which is dependent on urease expression (Schwartz and Allen 2006). Along these same lines, *M. bovis* BCG uses urease mediated phagosome pH neutralization to disrupt MHC-II endocytic trafficking in macrophages, thereby disrupting an important aspect of adaptive immunity (Sendide, et al. 2004).

The urease operon of *E. ictaluri* was discovered during an STM study in channel catfish, where a UreG and UreF mutant were found to be highly attenuated (Thune, et al. 2007). UreABCEFGD and both downstream transporters each have sigma 70 promoters, suggesting that UreA is translationally coupled to UreBCEFGD while the transporters are single gene transcripts. UreC contains 8 conserved histidine residues that form the essential nickel binding metallocenter at position 320, marking the active site. Additionally, *E. ictaluri* has a phenylalanine residue 7 sites upstream from histidine 320, followed by asparagine; both of which are associated with optimal activity at low pH like that seen in *Y. enterocolitica* (Booth 2006).

UreG is a highly conserved accessory protein and its role is generally thought to be GTP hydrolysis in concert with urease activation, involving the insertion of 2 nickel ions into the active site of UreC (Mobley, et al. 1995; Zambelli, et al. 2009). Purified UreG proteins in other bacteria (*K. aerogenes*, *M. tuberculosis*, *H. pylori*) contain motifs found in GTPases, in particular a P-loop motif (GXGKT) which is also present in *E. ictaluri*. This region is vital for *in vitro* activation of the urease complex. UreG binds both nickel and UreE, suggesting that nickel ions

are transferred from UreE to UreG to UreD to the active site (Boer, et al. 2010). The *ureG* *E. ictaluri* mutant was characterized further in a series of *in vitro* and *in vivo* experiments (Booth 2006). In wild type lysates, the presence of *E. ictaluri* UreA, UreC, and UreG at neutral pH suggests that expression is neither transcriptionally nor translationally regulated. In broth cultures, *E. ictaluri* Δ *ureG* was able to survive at pH 3 for 2 hours, similar to wild type, indicating that *E. ictaluri* uses other metabolic pathways for acid resistance. However at pH 5 in the presence of urea, wild type *E. ictaluri* is able to grow and can produce significant amounts of ammonia such that environmental pH is elevated to over 7. This effect is absent in the Δ *ureG* strain. Based on ammonia production the pH optimum of the *E. ictaluri* urease is between 2 and 3, while expression begins at pH 5. In HKDM, the Δ *ureG* strain was able to invade cells in similar numbers to the wild type, but could not replicate. Similarly, *in vivo* the Δ *ureG* strain could invade the pronephros like wild type, but could not replicate nor persist (Booth, et al. 2009).

Aside from its properties in pH regulation, ammonia can also contribute to bacterial pathogenicity directly, or via highly toxic derivatives. Ammonia is a highly water soluble gas which is protonated to form ammonium and has a $\text{NH}_3/\text{NH}_4^+$ pK_a of 9.2, which means that at physiologic pH the ratio of $\text{NH}_3/\text{NH}_4^+$ is one to 100; this has biological relevance where ammonia is thought to contribute as a buffer. Adverse effects of ammonia accumulation include the alteration of various biochemical reactions by mass-action effects and the competition of ammonium with K^+ and Na^+ transmembrane transport pathways. Ammonia is highly soluble in biological systems and is thought to travel via aqueous channels across cell membranes (Walsh 1998). The interaction of *H. pylori*'s urease, urea, and leukocyte myeloperoxidase can lead to monochloramine production, which is able to induce DNA damage. Additionally, ammonia and

ammonium hydroxide are directly cytotoxic and are inhibitory to normal immune function (Burne and Chen 2000).

In the model for *E. ictaluri* in the channel catfish, the naive monocyte or non-activated macrophage of the pronephros is the assay model for important reasons. Firstly, the pronephros is one of the first organs to be infected by *E. ictaluri*, where bacteria multiply rapidly in spite of the fact that the organ is almost entirely hematopoietic and has a very high myeloid cell population (Grizzle and Rogers 1976). Secondly, it is known that mature activated macrophages, like those elicited from the standard peritoneal lavage, are highly capable of phagocytosing and eliminating *E. ictaluri* (Shoemaker, et al. 1997). Given the fact that *E. ictaluri* can survive and replicate in naive HKDM, it is likely that *E. ictaluri* has found a replicative niche where exploitation of the macrophage is optimal. The use of naive macrophages, or those that are not previously exposed to the typical molecules (LPS, IFN- γ) known to activate macrophages, is standard in the following chapters.

From the preceding discussion, it should be understood that at present, the understanding of the molecular mechanisms regarding channel catfish (and almost all fish for that matter) monocytes and macrophages in the context of intracellular bacterial pathogens lags behind that of murine models by a substantial margin. With this in mind, the investigation of the model must begin at its foundations, being careful not to assume too much from investigations on the activation stages of macrophages in other species. Aside from the understanding that *E. ictaluri* has an acid activated urease that is of the utmost importance to intracellular survival and an AdiA that is of similar overall necessity to virulence, important gaps in the knowledge must be filled in.

HYPOTHESES

The following chapters follow a course of investigation aimed at the validation of several primary model assumptions. Of primary importance is the concept that *E. ictaluri* is able to alter the phagosomal pH to a less acidic or non-acidic range to facilitate replication. Of no less importance is the idea that there is an active interaction between the bacterium and the macrophage over the fate of intracellular arginine, culminating in the success or failure of the macrophage to eliminate *E. ictaluri*. The overall hypothesis is that *E. ictaluri* utilizes arginine and its metabolites for virulence in HKDM. The subhypotheses are these: 1) *E. ictaluri* is able to alter the HKDM phagosomal pH; 2) the *E. ictaluri* urease, at least one of the arginine decarboxylases, whether AdiA or SpeA, and possibly agmatinase are vital to this pH alteration; 3) channel catfish HKDM NO production, arginase function, and urea production are important for *E. ictaluri* pathogenesis.

The second chapter will describe the development of methods to assess intraphagosomal pH in *E. ictaluri* infected HKDM and the application of this method to *E. ictaluri* mutants that lack urease, AdiA, SpeA, or agmatinase function. This is the first study to show definitive evidence that *E. ictaluri* is able to maintain a neutral phagosome pH by using its urease and AdiA. The third chapter will focus on measuring NO production, urea production and arginase function in *E. ictaluri* infected HKDM.

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CHAPTER 2

MODIFICATION OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) MACROPHAGE PHAGOSOMAL PH BY *EDWARDSIELLA ICTALURI*

INTRODUCTION

Edwardsiella ictaluri is a Gram-negative rod-shaped bacterium and is the etiological agent of enteric septicemia of channel catfish (*Ictalurus punctatus* Rafinesque, 1818), otherwise known as ESC (Hawke 1979). This disease occurs commonly throughout channel catfish production areas and is a leading cause of mortality with substantial negative economic impact throughout the industry (USDA 2009a; USDA 2009b). ESC presents most commonly as an acute septicemia with a propensity for bacteria to infect the pronephros (head kidney), but also the liver, spleen, and intestine (Baldwin and Newton 1993; Hawke, et al. 1998). This is of particular relevance because *E. ictaluri* can enter, survive, and replicate in the phagosomes of pronephros derived macrophage (HKDM) primary cell cultures, making it a facultative pathogen of professional phagocytes (Booth, et al. 2006).

Using signature tagged mutagenesis (STM), (Thune, et al. 2007) recently identified several *E. ictaluri* virulence related genes using a live catfish challenge model. Of interest was the discovery of a urease operon and an arginine decarboxylase (AdiA) that were involved in virulence *in vivo*, suggesting that arginine metabolism is a vital aspect of this disease's pathogenesis in catfish. *E. ictaluri*, however, is not known to be urease positive in standard biochemical tests, so the nature and function of this urease was uncertain. Further investigation found that this urease is acid activated, has optimal activity at pH 2-3, can alter environmental pH from acid to neutral through ammonia production, and is necessary for bacterial replication in HKDM, but not for infection or survival (Booth, et al. 2009).

The utilization of urea produced from arginine for urease function in microbial pathogenesis is typified by *Helicobacter pylori*, a major cause of chronic gastritis, peptic ulcers, and gastric cancer worldwide. It is an impressively adapted pathogen that normally inhabits what was once thought uninhabitable; the human stomach, where daily pH ranges from 1 to 5 (Pflock, et al. 2006). Arginine is an essential amino acid for *H. pylori*, which uses bacterial arginase (RocF) to convert arginine into urea. Subsequently, the *H. pylori* urease converts urea into ammonia, which is required for successful macrophage infection (Allen 2007; Schwartz and Allen 2006). *Edwardsiella ictaluri* may utilize arginine in a similar fashion, using AdiA rather than arginase. Arginine decarboxylases can convert arginine to agmatine, which in turn can be converted to putrescine and urea by agmatinase.

Urea is utilized by urease to produce ammonia, bicarbonate, and carbon dioxide via the urease reaction (Burne and Chen 2000). Ammonia itself can act as a proton sink and bicarbonate is a cytoplasmic/periplasmic buffer; both of which can be utilized by bacteria for acid resistance. This has important implications to bacteria within the normally acidic macrophage phagosomal environment, where the ability of pathogens to alter pH can permit survival, intracellular replication, and disease progression (Huynh and Grinstein 2007). It has been suggested that *E. ictaluri* may use urease to alter phagosomal pH by ammonia production, thus preventing digestion and the activation of acid hydrolases, resulting in an environment conducive to replication (Booth, et al. 2009). In addition to AdiA, *E. ictaluri* has a second arginine decarboxylase, SpeA, and an agmatinase (SpeB). These data allowed development of a hypothetical model, where neutralization of phagosomal pH in HKDM by *E. ictaluri* occurs through the metabolism of arginine to urea, agmatine, and ammonia by bacterial arginine decarboxylase, SpeB, and urease (Fig 2.1). While it is known that *E. ictaluri* can use urease to

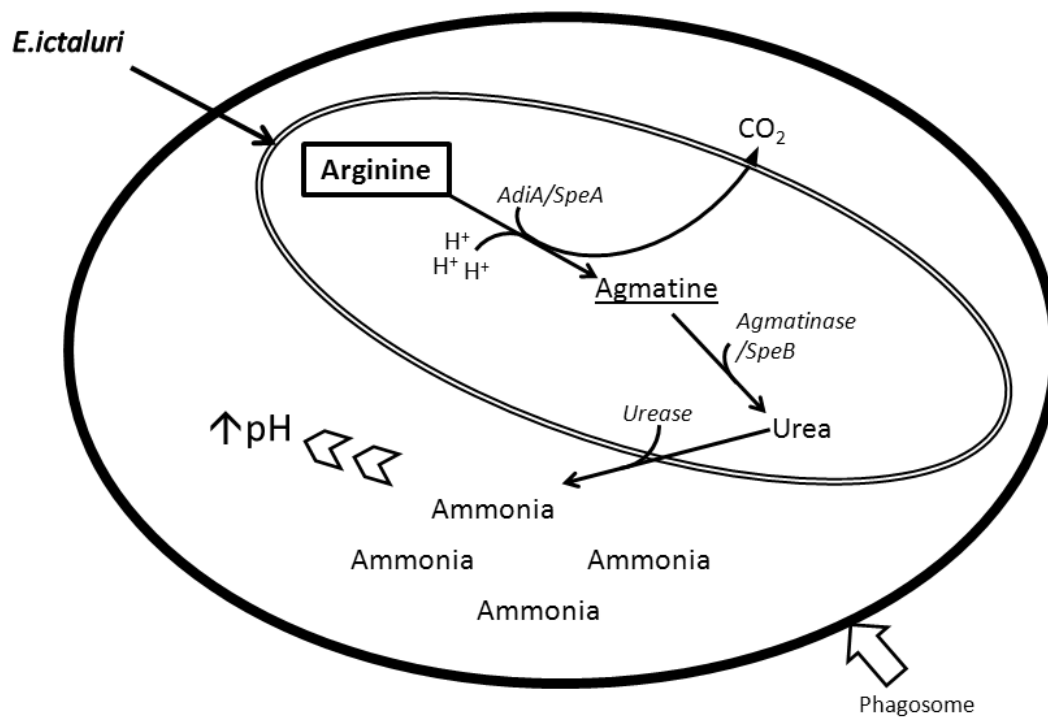


Figure 2.1. Proposed model for arginine utilization by *E. ictaluri* in HKDM phagosomes. Arginine is utilized by arginine decarboxylase (*AdiA* or *SpeA*) to produce agmatine. Agmatine is consumed by agmatinase (*SpeB*), providing urea to urease. Urease converts urea to ammonia, which consumes protons and alkalinizes *E. ictaluri* cell membranes and phagosome pH.

produce quantities of ammonia sufficient to increase environmental pH, and that this urease is necessary for replication in HKDM (Booth, et al. 2009), it has not been definitively shown that *E. ictaluri* uses its urease to alter phagosomal pH in HKDM. Furthermore, although an arginine decarboxylase mutant (Δ AdiA) was attenuated *in vivo* (Thune, et al. 2007) it has not been determined that AdiA is involved in regulation of phagosome pH. Consequently, the primary objectives of this study were to develop methods to evaluate the intraphagosomal pH of wild type *E. ictaluri* in HKDM and to use urease, AdiA, SpeA, and SpeB gene mutants to investigate their role in the model.

MATERIALS & METHODS

Bacterial Strains and Growth Conditions. Bacterial strains and plasmids are listed in Table 2.1. Unless otherwise noted, *Escherichia coli* was grown in Luria-Bertani broth (LB) at 37°C and *E. ictaluri* strains were grown in porcine brain heart infusion broth or LB-0.35% Mannitol (LB-Man) at 28°C. A defined minimal medium for *E. ictaluri* was used with minor modifications stated where pertinent (Collins and Thune 1996). Strain CC118 λ pir of *E. coli* was used to maintain the delivery plasmids and to isolate plasmid DNA prior to introduction into *E. coli* SM10 λ pir, the conjugation strain. Antibiotics were used in the following concentrations as needed: kanamycin (Km) at 50 $\mu\text{g ml}^{-1}$, colistin (Col) at 10 $\mu\text{g ml}^{-1}$, and ampicillin (Amp) at 200 $\mu\text{g ml}^{-1}$. When necessary, *E. ictaluri* CFU numbers were determined by making triplicate 10-fold serial dilutions in sterile phosphate buffered saline and drop plating 20 μl aliquots on Trypticase soy agar plates supplemented with 5% defibrinated sheep blood for colony counting.

TABLE 2.1. Bacterial strains and plasmids used in Chapter 2

Strain or plasmid	Description	Source or reference
<i>Escherichia coli</i>		
XL1-Blue MRF	(Δ mcrA)183 Δ (mcrCB-hsdSMR-mrr)173 end A1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacI ^q Z M15 Tn5 (Kan ^r)]	Stratagene, La Jolla CA
CC118 λ pir	Δ (ara-leu) araD Δ lacX74 galE galK phoA20 thi-1 rpsE rpoB argE (Am) recA λ pir lysogen	De Lorenzo 1990
SM10 λ pir	(aka Cold Spring Harbor TSM40) C600 derivative thi1 thr1 leuB6 supE44 tonA21 lacY1 recA-::RP4-2Mu KanR	De Lorenzo 1994
<i>Edwardsiella ictaluri</i>		
93-146	WT <i>E.ictaluri</i> strain isolated in 1993 from moribund channel catfish in a natural outbreak of ESC on a commercial farm	Louisiana Aquatic Diagnostic Laboratory
Δ speA::km mutant	93-146 strain with nucleotides 585-1505 of <i>speA</i> deleted and a kanamycin resistance cassette inserted	This paper
Δ speB::km mutant	93-146 strain with nucleotides 55- 779 of <i>speB</i> (agmatinase) deleted and a kanamycin resistance cassette inserted	This paper
Δ ureG::km mutant	93-146 strain with an STM tag L/M insertion in the <i>ureG</i> gene	Thune et al. 2007
Δ adiA::km mutant	93-146 strain with an STM tag L/M insertion in the <i>adiaA</i> gene	Thune et al. 2007
Plasmids		
pBluescript SK (-)	Phagemid cloning vector	Stratagene, La Jolla CA
pBBR1-MCS4	Plasmid cloning vector	Kovach 1995
pUT-miniTn5Km-MCS	pUT-miniTn5Km2 with multiple cloning site containing EcoRV, XbaI, and ApaI restriction enzyme sites	Thune et al. 2007
pRE107	Plasmid suicide vector, pGP704 derivative	Edwards 1998

SPF Channel Catfish. Channel catfish egg masses were obtained from a commercial producer with no history of ESC outbreaks. The eggs were disinfected with 100 ppm free iodine and hatched in closed recirculating systems in the specific pathogen free laboratory at the Louisiana State University School of Veterinary Medicine. Holding systems consist of four 350 liter round fiberglass tanks connected to a 45 liter biological bead filter (Aquaculture Systems Technologies, New Orleans, LA, USA). Water temperature was maintained at $28 \pm 2^{\circ}\text{C}$, and water quality parameters (including total ammonia nitrogen, total nitrate, pH, hardness, and alkalinity) were determined 3 times per week using a Hach kit (Hach company, Loveland, CO, USA). Water quality was adjusted to maintain optimal conditions. Fish were reared on commercial catfish feed at 2% body weight per day until used for HKDM harvest at one to 1.5 kg.

Generation and Edentification of *E.ictaluri* Mutants. The procedure used to produce insertion/deletion mutations of the genes of interest is depicted in figure 2.2 using the primers listed in table 2.2. Briefly, 5' and 3' target sequences were amplified with primers P1-P4, using primer linkers to add *EcoRI* cut sites in P3 and P4. Amplicons were digested with *EcoRI* and ligated to one another. This product was used as a template for primers P1 and P2 to produce a gene deletion with an internal *EcoRI* site. This construct was digested and ligated into pBluescript using *Sall* and *SacI* sites in the P1 and P2 primers. A Tn5 Km resistance cassette (*km*) was cloned from pUT-miniTn5Km-MCS and inserted into the *EcoRI* site of the $\Delta ureG::km$, $\Delta adiA::km$, $\Delta speA::km$, and $\Delta speB::km$ constructs. These were excised from pBluescript using *Sall* and *SacI* and the constructs were ligated into pRE107, a suicide vector for *E. ictaluri* encoding the *sacBI* gene, which is lethal to Gram-negative bacteria in the presence of sucrose (Edwards, et al. 1998). The vectors were conjugated into *E. ictaluri* and mutagenesis proceeded

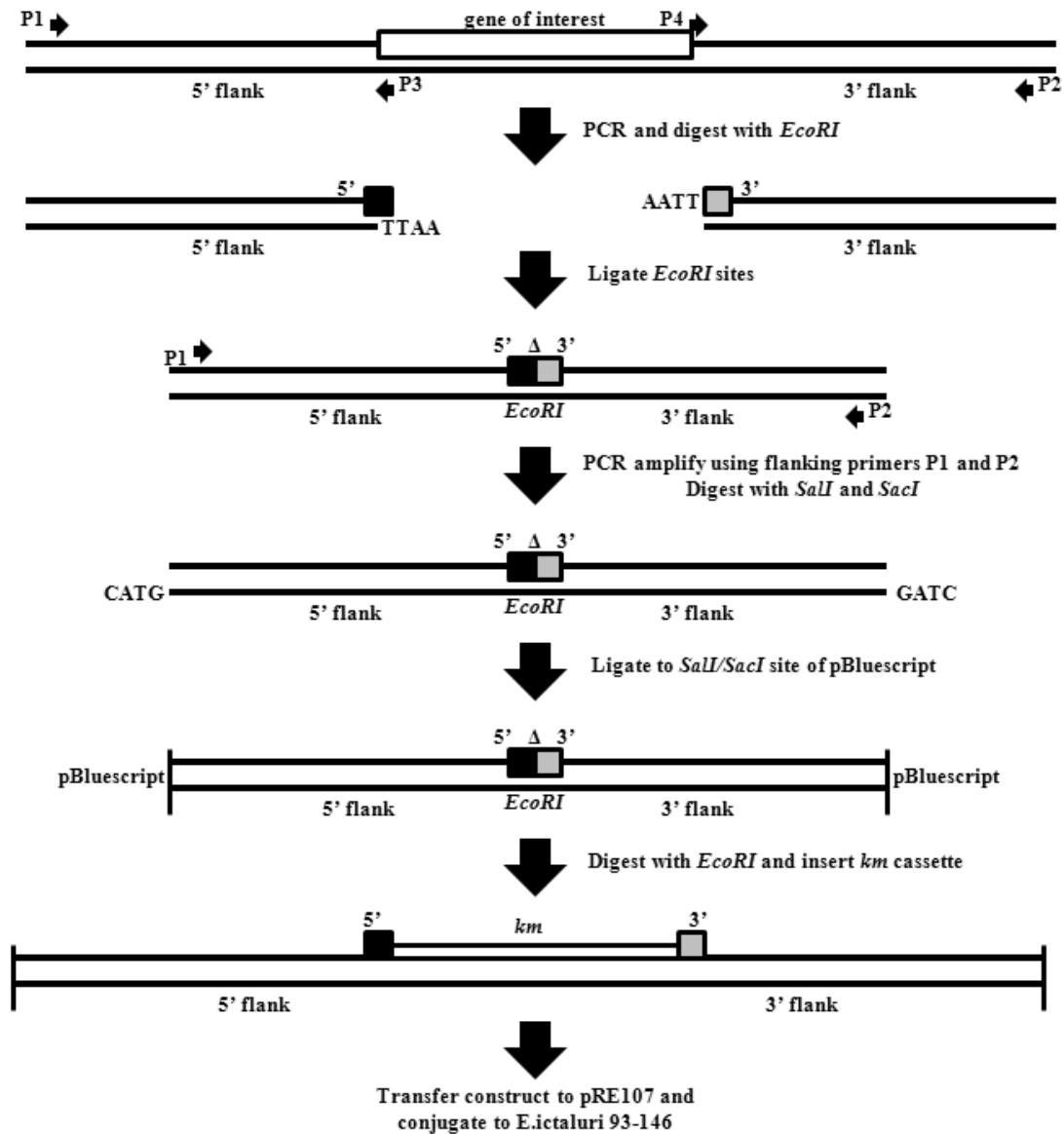


Figure 2.2. Outline of construction of gene knockouts with a kanamycin resistance cassette insertion.

TABLE 2.2. Primers used for mutagenesis in Chapter 2

Name	Primer Sequence 5'-3'	Description
Kan757	5'-TATATAGA <u>AATTC</u> GGAAGCCCTGCAAAGTAAA-3'	Primers for kanamycin
Kan 1635	5'-TATATAGA <u>AATTC</u> GCTCAGAAGAACTCGTCAA-3'	resistance cassette with <i>EcoRI</i> sites
SpeB P1	5'-ATATATG <u>TCGACT</u> GGGCAACATGCATAACCTGTTCG-3'	$\Delta speB::km$ mutant
SpeB P2	5'-ATATATG <u>AGCTC</u> AGAACTCTACGACCTGCAACAGGA-3'	
SpeB P3	5'-ATATATGA <u>AATTC</u> ACCAAAGGCGTTGGATACCAGAGA-3'	
SpeB P4	5'-ATATATGA <u>AATTC</u> TGGCCTGCAGGATCTGGATATTGT-3'	
SpeB shoulder FW	5'-ATTCGGATGGCACCATCGATCACT-3'	Primers for verification
SpeB shoulder RV	5'-GTCATTACCCAGTGGAAACACCGAT-3'	of construct
SpeA P1	5'-ATATATG <u>TCGAC</u> GCCCGTGTCTGATTTGATTGCACA-3'	$\Delta speA::km$ mutant
SpeA P2	5'-ATATATG <u>AGCTC</u> AGTTTCGTTTGGCAGTGCATTTTCGC-3'	
SpeA P3	5'-ATATATGA <u>AATTC</u> TAGCCATTGCAGACGATCACGCTA-3'	
SpeA P4	5'-ATATATGA <u>AATTC</u> TGGCCGATAAGCTGTACGTGAACT-3'	
SpeA shoulder FW	5'-ATGGCTTGGGTGTACCTCAGGATT-3'	Primers for verification
SpeA shoulder RV	5'-ACCAAAGGCGTTGGATACCAGAGA-3'	of construct

Underlined sequences are linkers incorporated into the primers used for cloning.

by allelic exchange. Conjugation cultures were plated onto LB-Man–Amp-Col agar to select for single crossover events (integration of the pRE107- *Δ::km* constructs), and resultant colonies were grown on LB-Man-Col agar and then LB-Man-Col-sucrose (5%) agar to select for a second crossover and removal of the pRE107 backbone. Appropriate recombinants were selected for an Amp^s phenotype and verified by PCR and DNA sequencing. The *ΔureG::kan* and *ΔadiA::kan* mutants (234AB and 84LM) were generated by STM and verified in a previous study (Thune, et al. 2007).

Labeling Bacteria/ Oregon Green/LysoTracker Red. Oregon Green 514 carboxylic acid succinimidyl ester (OG) and LysoTracker Red DND-99 1mM (LTR) were acquired from Invitrogen, Carlsbad, CA, USA. Staining of bacteria by OG was according to a previously described protocol (Porte, et al. 1999). Briefly, late log phase cultures (10⁹ bacteria/ml) were washed twice in sterile saline and suspended in 1 ml of saline with 0.05% Tween 80. Ten microliters of 10 mg/ml OG (reconstituted in dimethylsulfoxide) was added and the sample was vortexed briefly. Incubation for 30 minutes at 4°C in the dark was followed by centrifugation and the addition of Tris-HCl (pH 8.3) to 100 mM final concentration. Resuspension of bacteria and a second 4°C incubation for 15 minutes was followed by 2 washes in saline prior to opsonization with autologous channel catfish serum for 30 minutes at room temperature. Opsonized bacteria were then used for infection experiments. Killed bacteria were produced by washing bacteria twice in saline, followed by incubation at 70°C for 30 minutes; killing was verified by plating on blood agar. LysoTracker Red was diluted according to manufacturer's directions and added to HKDM media at a 30 nM concentration 30 minutes prior to infection. . LysoTracker Red at this concentration was maintained in the culture media until it was changed to saline for microscopy.

Infection and Intracellular Viability of Mutant and OG Stained *E. ictaluri* in HKDM. A

standard gentamicin survival assay using HKDM was used as described by (Booth 2006) to evaluate the ability of wild type *E. ictaluri* (WT), $\Delta adiA::km$, $\Delta speA::km$, and $\Delta speB::km$ mutants, or OG stained WT to enter, survive, and replicate in HKDM . Briefly, adult channel catfish were euthanized via overdose with tricaine methanesulfonate (200 mg/liter) and bled via phlebotomy to exsanguination. The pronephros was removed using sterile procedure and macerated in a tissue sieve (stainless steel mesh 280 and 140 μ m) to collect free cells. Cell viability was determined by Trypan blue exclusion (Booth 2006). Dissociated cells were suspended to a final concentration of 1×10^7 live cells/ml in channel catfish macrophage medium (CCMM) consisting of catfish RPMI [(RPMI 1640 sans phenol red & L-glutamine, Lonza, Walkersville, MD, USA) containing 1x glutamine substitute (GlutaMAX –I CTS, Gibco, Invitrogen Corporation, Carlsbad, CA, USA)], 15 mM HEPES buffer (GIBCO)], with 0.18% sodium bicarbonate solution (GIBCO), 0.05 mM 2-beta-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), and 5% heat inactivated pooled channel catfish serum. Media was diluted to a tonicity of 240 mosmol/kg H₂O by adding 1 part sterile deionized/distilled water. One ml of cell suspension was added to each well of a 24 well plate (Biocoat poly-d-lysine plates, Becton Dickinson Labware, Bedford, MA, USA), allowed to adhere for 16 hours (overnight) at 28°C with 5% CO₂, and washed 3x with catfish RPMI before returning to fresh CCMM for infection. HKDM wells were infected at a multiplicity of infection (MOI) of 1 bacterium: 10 HKDM using bacteria previously opsonized for 30 minutes in autologous serum. Wells were then centrifuged (400 g for 5 minutes) to synchronize infection and incubated for 30 minutes. One hundred μ g/ml gentamicin was added for one hour to kill extracellular bacteria. Cells were washed once in catfish RPMI and placed in CCMM with a static dose of 0.5 μ g/ml gentamicin. After 0, 4, 8, and

10 hours post infection at 28°C with 5% CO₂, HKDM were washed with RPMI and lysed by adding 100 µl of 1% Triton X-100 (Fisher Scientific, Fair Lawn, NJ, USA). Media was serially diluted and plated on blood agar to determine increase from timepoint zero.

Fluorescent Microscopy for Vacuolar pH Determination. The pH sensitivity of OG fluorescence in the weakly acidic range coupled with its fluorescence characteristics ($\lambda_{\text{ex}}=510$ nm peak excitation, $\lambda_{\text{ex}}=450$ nm insensitive) allows ratiometric pH estimation in cellular vacuoles (Invitrogen). When exposed to 510 nm light, OG fluorescent brightness varies with pH, while at 450 nm it is relatively nonreactive. The microscope used exposes OG stained bacteria to 510 nm and 450 nm ultraviolet light in rapid succession and a ratio of the measured fluorescence for any specified object at 510 nm to that at 450 nm can be generated by the software package. Such ratios can then be used for comparative purposes. HKDM were harvested and cultured as described above for the gentamicin assay, except 1×10^7 cells were plated onto 35 mm poly-D-lysine coated, No.1.5 borosilicate German glass bottom dishes, (MatTek, Ashland, MA, USA). HKDM were infected with bacteria at a 10:1 MOI, centrifuged, allowed to settle for 10 minutes, then washed in catfish RPMI 3 times and placed in CCMM with a static gentamicin dose (0.5 µg/ml). Cells used to determine pH calibration data with live imaging were incubated in ionophore calibration solutions while cells used to measure experimental data were incubated in saline for microscopic examination. For calibration, infected HKDM were incubated in 140 mM KCl, 1 mM MgCl₂, 0.2 mM EGTA, 20 mM MES, adjusted to pH 4, 5, and 6 with NaOH and containing 5 µg/ml nigericin and 5 µM monensin to collapse pH gradients across membranes (Demaurex and Grinstein 2006). *In situ* calibration curves were made for each bacterial strain experiment for each day and each experiment was repeated 3 times, each on different days with different fish and bacterial cultures. Using the same dish for all pH calibrations, HKDM were

bathed for 8 minutes in each solution and multiple images were acquired using a dual exciter filter (Chroma) to generate ratiometric data before changing to the next pH solution. For experimental data a separate cell dish was incubated in saline and was imaged similarly, 60-80 minutes post infection. For each image, background was identified as the grey/black level corresponding to areas devoid of cells and biological material and was subtracted from the image prior to ratio data gathering. Individual bacteria were chosen manually from images according to proximity within the confines of the HKDM cell membranes, identifiability as bacteria (shape, size), being separate from other highly reactive cellular fluorescent material (including tightly clustered bacteria or superimposed structures), and being in relative focus. A ratio was thus generated for each bacterium by the computer software. Approximately 50-100 bacteria were chosen for each group for each experiment (pH 4, 5, 6 and experimental groups).

For LTR, cells were processed as for OG, except 30 nM LTR was added to the dish 60 minutes prior to infection and remained until immediately prior to imaging. An additional filter (Zeiss 64 HE) was used for the appropriate ultraviolet range. One hundred bacteria were counted from images taken at 60 minutes post infection, and the proportion of those stained red was assessed by contrast and brightness adjustments for the appropriate channel.

Cells were visualized with a Zeiss Observer.Z1 microscope with CO₂Module S and TempModule S (Carl Zeiss MicroImaging GmbH, Jena, Germany) using a 63X oil objective in a stage insert (Heating Insert P, PeCon GmbH, Erbach, Germany) equipped to supply humidified CO₂ and maintain temperature at 5% and 28°C, respectively. Illumination was provided by a Lambda DG-4, 175 Watt Xenon arc lamp (Sutter Instrument Co., Novato, CA, USA). Cubes used are as follows: Chroma 71001 dual exciter (green: 440/20, 495/10), 535/25 emitter (Chroma Technology Corporation, Bellows Falls, VT, USA) and Zeiss filter set 64 HE exciter 587/25,

emitter 647/70 (red: Carl Zeiss, Germany). Digital images were captured using Zeiss AxioVision software version 4.8.1 and analyzed with the Physiology Acquisition Module (Carl Zeiss, Germany).

Statistical Analysis. Data for gentamycin survival assays in HKDM were determined by analysis of variance (ANOVA) using the General Linear Methods Procedure (Proc GLM; SAS 9.3, SAS Institute Inc. 2011). Fold increase was determined by dividing the CFU/well from timepoints past time zero by the mean CFU/well at time zero for each strain. Mean fold replication was then calculated with standard errors of the means. When the model indicated significance at $P \leq 0.05$, a least square means procedure was used for pairwise comparison of interaction effects.

RESULTS

Generation of $\Delta speA::km$ and $\Delta speB::km$ Mutants. Mutations in *E. ictaluri* $\Delta speA::km$ and $\Delta speB::km$ were made by allelic exchange (Figure 2.2). An internal portion was deleted and a kanamycin (Km) resistance gene cassette was inserted, yielding $\Delta speA::km$ and $\Delta speB::km$ *E. ictaluri*. Both *speA* and *speB* are present sequentially on the same DNA strand, separated by 210 bases, and are oriented in the same direction with separate -10 and -35 putative promoter sites. There are no known genes associated with them on either strand, so mutation does not cause polarity issues. Mutation of *speA* resulted in removal of amino acids 195-502 from the sequence, producing a truncated protein of 673 amino acids, with the central 307 amino acids replaced by a 299 amino acid Km cassette, leaving native shoulders of 195 and 179 amino acids. Mutation of *speB* resulted in removal of amino acids 18-260 from the sequence, producing a 366 amino acid

protein with the central 242 amino acids removed and replaced by a 303 amino acid Km cassette, leaving native shoulders of 18 and 45 amino acids.

Intracellular Replication in Channel Catfish Macrophages. Survival and replication of OG stained WT, $\Delta adiA::km$, $\Delta speA::km$, and $\Delta speB::km$ versus WT in channel catfish HKDM, as determined by the gentamycin survival assay, are presented in Figures 2.3, 2.4, and 2.5, respectively. The WT strain and OG stained WT increased similarly at 4, 8 and 10 h postinfection and fold increase in replication was not significantly different at any timepoint (Figure 2.3). The $\Delta adiA::km$ mutant showed significantly attenuated replication in HKDM compared to WT at 8 and 12 hours (Figure 2.4). Disruption of *speA* or *speB*, like OG stained WT, did not have a significant effect on initial uptake or replication of *E. ictaluri* in HKDM compared to WT (Figure 2.5).

In vivo Determination of Bacterial Membrane pH in HKDM. Staining of bacteria enabled visualization within macrophages (Figure 2.6). OG staining clearly outlined bacterial membranes. In addition, multifocally, OG staining was diffuse within irregular vacuoles and phagosomes; such organelles often contained bacteria or bacterial remnants. In LTR stained HKDM, bacteria that experienced environmental acidification were red/pink (Figure 2.7). These data show acidification and degradation/killing of *E. ictaluri* in HKDM phagosomes.

WT (*E. ictaluri*). The pH of vacuoles containing WT at 1 hour post-infection is represented in Figures 2.8 and 2.9. For the majority of WT, pH was significantly higher than pH 6. In figure 2.8, it can be seen that there is a relatively wide spread of data in the Exp group, indicating that WT in HKDM experience a very broad pH range, in some cases well above pH 6 but also equal to pH 4. Because infection was synchronized, this suggests that there is marked heterogeneity in

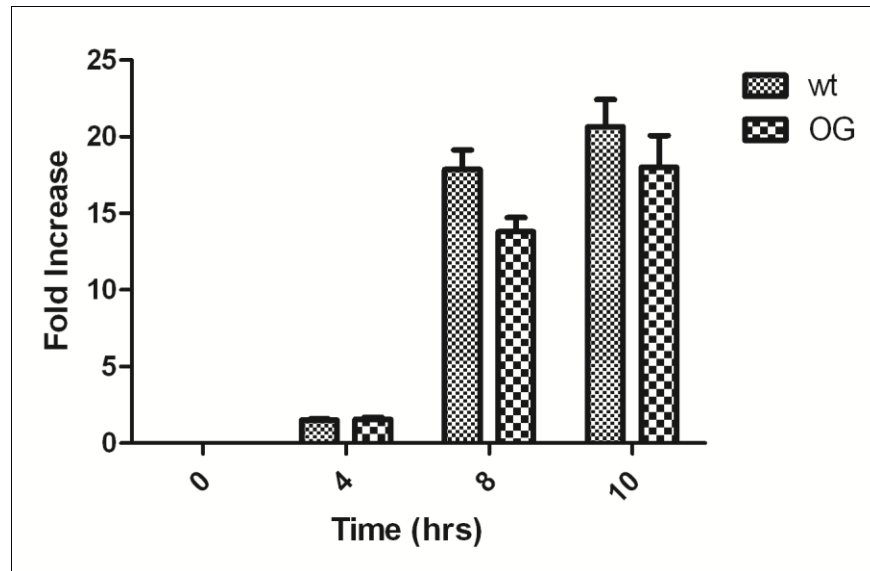


Figure 2.3. Intracellular survival and replication of WT and OG stained WT (OG) in channel catfish HKDM. WT and OG increased approximately 20 fold by 10 hours post infection. Results for WT and OG were not significantly different from one another at any timepoint ($P \leq 0.05$). Results are presented as means and standard errors of the means for 3 wells per treatment and are representative of 3 replicate experiments.

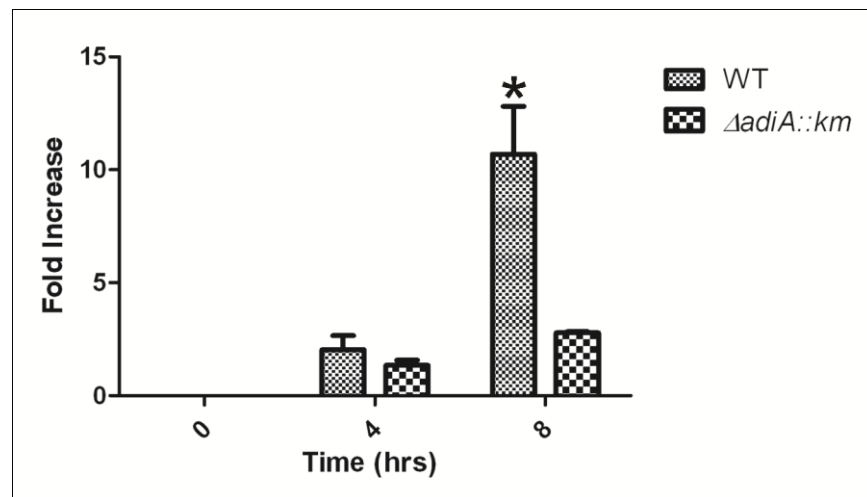


Figure 2.4. Intracellular survival and replication of WT and $\Delta adiA::km$ in channel catfish HKDM. WT increased approximately 10 fold by 8 and 12 hours post infection while $\Delta adiA::km$ replication did not increase significantly. (*) indicates $P \leq 0.01$ versus WT 4 hour and all $\Delta adiA::km$ timepoints. Results are presented as means and standard errors of the means for 3 wells per treatment and are representative of 2 replicate experiments.

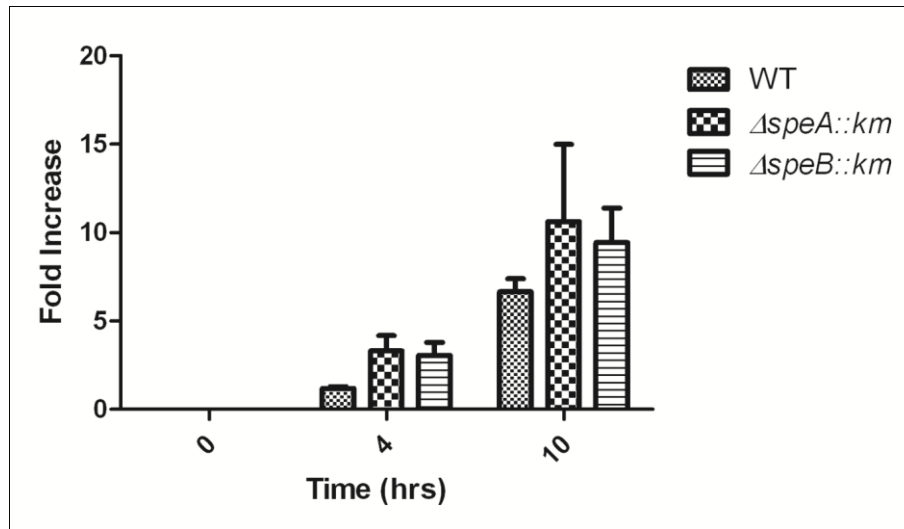


Figure 2.5. Intracellular survival and replication of WT and $\Delta speA::km$ and $\Delta speB::km$ in channel catfish HKDM. WT and mutants increased approximately 7-10 fold by 10 hours post infection. Results for WT and mutants were not significantly different from one another at any timepoint ($P \leq 0.05$). Results are presented as means and standard errors of the means for 3 wells per treatment and are representative of 3 replicate experiments.

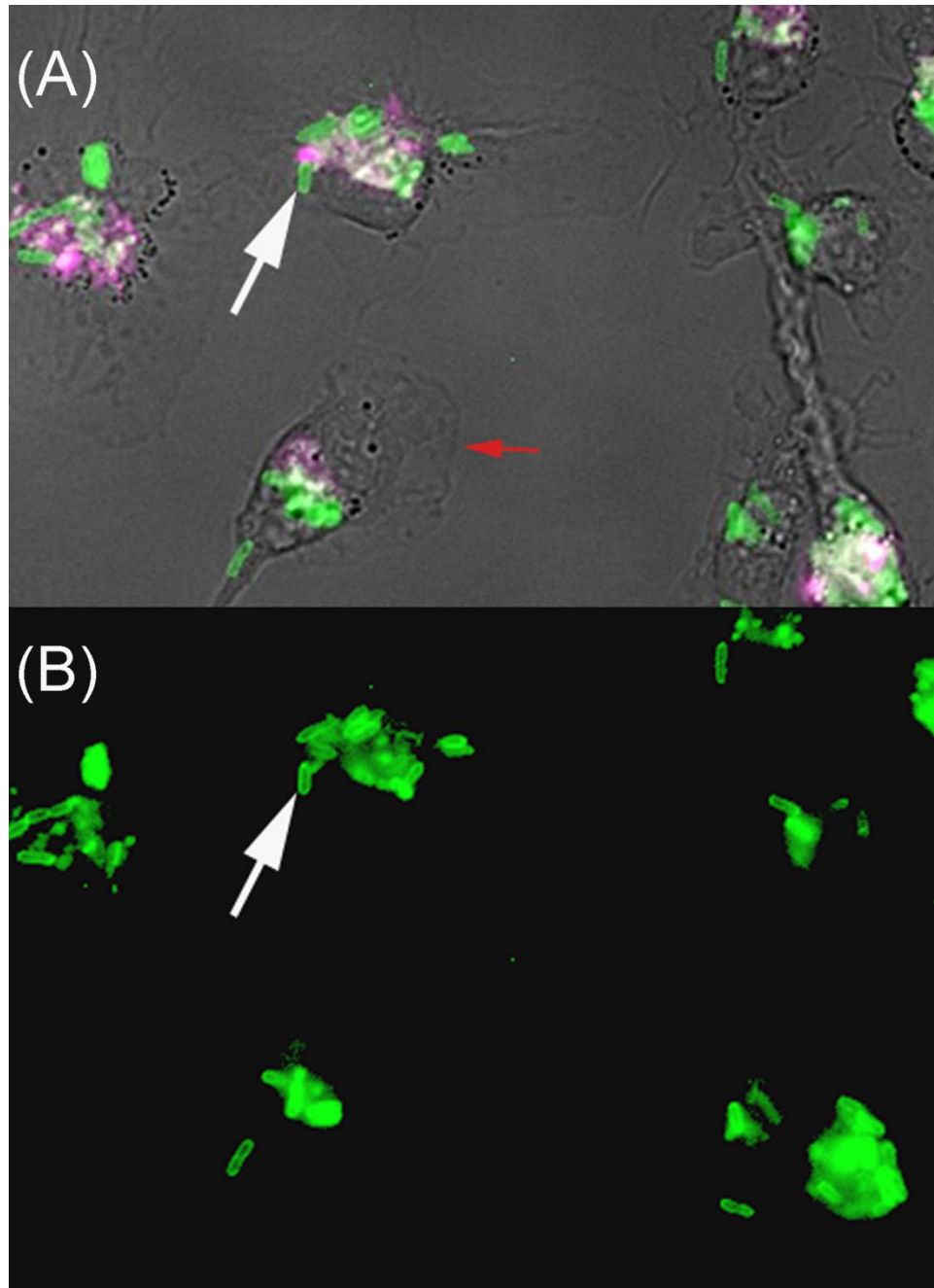


Figure 2.6. Photomicrographs of live HKDM infected with WT *E. ictaluri*, 1 hour post infection stained with Oregon green and LysoTracker Red. (A) Individual macrophages (red arrow) contain numerous OG stained bacteria. In close association, but distinct from WT, are multiple pink vacuoles containing LTR, which indicates acidification. Three light channels were used; Nomarski (white), green, and red. (B) Same image as A, with only the green channel. White arrow shows individual bacteria outlined by the OG staining procedure.

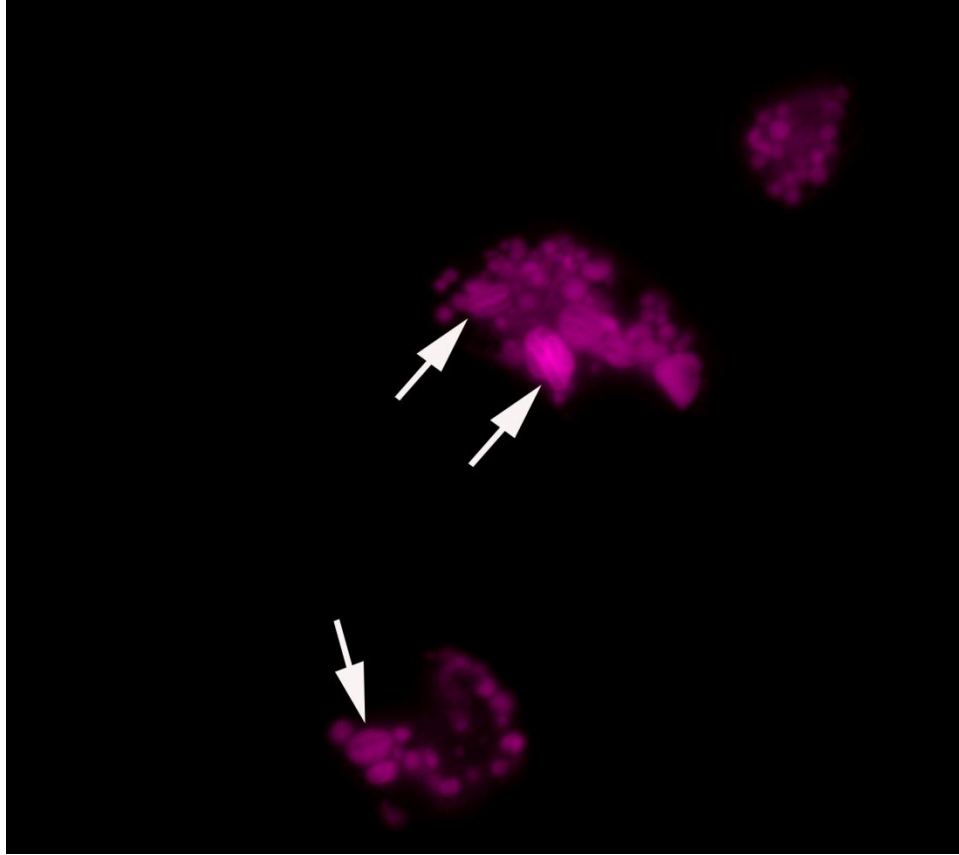


Figure 2.7. Photomicrograph of live HKDM infected with WT *E. ictaluri*, 1 hour post infection stained with LTR. Two macrophages are present, each containing bacteria. LTR fluoresces red when acidified. White arrows show bacteria in acidified environments. Additionally, several varisized irregular vacuoles show acidification.

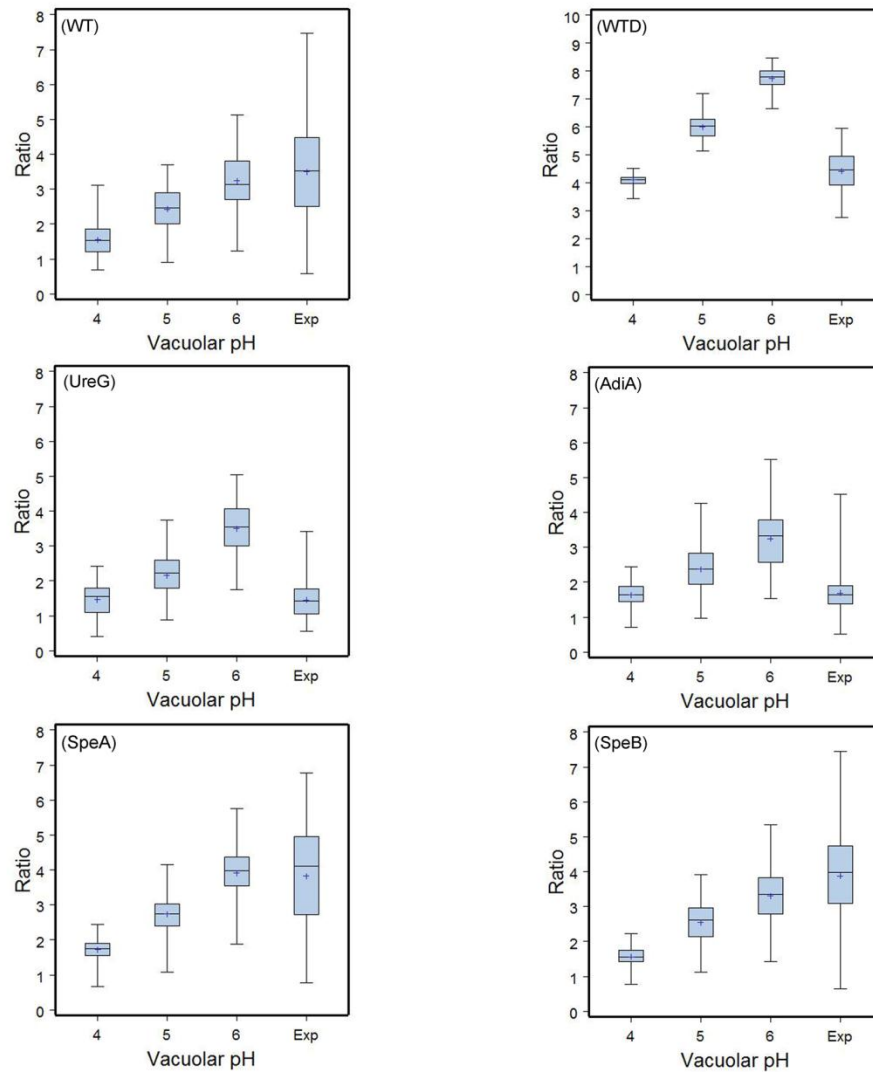


Figure 2.8. Intracellular determination of vacuolar pH in *E. ictaluri* infected channel catfish HKDM one hour post infection using fluorescent bacterial cell membrane staining (box-whisker plots). Infecting strains are designated as: wild type (WT), heat killed WT (WTD), $\Delta ureG::km$, $\Delta adiA::km$, $\Delta speA::km$, and $\Delta speB::km$. The box-whisker plots showing data range (whiskers), interquartile range (the mid 50% of data points, in blue), and the mean (designated +). Vacuolar pH at 4, 5, and 6 represents the artificial manipulation of phagosome pH by using ionophore calibration solutions to generate fluorescent ratio values of intraphagosomal bacteria at pH 4, 5, and 6. “Exp” represents the measurement of fluorescent ratios of bacterial cell membranes when macrophages are bathed in saline and phagosomal pH is not artificially altered. Ratio is generated by microscope software for relative comparisons and is based on the fluorescence characteristics of OG 514. Results are combined from 3 replicate studies.

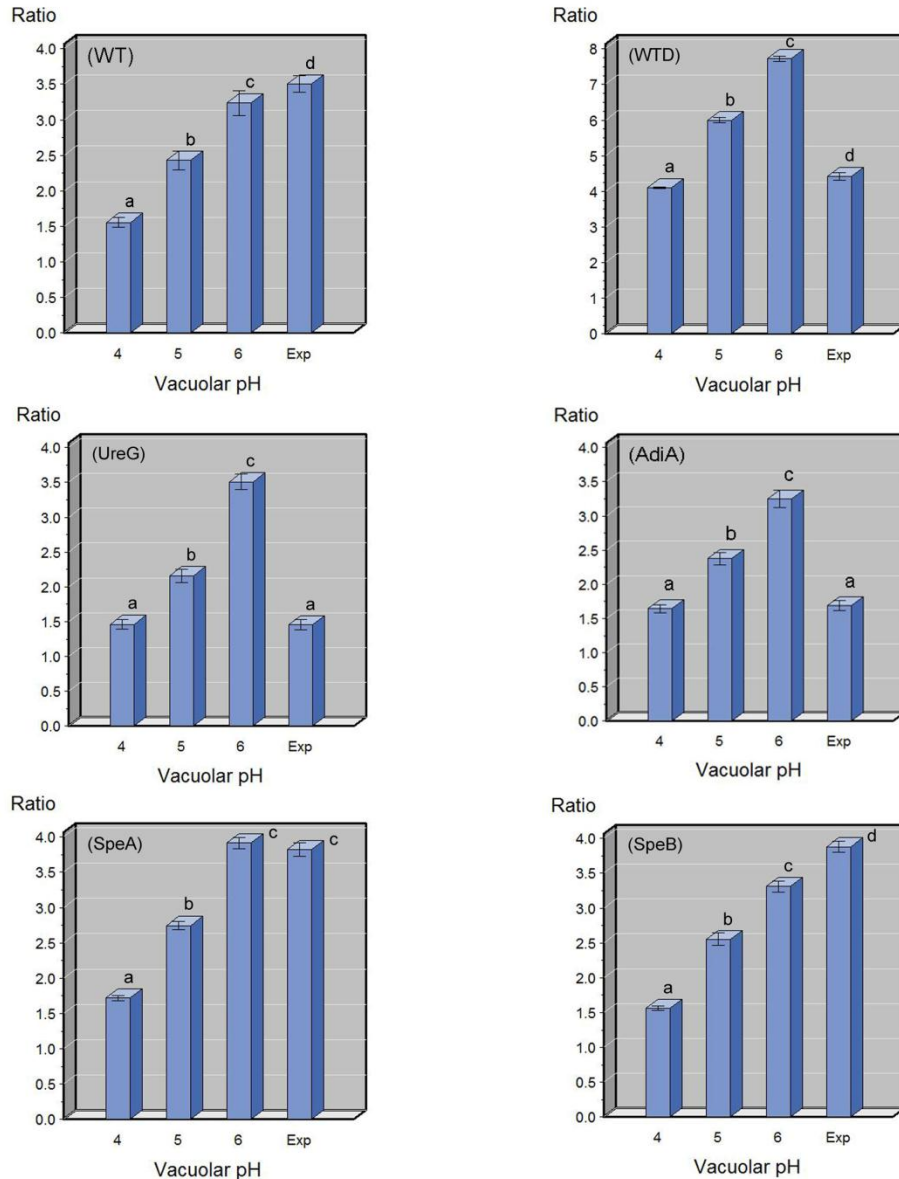


Figure 2.9. Intracellular determination of vacuolar pH in *E. ictaluri* infected channel catfish HKDM one hour post infection using fluorescent bacterial cell membrane staining (bar graphs). Infecting strains are designated as: wild type (WT), heat killed WT (WTD), $\Delta ureG::km$, $\Delta adiA::km$, $\Delta speA::km$, and $\Delta speB::km$. The same data as figure 6, presented as means and standard errors of the means (bars). Different letters atop columns designate significant differences in means. Vacuolar pH at 4, 5, and 6 represents the artificial manipulation of phagosome pH by using ionophore calibration solutions to generate fluorescent ratio values of intraphagosomal bacteria at pH 4, 5, and 6. “Exp” represents the measurement of fluorescent ratios of bacterial cell membranes when macrophages are bathed in saline and phagosomal pH is not artificially altered. Ratio is generated by microscope software for relative comparisons and is based on the fluorescence characteristics of OG 514. Results are combined from 3 replicate studies.

successful phagosome pH regulation by *E. ictaluri*, possibly based on the relative maturation of the macrophage. LTR staining of bacteria supported OG data, finding that only 22% had acidified (Table 2.3).

Dead WT *E. ictaluri*. By one hour post infection, the majority of bacteria had been digested, based on the abundant presence of varisized diffusely stained OG filled vacuoles and irregular OG stained bacteria remnants. Of the dead bacteria that were readily recognizable, they were often closely aggregated into large bundles within phagosomes. The majority of vacuoles containing dead bacteria had a pH between 4 and 5, which is in agreement with other studies concerning typical phagolysosome acidity (Mukherjee and Maxfield 2009). Likewise, LTR staining found that the vacuoles containing dead bacteria were acidified (Table 2.3). Bacterial killing was verified by plating bacterial stock.

***E. ictaluri* mutants.** The pH experienced by $\Delta ureG::km$ in macrophages at 1 hour post infection is represented in Figures 2.8 and 2.9. Unlike WT, the pH of vacuoles containing $\Delta ureG::km$ was not significantly different than pH 4. From the box whisker plots in A, it can be seen that there are multiple vacuoles with ratios that are consistent with pH 5 and 6. This suggests that while many vacuoles are highly acidified, other vacuoles maintain a pH that is only moderately acidified. LTR data showed that almost all bacteria were in acidified vacuoles by 1 hour post infection.

The vacuolar pH for $\Delta adiA::km$ is similar to that of $\Delta ureG::km$. Not only were the $\Delta adiA::km$ pH ratios not significantly different from pH 4, but the whisker range also indicates that multiple bacteria containing vacuoles had neutral pH levels. LTR data also indicates the

TABLE 2.3. LysoTracker Red Assay for Acidification Results

Name	<u>% Bacteria Acidified</u>
93-146 wild type <i>E. ictaluri</i>	22
93-146 wild type <i>E. ictaluri</i> heat killed	100
$\Delta ureG::km$	99
$\Delta adiA::km$	100
$\Delta speA::km$	12
$\Delta speB::km$	18

vacuoles of almost all *ΔadiA::km* bacteria were acidified. Vacuolar pH for *ΔspeA::km* is not significantly different than pH6.

DISCUSSION

The maintenance of appropriate phagosomal pH is vital throughout phagosome maturation in order to properly assemble a functional microbicidal organelle (Mukherjee and Maxfield 2009). Some pathogens have the ability survive in macrophages by circumventing their normal microbicidal responses and are termed intracellular pathogens. Various strategies to achieve this effect are found, including phagosomal escape, phagosome maturation inhibition, misdirection of phagosome maturation, and survival within acidified phagolysosomes (Huynh and Grinstein 2007; Stavru, et al. 2011; Thompson and Holden 2009). Modulation of phagosomal pH is a tactic employed by *H. pylori*, *Mycobacterium tuberculosis*, and *Legionella pneumophila* to inhibit or alter phagosome maturation for the pathogen's benefit (Huynh and Grinstein 2007; Stavru, et al. 2011).

Of these, *H. pylori* is known to have extracellular and intracellular acid survival/acclimation mechanisms where urease function is a vital feature. Bacterial ureases are operon encoded, multiprotein enzymes that hydrolyze urea to ammonia and carbamate, the latter of which spontaneously decomposes into more ammonia and carbonic acid. In *H. pylori* the cytoplasmic urease, in conjunction with an inner membrane histidine kinase, an acid activated urea channel, and 2 carbonic anhydrases, allow the bacterium to maintain a neutral cytoplasmic pH and near neutral periplasmic pH in 2 main ways: (i) the ammonia produced rapidly consumes protons in the cytoplasm and periplasm, and (ii) the carbonic acid is converted to bicarbonate in the periplasm (Scott, et al. 2010). In this way, *H. pylori* not only can thrive in stomach acid, but

also within altered macrophage phagosomes called megasomes, which are dependent on urease expression. It is estimated, however, that only 1% of the bacteria enter the lamina propria and become intracellular (Schwartz and Allen 2006).

Edwardsiella ictaluri is a natural pathogen of channel catfish and is able to replicate in channel catfish macrophages (Booth, et al. 2006). *E. ictaluri* also encodes an acid activated urease that is necessary for replication in HKDM and for virulence *in vivo* (Booth, et al. 2009). This urease is able to generate ammonia sufficient to increase environmental pH from 5 to over 7 in broth cultures. Based on these data, it was hypothesized that the *E. ictaluri* urease functions to neutralize phagosomal pH in catfish macrophages in a similar fashion (Booth, et al. 2009). The data in this study confirms a role for urease in regulation of phagosomal pH. Wild type *E. ictaluri* is able to maintain an intraphagosomal pH above 6 for at least an hour post infection, while phagosomes containing a urease mutant or heat killed WT bacteria acidified to below 5. These findings are in accordance with previous data reporting the ability of *E. ictaluri* to replicate within macrophages and the necessity of urease for intracellular replication. Measurements also show that some vacuoles containing urease mutants maintain a pH in the 5-6 range (Figure 2.7), possibly explaining the low grade replication of the urease mutant in HKDM (Booth, et al. 2009).

While it is known that some pathogens, such as *Salmonella* and *Legionella monocytogenes*, require phagosome acidification for optimal upregulation of virulence mechanisms, whether or not this occurs in *E. ictaluri* it is not yet clear (Beauregard, et al. 1997; Mukherjee and Maxfield 2009). Based on the acid mediated upregulation of the *E. ictaluri* type III secretion system (T3SS), and the type VI secretion system, as well as the acid activation of the urease at low pH, it is likely that an initial acidification occurs in order to initiate activity of

these virulence factors (Booth, et al. 2009; Rogge and Thune 2011). Further studies are needed to determine if initial acidification is required for *E. ictaluri* HKDM replication, and to understand the progression of phagosomal pH change over time.

Similar to urease, our experiments indicate that AdiA is also important for *E. ictaluri* phagosome pH regulation. The $\Delta adiA::km$ mutant failed to prevent phagosome acidification (Figure 2.9) and like the urease mutant can survive, but cannot replicate, in HKDM. This may be due to the ability of AdiA to function in acid resistance by the removal of carboxyl groups from arginine in a reaction that consumes protons and produces carbon dioxide (which can be converted to bicarbonate) as a byproduct (Viala, et al. 2011). In *Salmonella* serovar Typhimurium, AdiA promotes acid survival most efficiently at pH 2.3, but confers little advantage at pH 4.5 where ornithine and lysine decarboxylases are more efficient (Viala, et al. 2011). In *E. ictaluri*, the role AdiA may have in acid resistance is not clear, but the acid activated urease is upregulated at pH 5 and can produce ammonia to neutralize acidity at pH 5 exposure, but not 4.5. By using urease and AdiA in tandem *E. ictaluri* may, like *H. pylori*, have 2 different acid resistance mechanisms to respond to differing degrees of acidity (Scott, et al. 2010).

SpeA is the second arginine decarboxylase in *E. ictaluri* and genetic deletion showed little evidence that HKDM replication or phagosome pH regulation was affected. The phagosomal pH measured by OG in Figure 2.9 shows bacterial pH is not significantly different than pH 6, which indicates that SpeA does not contribute to the regulation of vacuolar pH. The $\Delta speA::km$ mutant can also replicate in catfish HKDM like WT (Figure 2.4) and LTR staining indicates acidification similar to WT. Therefore it is likely that SpeA is a biosynthetic decarboxylase that functions at a more neutral pH, while AdiA is an acid upregulated

decarboxylase, similar to *Salmonella* serovar Typhimurium, *E. coli*, and others (Viala, et al. 2011). Therefore, loss of SpeA would not be expected to affect intracellular replication.

Agmatinase, like SpeA, was not found to be involved in intracellular virulence in these experiments. The intracellular pH of the *ΔspeB::km* mutant was significantly higher than pH 6 and replication in catfish macrophages was similar to WT (Figure 2.10). This indicates that the source of urea for *E. ictaluri*'s urease is elsewhere and the most likely source is the catfish encoded arginase (Booth, et al. 2009). Arginases are trimeric manganese metalloenzymes found in prokaryotes and eukaryotes that consume arginine to produce urea and ornithine (Peranzoni, et al. 2008). *Helicobacter pylori* can utilize its own encoded arginase to produce urea, but arginase is not present in the *E. ictaluri* genome, so macrophage arginase would be the most likely source of urea. In macrophages, arginase upregulation and function are associated with alternative macrophage activation, a state in which tissue regeneration, cell proliferation, and a reduction in inflammation are enhanced (Grillo and Colombatto 2004; Munder 2009). In terms of microbe-macrophage interaction after phagocytosis, arginase and nitric oxide synthase (NOS) compete for available arginine. This competition can result in the dysregulation of phagocyte antimicrobial mechanisms, most directly through a reduction in NO production (Gobert, et al. 2001). While *H. pylori* produces urea for itself via its own arginase, in the case of *E. ictaluri* the upregulation of macrophage arginase would serve this purpose; providing substrate for a phagosome neutralizing urease as well as preventing macrophage NO formation. This situation has been hypothesized to occur with *Mycobacterium bovis* BCG and *H. pylori* (Schwartz and Allen 2006; Sendide, et al. 2004).

In conclusion, *E. ictaluri* utilizes urease and AdiA to neutralize phagosomal pH and enable replication in channel catfish HKDM (Collins and Thune 1996). Furthermore, a

secondary arginine decarboxylase (SpeA) and agmatinase (SpeB) are not associated with phagosome pH neutralization and are not required for replication or survival in HKDM. Therefore it is likely that the macrophage arginase is the source of the urea utilized by *E. ictaluri* for vacuolar pH regulation and intracellular replication (Figure 2.10).

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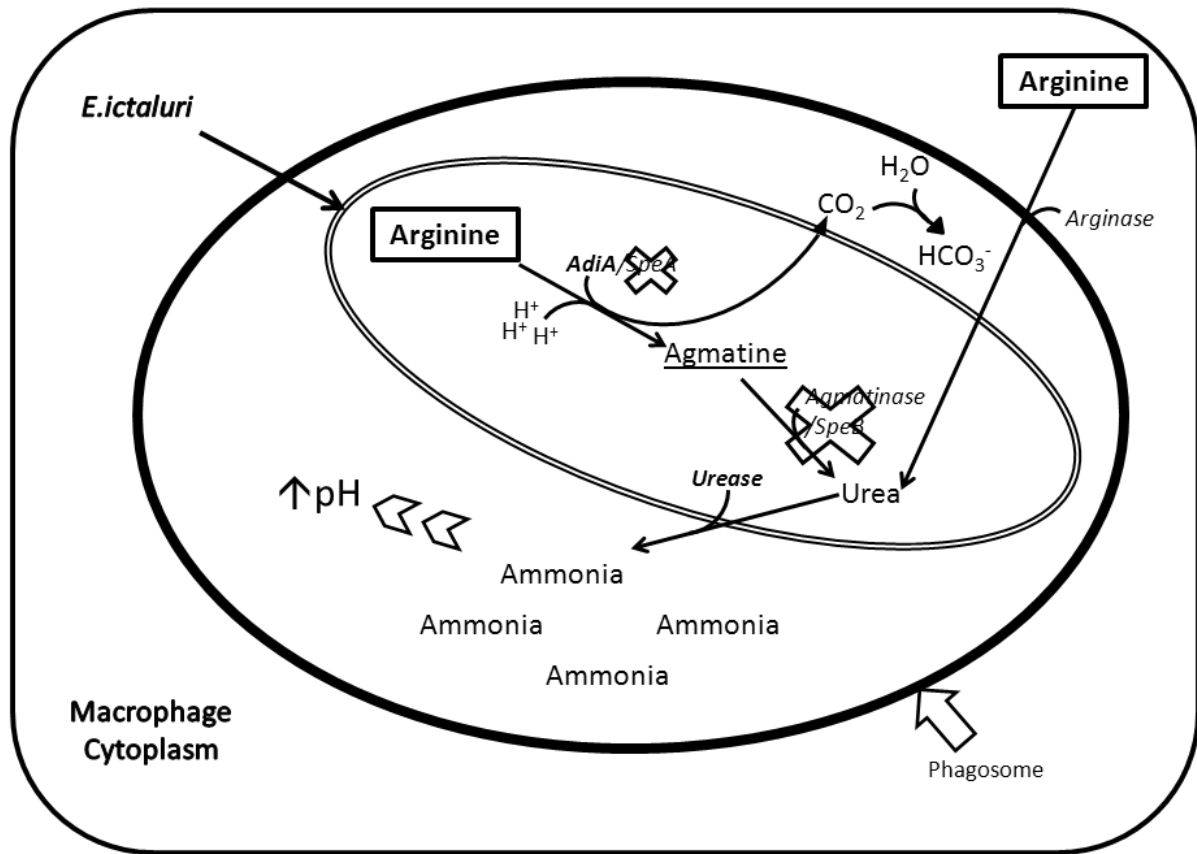


Figure 2.10. Revised model for arginine utilization by *E. ictaluri* in HKDM phagosomes. Arginine is utilized by *AdiA* to produce agmatine and carbon dioxide. Macrophage arginase converts arginine to urea. Urease converts urea to ammonia, which consumes protons and neutralizes *E. ictaluri* phagosomal pH.

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CHAPTER 3

THE METABOLISM OF ARGININE IN *EDWARDSIELLA ICTALURI* INFECTED CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) MACROPHAGES

INTRODUCTION

Edwardsiella ictaluri is a Gram-negative bacterium and the etiological agent of enteric septicemia of catfish (ESC), arguably the most important disease of farmed channel catfish (*Ictalurus punctatus* Rafinesque, 1818) since its first report from diseased fish in 1976 (Hawke 1979). The fulminant form of ESC is a septicemia characterized by rapid infection of internal organs such as the liver, spleen, metanephros (kidney), and pronephros (head kidney) by invasion through the gut or gills (Baldwin and Newton 1993; Nusbaum and Morrison 1996; Thune, et al. 1993; USDA 2009a; USDA 2009b). By the time clinical and gross signs are evident, there is widespread granulomatous inflammation characterized by coalescing to diffuse macrophage infiltration in multiple organs, particularly the liver, spleen, and kidneys (Blazer, et al. 1985; Shotts, et al. 1986). The pronephros is one of the first organs infected by *E. ictaluri* and can be infected as early as 15 minutes postinoculation and remain infected throughout the course of the disease (Baldwin and Newton 1993). This organ is the functional equivalent of mammalian bone marrow where myeloid progenitors, monocytes, and mature macrophages exhibit varying levels of phagocytic and microbicidal capabilities (Belosevic, et al. 2006). Since *E. ictaluri* is able to infect, survive, and replicate in pronephros derived macrophages (HKDM) harvested from naive *I. punctatus*, this cell type may serve as a replicative niche *in vivo* (Booth, et al. 2006).

Edwardsiella ictaluri is known to have an acid activated urease that is necessary for HKDM replication and phagosome pH neutralization (Booth, et al. 2009, this work). *In vitro*,

this urease is able to produce sufficient ammonia to alter environmental pH from 5 to 7 (Booth, et al. 2009). Furthermore, *E. ictaluri* encodes an arginine decarboxylase (AdiA) that is also required for HKDM replication and for phagosome pH neutralization (this work). AdiA acts to produce agmatine and carbon dioxide from arginine, and agmatine can be further broken down to urea and putrescine by agmatinase (Lu 2006). Previous work, however, indicates that agmatinase is not involved in HKDM replication and is therefore not an important source of urea for *E. ictaluri* urease function in HKDM. Although agmatine deiminase (*aguA*) is a potential source of ammonia that may contribute to pH regulation, BLAST analysis with AguA protein sequences from *Aeromonas hydrophila* subsp. *hydrophila* (GenBank: ABK36295.1), *Yersinia enterocolitica* subsp. *enterocolitica* (NCBI ref. YP_001007597.2), *Francisella novicida* (GenBank: AEB28349.1), and *Serratia proteamaculans* (GenBank: ABV41583.1) indicates that it is not present in the *E. ictaluri* genome. Since *E. ictaluri* doesn't encode arginase, the most likely source of urea is the macrophage arginase (Booth, et al. 2009).

Arginase is a trimeric manganese metalloenzyme that hydrolyzes arginine to urea and ornithine (Peranzoni, et al. 2008). In myeloid cells the main enzyme competing for available arginine is nitric oxide synthase (NOS), which converts arginine to nitric oxide (NO) and L-citrulline (Munder 2009). Nitric oxide synthase is a high output enzyme that has 4 major antimicrobial functions; toxic effects directly attributable to NO, negation/frustration of microbial virulence mechanisms, NOS dependent effects aside from NO, and immunostimulation by NO (Bogdan 2009). Nitric oxide synthase activity also depends on arginine availability; deficiency leads to decreased NO through substrate depletion but also leads to reduced protein expression (Bogdan 2009; Chaturvedi, et al. 2007; Pautz, et al. 2010).

The interplay between arginase and NOS may partly regulate the outcome of microbial infections due to their association with different macrophage activation states and inflammatory responses. For optimal function, macrophages must be *activated*, which is to say that they exhibit enhanced antimicrobial and antineoplastic capacities. This activation is conceptually split into 2 possible pathways, namely *classical* and *alternative* (Zhang and Mosser 2009). Classically activated macrophages exhibit increased microbicidal and pro-inflammatory capacities that are associated with interferon gamma (IFN- γ), interleukins (IL) 1, 12, and 23, and tumor necrosis factor alpha (TNF- α). They are also associated more broadly with a T cell helper type 1 (Th1) immune response, which is involved with cell-mediated immunity and antimicrobial responses like increased NO production (Gaffen and Hajishengallis 2008; Zhang and Mosser 2009). Alternatively activated macrophages are associated with a different cytokine subset which includes IL-4, IL-5, IL-10, and IL-13. This activation status is correlated with a Th2 immune response, characterized by increased arginase activity, weak NO production, strong antibody production, antiparasitic inflammatory reactions, and inhibition of certain microbicidal functions (Grillo and Colomatto 2004; Munder 2009; Zhang and Mosser 2009). This polarization of arginine utilization by NOS and arginase within macrophages leads to substrate limitation, altered NO production, and pro-inflammatory or anti-inflammatory tissue environments (Chang, et al. 1998; Munder 2009).

Pathogenic microbes can take advantage of this situation primarily through arginase modulation to regulate or counteract the antimicrobial effects of NO (Bogdan 2009; Chang, et al. 1998; Gobert, et al. 2001). *Helicobacter pylori* can use its own arginase, RocF, to deplete extracellular arginine, leading to reduced NO production by co-cultured macrophages (Chaturvedi, et al. 2007; Gobert, et al. 2001). Alternatively, *H. pylori* can upregulate macrophage

arginase to induce apoptosis (Gobert, et al. 2002). *Salmonella* serovar Typhimurium can also upregulate host macrophage arginase, leading to antagonism of NOS, reduction of NO, and maintenance of virulence *in vivo* (Lahiri, et al. 2008).

Since *E. ictaluri* requires urease activity for intracellular replication, there must be a source of urea for *E. ictaluri* in the phagosome. Possibilities include the production of urea from arginine via the activity of bacterial arginine decarboxylase and agmatinase, or from the activity of arginase encoded by HKDM. In either situation, arginine is the source of urea production. Examination of arginine metabolism in infected HKDM led to the identification of 3 possible enzymes involved in arginine metabolism; macrophage NOS, macrophage arginase, and *E. ictaluri* AdiA (Figure 3.1). Depending on the modulation of arginine catabolism by *E. ictaluri* and the activation status of the macrophage, the majority of arginine may be diverted to NOS or arginase. If arginine is primarily made available to NOS then NO production increases, leading to reactive nitrogen species production and antimicrobial activity. Contrariwise, if arginine is primarily made available to arginase, urea and ornithine production increases, leading to increased polyamine (anti-inflammatory molecules) production and urea for the *E. ictaluri* urease.

Consequently, primary objectives of this study were to better understand the overall regulation of arginine in this model by examining NO production, urea production, and the contribution of arginase to the pathogenesis of *E. ictaluri* in HKDM.

MATERIALS & METHODS

Bacterial Strains and Growth Conditions. Bacterial strains and plasmids are listed in Table 3.1. Unless otherwise noted, *Escherichia coli* was grown in Luria-Bertani broth (LB) at 37°C

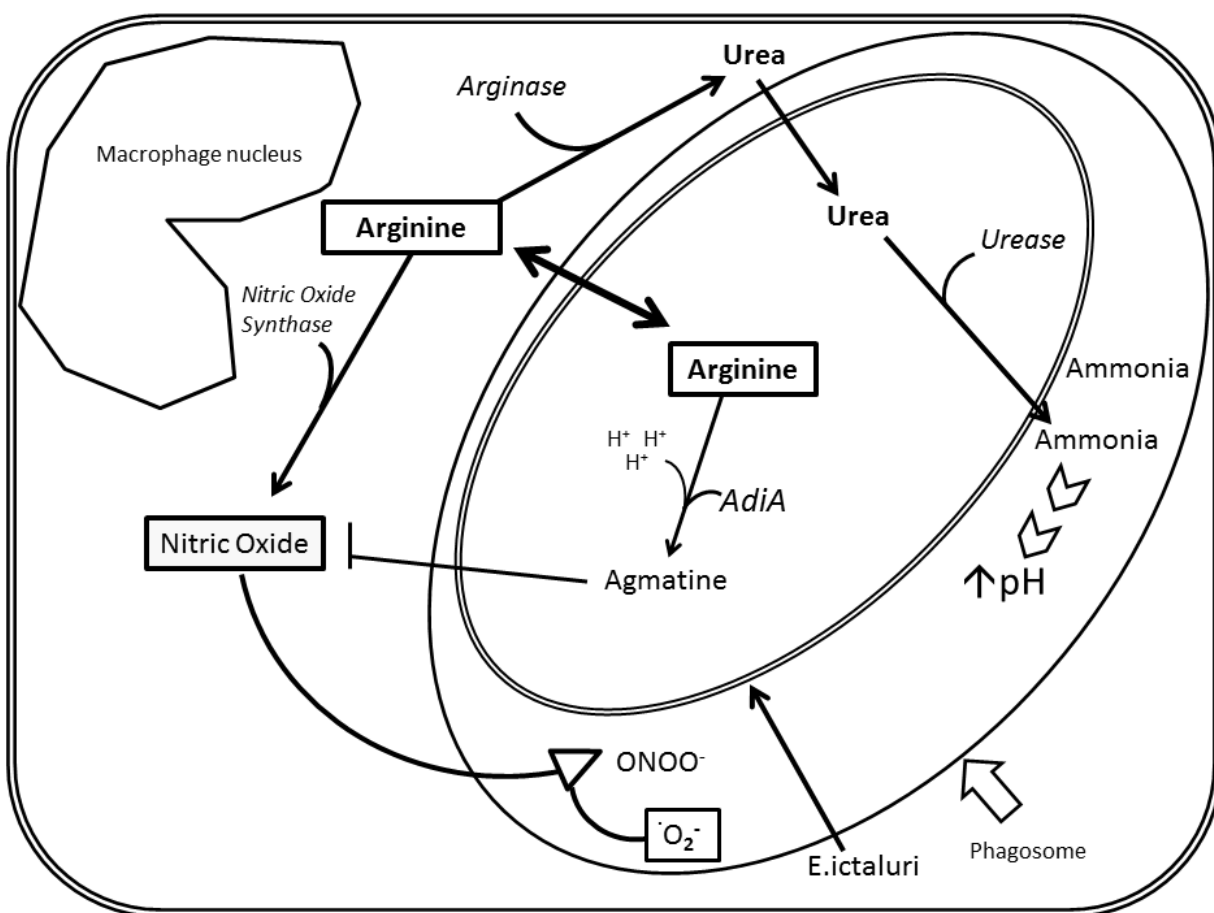


Figure 3.1. Hypothetical model for the interaction of *E. ictaluri* with catfish macrophages. Arginine can be utilized by HKDM nitric oxide synthase and arginase, or the bacterial arginine decarboxylase (AdiA).

TABLE 3.1. Bacterial strains used in Chapter 3

Strain or plasmid	Description	Source or reference
<i>Escherichia coli</i>		
QC779	F-, $\Delta(\text{argF-lac})169$, λ , $\phi(\text{sodB-kan})1(-\Delta 2)$, IN(rrnD-rrnE)1, rpsL179(strR), sodA25::MudPR13	Carlioz and Touati 1986
<i>Edwardsiella ictaluri</i>		
93-146	WT <i>E.ictaluri</i> strain isolated in 1993 from moribund channel catfish in a natural outbreak of ESC on a commercial farm	Louisiana Aquatic Diagnostic Laboratory
$\Delta\text{ureG}::\text{km}$	93-146 strain with an STM tag L/M insertion in the <i>ureG</i> gene	Thune et al. 2007
$\Delta\text{adiaA}::\text{km}$	93-146 strain with an STM tag L/M insertion in the <i>adiaA</i> gene	Thune et al. 2007

and *E. ictaluri* strains were grown in porcine brain heart infusion broth or LB-0.35% Mannitol (LB-Man) at 28°C. An *E. coli* superoxide dismutase (SOD) double mutant, QC779, was used as a killing control (Carlloz and Touati 1986). A defined minimal media for *E. ictaluri* was used with minor modifications stated where pertinent (Collins and Thune 1996). Antibiotics were used in the following concentrations: kanamycin (Km) at 50 µg ml⁻¹, colistin (Col) at 10 µg ml⁻¹, and ampicillin (Amp) at 200 µg ml⁻¹. When necessary, *E. ictaluri* colony forming (CFU) unit numbers were determined by making triplicate 10-fold serial dilutions in sterile phosphate buffered saline (PBS) and drop plating 20 µl aliquots on Trypticase soy agar plates supplemented with 5% defibrinated sheep blood for colony counting.

SPF Channel Catfish. Channel catfish egg masses were obtained from a commercial producer with no history of ESC outbreaks. The eggs were disinfected with 100 ppm free iodine and hatched in closed recirculating systems in the specific pathogen free laboratory at the Louisiana State University School of Veterinary Medicine. Holding systems consist of 350 liter round fiberglass tanks connected to a 45 liter biological bead filter (Aquaculture Systems Technologies, New Orleans, LA, USA). Water temperature was maintained at 28 ± 2°C, and water quality parameters (including total ammonia nitrogen, nitrite, total nitrate, pH, hardness, and alkalinity) were determined 3 times per week using a Hach kit (Hach company, Loveland, CO, USA). Water quality was adjusted to maintain optimal conditions. The fish were reared on commercial catfish feed at 2-3% body weight per day until used for HKDM harvest at 1 to 1.5 kg.

Labeling Bacteria/ Oregon Green/LysoTracker Red. Oregon Green 514 carboxylic acid succinimidyl ester (OG) was acquired from Invitrogen, Carlsbad, CA, USA. Staining of bacteria by OG was according to Porte et al. (Porte, et al. 1999). Briefly, log phase cultures (10⁹ bacteria/ml) were washed twice in sterile saline and suspended in 1 ml of saline with 0.05%

Tween 80. Ten microliters of 10 mg/ml OG was added and the sample was briefly vortexed. Incubation for 30 minutes at 4°C in the dark was followed by centrifugation and addition of Tris-HCl (pH 8.3) to a 100 mM final concentration. Resuspension of bacteria and a second 4°C incubation for 15 minutes was followed by 2 washes in saline prior to opsonization with autologous channel catfish serum for 30 minutes at room temperature. Opsonized bacteria were then used for infection experiments. Killed bacteria were produced by washing bacteria twice in saline, then incubation at 70°C for 30 minutes. Killing was verified by plating aliquots on blood agar.

Infection and Intracellular Viability of Mutant and OG Stained *E. ictaluri* in HKDM. A

standard gentamicin survival assay using HKDM was used to evaluate the ability of OG stained *E.ictaluri* to enter, survive, and replicate in norvaline treated HKDM (Booth, et al. 2009).

Briefly, adult channel catfish were euthanized via overdose with tricaine methanesulfonate (200 mg/liter) and then bled via phlebotomy to exsanguination. The pronephros was removed using sterile procedure and macerated in a tissue sieve (stainless steel mesh 280 and 140 µm) to collect free cells. Cell viability was determined by Trypan blue exclusion. Dissociated cells were suspended to a final concentration of 1×10^7 live cells/ml in channel catfish macrophage medium (CCMM) consisting of: catfish RPMI [(RPMI 1640 sans phenol red & L-glutamine, Lonza, Walkersville, MD, USA) diluted to a tonicity of 240 mosmol/kg H₂O by adding 1 part sterile deionized/distilled water, 1x glutamine substitute (GlutaMAX –I CTS, Gibco, Invitrogen Corporation, Carlsbad, CA,USA)], 15 mM HEPES buffer (GIBCO)], with 0.18% sodium bicarbonate solution (GIBCO), 0.05 mM 2-beta-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), and 5% heat inactivated pooled channel catfish serum. One ml of cell suspension was added to each well of a 24 well plate (Biocoat poly-d-lysine plates, Becton

Dickinson Labware, Bedford, MA, USA) and allowed to adhere for 16 hours at 28°C with 5% carbon dioxide, then washed 3 times with catfish RPMI before returning to fresh CCMM for infection. HKDM wells were infected at a 1:10 MOI (bacteria: macrophage multiplicity of infection) with bacteria opsonized previously for 30 minutes in autologous serum. Wells were then centrifuged (400 g for 5 minutes) to synchronize infection and incubated for 30 minutes. One hundred µg/ml gentamicin was then added for 1 hour to kill extracellular bacteria. Cells were then washed once in catfish RPMI and placed in CCMM with 0.5 µg/ml gentamicin (static dose). After 0, 4, and 10 hours at 28°C with 5% CO₂, HKDM were washed with RPMI and lysed by adding 100 µl of 1% Triton X-100 (Fisher Scientific, Fair Lawn, NJ, USA). Media was serially diluted and plated on blood agar to determine increase from timepoint zero.

Fluorescent Microscopy for Vacuolar pH Determination. The pH sensitivity of OG fluorescence in the weakly acidic range coupled with its fluorescence characteristics ($\lambda_{\text{ex}}=510$ nm peak excitation, $\lambda_{\text{ex}}=450$ nm insensitive) allows ratiometric pH estimation in cellular vacuoles (Invitrogen). When exposed to 510 nm light, OG fluorescent brightness varies with pH, while at 450 nm it is relatively nonreactive. The microscope used exposes OG stained bacteria to 510 nm and 450 nm ultraviolet light in rapid succession and a ratio of the measured fluorescence for any specified object at 510 nm to that at 450 nm can be generated by the software package. Such ratios can then be used for comparative purposes. HKDM were harvested and cultured as described above for the gentamicin assay, except cells were plated onto 35 mm glass bottom dishes (poly-d-lysine coated, No.1.5 glass, MatTek, Ashland, MA, USA) and were incubated overnight in 10 mM norvaline (Sigma, St. Louis MO). HKDM were infected with bacteria at a 10:1 MOI, centrifuged, allowed to settle for 10 minutes, then washed in saline 3 times and placed in CCMM with a static gentamicin dose (0.35 µg/ml) and 10 mM norvaline. Cells used to

determine pH calibration data with live imaging were incubated in ionophore calibration solutions while cells used to measure experimental data were incubated in saline with 10 mM norvaline. For calibration, infected HKDM were incubated for 8 minutes in progressively more acidic solutions of defined pH (140 mM KCl, 1 mM MgCl₂, 0.2 mM EGTA, 20 mM MES, 10 mM norvaline, adjusted to pH 4, 5, and 6 with NaOH) containing 5 µg/ml nigericin and 5 µM monensin to collapse pH gradients across membranes (Demaurex and Grinstein 2006). *In situ* calibration curves were made for each bacterial strain experiment for each day. After 8 minutes in each pH solution, several images were acquired using a dual exciter filter (Chroma) to generate ratiometric data before changing pH solution. For experimental data, cells incubated in saline were imaged similarly after 60-80 minutes post-infection. For each image, background was identified as the grey/black level corresponding to areas devoid of cells and was subtracted from the image prior to ratio data gathering. Individual bacteria were chosen manually from images according to: proximity within the confines of the HKDM cell membranes, identifiability as bacteria (shape, size), being separate from other highly reactive cellular fluorescent material (including tightly clustered bacteria or superimposed structures), and being in relative focus. A ratio was thus generated for each bacterium. Approximately 50-100 bacteria were chosen for each group (pH 4, 5, 6, and experimental groups).

Cells were visualized with a Zeiss Observer.Z1 microscope with CO₂Module S and TempModule S (Carl Zeiss MicroImaging GmbH, Jena, Germany) using a 63x oil objective in a stage insert (Heating Insert P, PeCon GmbH, Erbach, Germany) equipped to supply humidified carbon dioxide and maintain temperature at 5% and 28°C, respectively. Illumination was provided by a Lambda DG-4, 175 Watt Xenon arc lamp (Sutter Instrument Co., Novato, CA, USA). Cubes used are as follows: Chroma 71001 dual exciter (440/20, 495/10), 535/25 emitter

(Chroma Technology Corporation, Bellows Falls, VT, USA) and Zeiss filter set 64 HE exciter 587/25, emitter 647/70 (Carl Zeiss, Germany). Digital images were captured using Zeiss AxioVision software version 4.8.1 and analyzed with Physiology Acquisition Module (Carl Zeiss, Germany).

Nitric Oxide Assay. The stable breakdown products of nitric oxide (NO), nitrite (NO_2^-) and nitrate (NO_3^-), were measured using a standardized kit for colorimetric determination of nitrite and nitrate (enzymatic reduction) in biological samples based on the Griess reaction (DetectX Nitric Oxide colorimetric detection kit, Arbor Assays, Ann Arbor, MI, USA). HKDM were harvested and were incubated overnight as previously described. The next day, cells were washed twice in catfish RPMI and once in Krebs-Ringer bicarbonate buffer (KRBB; 119 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , 0.5 mM L-arginine, 0.5 mM L-glutamine, 5 mM D-glucose, 20 mM HEPES, 5% BSA, pH 7.4) before incubation in KRBB. HKDM were infected with wild type *E. ictaluri* (WT) and mutant strains as well as superoxide dismutase (QC779) mutant *E. coli* to serve as a control in addition to non-infected HKDM. Media was recovered from infected wells after 24 hours, filtered through a 10 K molecular weight cut-off filter (Amicon Ultra 0.5 ml 10K centrifugal filter, Millipore, Billerica, MA, USA), and stored at -29°C until tested. Samples were analyzed according to kit protocol, with enzymatic reduction of nitrate to nitrite. Samples were read at 543 nm on a SpectraMax M2 microplate reader using SoftMax Pro software version 4.8 (Molecular Devices, Sunnyvale, CA, USA).

Urea Assay. HKDM were harvested and incubated as described previously. HKDM were infected with WT and $\Delta\text{ureG}::km$, in addition to non-infected HKDM. Cells were incubated for 24 hours prior to supernatant and cell lysate collection. Media samples were frozen at -70°C until

testing. Urea levels were quantitatively measured by using a standardized kit for colorimetric determination of urea in biological samples (DetectX Urea Nitrogen colorimetric detection kit, Arbor Assays, Ann Arbor, MI, USA). Samples were analyzed according to kit protocol and read at 450 nm on a SpectraMax M2 microplate reader using SoftMax Pro software version 4.8 (Molecular Devices, Sunnyvale, CA, USA).

Norvaline Mediated for HKDM Arginase Inhibition, Gentamycin Exclusion Assay. HKDM were harvested as previously described. Cells were incubated overnight in 10 mM norvaline (Sigma, St. Louis MO) and were infected with WT. After infection cells were returned to media with norvaline until lysis at timepoints 0, 4, and 10 hours postinfection for bacterial enumeration. HKDM infected cells without norvaline served as negative controls for comparison.

Statistical Methods. For nitrate/nitrite and urea assays, the data were analyzed using the General Linear Methods Procedure for least means (Proc GLM; SAS 9.3, SAS Institute Inc. 2011). Tukey's Studentized Range (HSD) test was used for pairwise comparisons. For HKDM gentamycin exclusion assays, fold increase was determined by dividing the colony forming units/well from timepoints past time zero, by the mean colony forming units /well at time zero for each strain. Mean fold replication was then calculated with standard errors of the means. Data were then analyzed by analysis of variance (ANOVA). In addition, individual colony forming units counts were log transformed and analyzed by Proc GLM and Tukey's HSD for pairwise comparisons. For pH measurement data in OG stained *E. ictaluri*, Proc GLM and ANOVA were used. When the model indicated significance at $P \leq 0.05$, a least square means procedure was used for pairwise comparison of interaction effects.

RESULTS

NO Measurement in *E. ictaluri* Infected HKDM. Nitric oxide production was determined by measuring the NO breakdown products nitrite and nitrate. Total nitric oxide (nitrite plus nitrate) levels were not significantly different between positive (QC779) controls, negative (HKDM only) controls, and bacterial strains (wild type *E. ictaluri*, $\Delta ureG::km$, $\Delta adiA::km$) at 24 hours post infection. In all tests nitrite levels were at the low end of the test detection range. Levels detected in positive and negative controls were not significantly different from one another. Nitrite levels in *E. ictaluri* infected HKDM were not significantly different from uninfected HKDM.

Urea Production in *E. ictaluri* Infected HKDM. Urea production was determined by direct assay for urea nitrogen. Urea levels were at the low end (~0.15 mg/dL) of assay detection range. Measurements from bacterial strains (wild type *E. ictaluri*, $\Delta ureG::km$, $\Delta adiA::km$, QC779) and controls (HKDM only) were not significantly different from one another.

Effect of Arginase Blocking by Norvaline on *E. ictaluri* Replication in HKDM. The results on the ability of *E. ictaluri* to replicate in norvaline treated HKDM (arginase blocking) are presented in Figure 3.2. The inhibition of HKDM arginase prevented *E. ictaluri* replication at 10 hours post infection ($p \leq 0.0001$), while WT increased 15-20 times in that period.

Measurement of *E. ictaluri* Intracellular pH in Norvaline Treated HKDM. When HKDM were treated with norvaline to inhibit arginase, WT pH was not significantly different from pH 4 (Figure 3.3). There are multiple bacteria with ratios comparable to those seen in the pH 5 to 6 range, which is consistent with WT survival data (Figure 3.2). This indicates that arginase

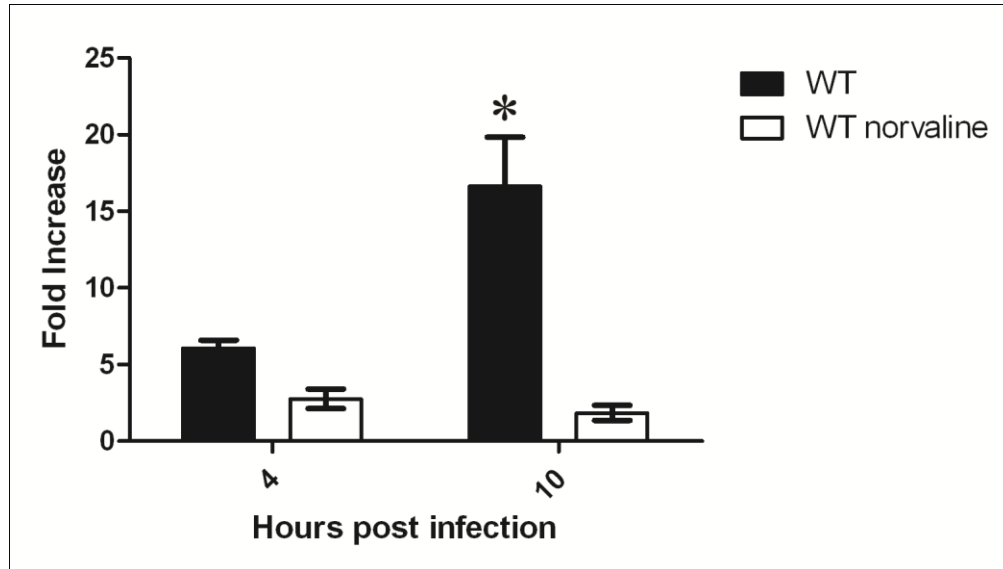


Figure 3.2. Intracellular survival and replication of *E. ictaluri* in HKDM and norvaline treated HKDM. WT *E. ictaluri* increased over 15 fold after 10 hours, while norvaline treatment to block arginase function prevented WT replication. (*) indicates $P \leq 0.0001$ versus all other time points and treatments. Norvaline treatment did not prevent WT survival. Results are presented as means and standard errors of the means for 3 wells per treatment per time point and are representative of 3 replicate studies.

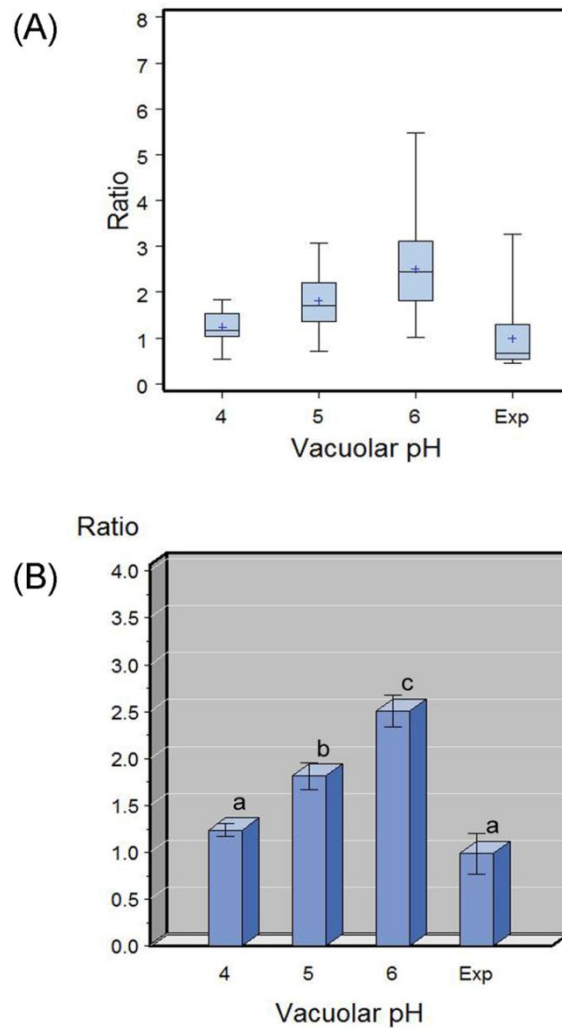


Figure 3.3. Intracellular (phagosome) determination of WT vacuole pH in channel catfish HKDM treated with 10 mM L-norvaline 1 hour post infection using fluorescent bacterial cell membrane staining. (A) The box-whisker plots showing data range (whiskers), interquartile range (the mid 50% of data points, in blue), and the mean (designated +). (B) The same data as A, presented as means and standard errors of the means (bars). Different letters atop columns designate significant differences in means. Vacuolar pH at 4, 5, and 6 represents the artificial manipulation of phagosome pH by ionophore calibration solutions to generate fluorescent ratio values of intraphagosomal bacteria at pH 4, 5, and 6. “Exp” represents the measurement of fluorescent ratios of bacterial cell membranes when macrophages are bathed in saline with norvaline and phagosomal pH is not artificially altered. Ratio is generated by microscope software for relative comparisons and is based on the fluorescence characteristics of OG 514. Results are combined from 2 replicate studies.

function is vital to WT HKDM phagosome pH neutralization, which is typically above pH 6 (this work).

DISCUSSION

Macrophages have the ability to differentially regulate arginine metabolism via NOS and arginase in association with different activation states, which has important implications for cells and intracellular pathogens. The NOS pathway, associated with a Th1 immune response, leads to increased bacterial killing and disruption of virulence mechanisms employed by intracellular pathogens (Bogdan 2009). The arginase pathway leads to cell proliferation, anti-inflammation responses, and is associated with a Th2 immune response (Munder 2009).

NOS is a high output enzyme found in macrophages and has 4 major antimicrobial functions; toxic effects directly attributable to NO, negation/frustration of microbial virulence mechanisms, NOS dependent effects aside from NO, and immunostimulation by NO (Bogdan 2009). In resting murine macrophages, arginase was found to consume 96% of available arginine, while NOS consumed 0.7%. Upon activation, NOS consumption of arginine increased to 29% (Granger, et al. 1990). Similarly, in freshly isolated carp pronephros macrophages, stimulation with lipopolysaccharide (LPS) led to a significant increase in NO production (Joerink, et al. 2006). Using freshly isolated channel catfish HKDM, we found no evidence for NOS upregulation/NO production, probably due to the use of non-stimulated macrophages in our model, which specifically studies the effects of intracellular *E. ictaluri* in naive macrophage populations. Minimal NO production in non-stimulated macrophages is a common finding in murine and fish macrophage studies, using both cell lines and primary cultures (Arts, et al. 2010; Chaturvedi, et al. 2007; Grayfer, et al. 2011). In addition, the degree of HKDM maturity may

also hamper NO production as immature goldfish monocytes cannot produce significant amounts of NO even when stimulated (Plouffe, et al. 2005). In channel catfish, the capacity for naive HKDM to produce NO has not been reported, but LPS-activated peritoneal wash macrophages were able to produce NO, which increased significantly with arginine supplementation (Buentello and Gatlin III 1999). Additionally, in pronephros homogenates from *E. ictaluri* infected catfish 5 days post-infection, NO production was increased almost 100 fold versus control (Schoor and Plumb 1994). These results show the capacity for remarkable NOS upregulation in the cells of the pronephros. However, it is important to keep in mind that small, but biologically relevant, amounts of NO may be below the detection limit of the Griess reaction (Gantt, et al. 2001).

Arginase is a manganese metalloenzyme that hydrolyzes arginine to urea and ornithine. In order to assess the contribution of HKDM arginase to *E. ictaluri* pathogenesis, we used the arginase inhibitor, norvaline, to inhibit urea production. Norvaline is able to inhibit arginase activity in J774.1 murine macrophages by 55% due to norvaline's structural similarity to ornithine, a natural arginase inhibitor (Chang, et al. 1998). Treatment of *E. ictaluri* infected HKDM with norvaline prevented intracellular replication, while WT could replicate over 15 fold in 10 hours (Figure 3.2). In addition, using fluorescence based pH measurements, norvaline treatment resulted in acidification of the *E. ictaluri* containing phagosome to pH 4, whereas under standard conditions pH would be above 6 (previous chapter). These data show that cellular arginase is required for *E. ictaluri* replication and phagosome pH regulation in HKDM. Similar data were seen using a urease deficient strain ($\Delta ureG::km$), indicating that the urea produced by HKDM arginase is the primary source of urea that the *E. ictaluri* urease uses to produce ammonia and neutralize phagosome pH. In addition, we measured total urea production by

HKDM to further assess arginase activity. After 24 hours post infection, urea levels in infected HKDM were not different from controls and urea levels were low. Similar data were seen in unstimulated J774.1 cells infected with *Mycobacterium bovis* BCG and in controls. When cells were then activated with LPS and IFN- γ , increased arginase expression and significant increases in urea production were found (Talaue, et al. 2006). The urea present in HKDM is biologically relevant, however, because arginase inhibition and urease mutation both lead to the same *E. ictaluri* phenotype, with a highly acidified vacuole and an inability to replicate.

In conclusion, intracellular *E. ictaluri* requires HKDM arginase to generate the urea needed for pH neutralization and replication in the *E. ictaluri* containing vacuole. Within naive channel catfish macrophages, *E. ictaluri* may upregulate cellular arginase in a manner similar to *Salmonella* serovar Typhimurium, which deprives the macrophage of substrate for antimicrobial activity (Lahiri, et al. 2008). Alternatively, *E. ictaluri* may take advantage of an intracellular niche in immature macrophages where arginase activity prevails and NOS activity is low. Such cells would have reduced or inefficient capacity for degradation and killing. In figure 3.4, the model has been amended to represent the current understanding of arginine regulation in the *E. ictaluri*-HKDM phagosome dynamic based on the data presented here.

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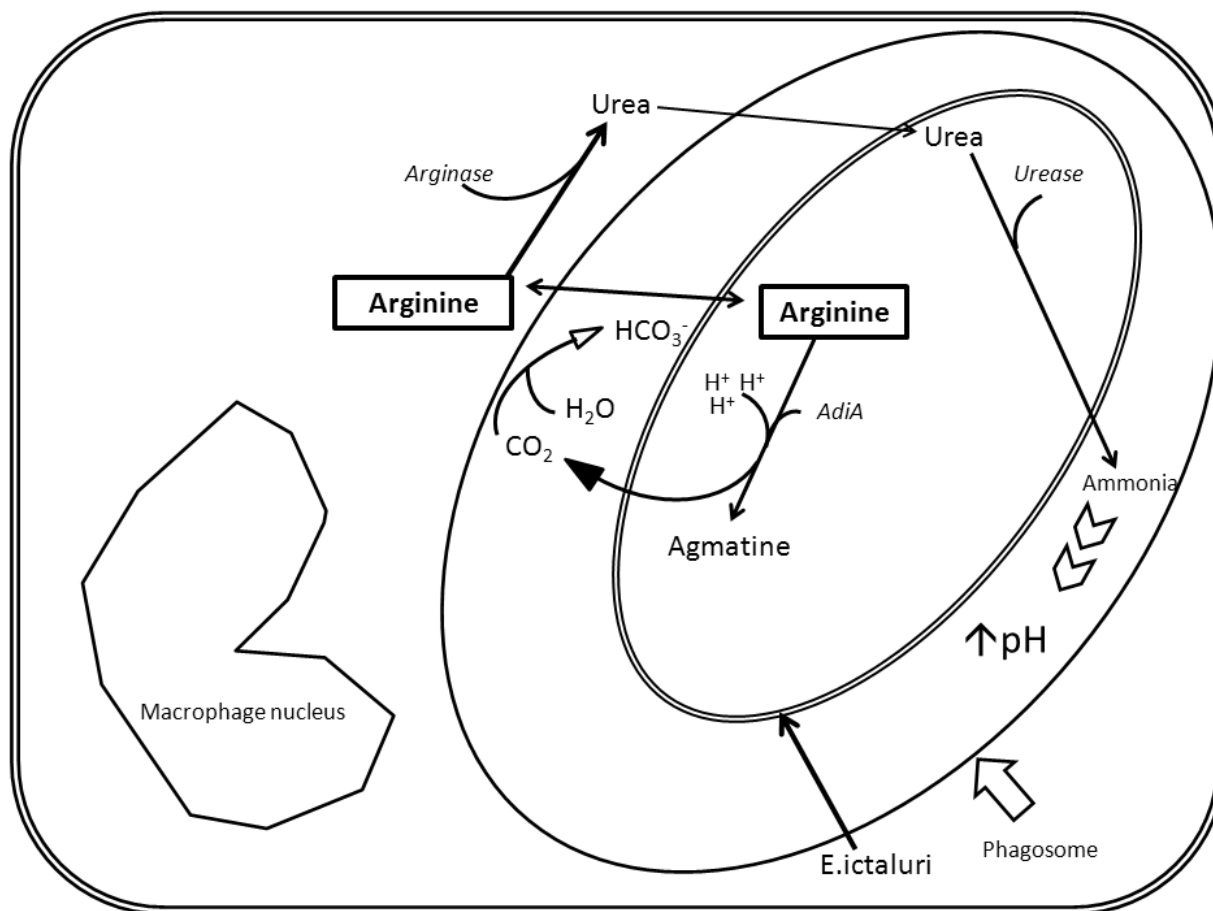


Figure 3.4. Model for the metabolism of arginine in channel catfish HKDM. Arginine is used primarily by HKDM arginase to produce urea and ornithine. The urea produced is used by the *E. ictaluri* urease for the generation of ammonia. Arginine decarboxylase (AdiA) converts arginine to agmatine, consuming protons in the process.

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CHAPTER 4

CONCLUSIONS

Edwardsiella ictaluri is an interesting bacterium. It belongs to a rather short list of known macrophage pathogens and is able to neutralize phagosome pH, permitting intracellular replication that eventually leads to macrophage death (Booth, et al. 2006). The host of choice is the channel catfish, an ectothermic poikilotherm whose immune response varies with environmental temperature (Lorenzen, et al. 2009). When *E. ictaluri* enters the fish bloodstream, numerous organs are infected within minutes of exposure (Baldwin and Newton 1993). In particular the pronephros, or head kidney, is situated almost immediately downstream from the gills (a likely route of infection) and receives abundant blood from the dorsal aorta and the common cardinal veins. The pronephric parenchyma itself contains islands of erythroid and myeloid cells in all stages of maturity surrounded by blood filled sinuses (Grizzle and Rogers 1976). Thus, *E. ictaluri* is able to rapidly gain access to the widest possible variety of myeloid cell types and stages, especially that of the monocyte/macrophage line, which acquire phagocytic and antimicrobial capabilities both incrementally and to varying degrees as they mature.

The *Edwardsiella ictaluri* urease and arginine decarboxylase (AdiA) are required for replication in channel catfish head kidney derived macrophages (HKDM) (Figure 4.1). These enzymes provide *E. ictaluri* with the capacity to neutralize phagosome pH to over 6, which is permissive for bacterial replication and would indicate that a source of arginine and a source of urea are required for *E. ictaluri* replication in HKDM. Arginine enters macrophages through the cationic amino acid transporters (CAT)1 and CAT2, providing a pool of substrate for nitric oxide

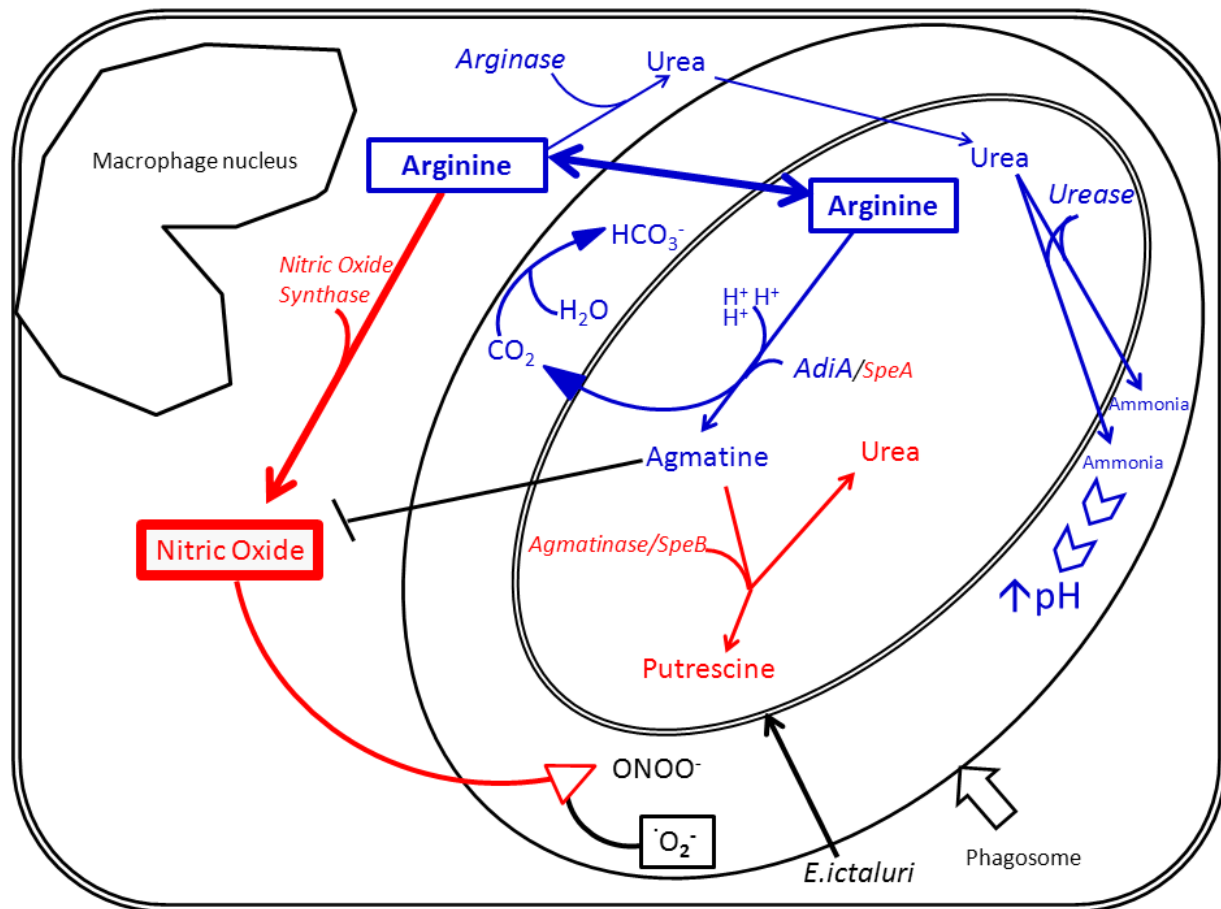


Figure 4.1. Model for the role of arginine in the pathogenesis of *E. ictaluri* infected HKDM based on findings in this study. Pathways highlighted in blue are supported by data, while the data indicates that pathways highlighted by red are not involved.

synthase and arginase. In phagosomes, *E. ictaluri* could also utilize the macrophage CAT system for arginine import, as happens with *Salmonella enterica*, where macrophage CAT is diverted to the *Salmonella* containing vacuole (Das, et al. 2010).

There are 3 potential sources of urea for HKDM and *E. ictaluri in vivo*. The first is from nitrogenous waste metabolism exogenous to HKDM, the second is from macrophage (arginase), and the third from bacterial sources (agmatinase) (Lu 2006; McDonald, et al. 2006). It is likely that exogenous urea is insufficient for *E. ictaluri*'s needs in HKDM *in vivo*. Ammonia and urea are metabolites derived from protein degradation mechanisms in the liver. Ammonia is highly toxic but easily and rapidly excreted in water through the gills and is thus preferred. Urea is less toxic, but it requires more energy to produce, and cell membranes are relatively impermeable to urea. Urea is primarily excreted through the skin and gills by poorly understood mechanisms and is retained at relatively low levels in the body, 0.93 mM for *I. punctatus* blood (Francis-Floyd 1993; Walsh 1998). Significant data indicates that the *E. ictaluri* agmatinase, SpeB, is not associated with HKDM replication or phagosome pH modulation, indicating it is not an important source of urea, and, *E. ictaluri* has no encoded arginase. These data indicate that the macrophage arginase activity provides a likely source of urea for *E. ictaluri* and the norvaline experiments confirm that this is the case.

The pathway for urea entry into *E. ictaluri* within the HKDM phagosome is unknown, but may be surmised. Macrophages have no known urea specific transporters, but they do have aquaporins, which can nonspecifically transport urea (Aharon and Bar-Shavit 2006; Litman, et al. 2009). Furthermore, aquaporins are known to integrate into *Salmonella* containing vacuoles in macrophages (Radtke and O'Riordan 2008) and a similar situation is possible for *E. ictaluri* in HKDM. Once in the phagosome, urea can be internalized by *E. ictaluri* through its encoded urea

transporter, UreI, which is part of the urease operon (Booth, et al. 2009). Urease contributes to phagosome pH neutralization first through the production of ammonia, which sequesters protons by ammonium formation. Secondly, carbon dioxide is produced which, under permissive pH conditions, can be converted to bicarbonate, a powerful buffer (Huynh and Grinstein 2007).

Two potential purposes for AdiA are evident; as an acid resistance pathway enzyme and as a source of agmatine. The conversion of arginine to agmatine and carbon dioxide by AdiA consumes protons. Although the function of carbon dioxide as a buffer was already mentioned (Viala, et al. 2011), the function of agmatine in the model is uncertain. It has little known use in bacteria, save as a precursor to polyamine synthesis (Satriano 2003). However, it still may be of use to *E. ictaluri* in HKDM, due to the important regulatory effects this molecule has in eukaryotic cells. Agmatine can inhibit NOS and suppress the production of polyamines which are cytoprotective, antiproliferative, and antiapoptotic (Sastre, et al. 1998; Satriano 2004). For intracellular pathogens like *E. ictaluri*, agmatine may serve as a virulence factor in a somewhat similar manner to *H. pylori* in the stomach (Molderings, et al. 1999). If agmatinase does function in putrescine production in HDKM, the overall contribution of polyamines to pathogenesis is uncertain.

No evidence for NO production was found in HKDM. As discussed in chapter 3, this may be due to the inability of non-stimulated HKDM to produce NO. In goldfish pronephros macrophages, immature monocytes have phagocytic capacities similar to mature macrophages, but have a low capacity for phagolysosome formation and reaction oxygen intermediate production (Rieger, et al. 2010). Mature macrophages, in the absence of activation, showed a similar uncoupling of phagocytosis from killing/degradation mechanisms. A comparable situation may be present in channel catfish HKDM, accounting for the lack of NO production.

Once inside a non-activated HKDM phagosome, *E. ictaluri* may find itself in a cell where arginine metabolism is monopolized by arginase, like that found in murine models (Granger, et al. 1990). If the infected HKDM also have uncoupled phagocytosis and killing mechanisms, *E. ictaluri* has a clear advantage over the macrophage for survival.

We began with 3 hypotheses: that *E. ictaluri* is able to alter the HKDM phagosome pH, that *E. ictaluri* urease uses AdiA, SpeA or SpeB for phagosome pH alteration, and that HKDM NO production, arginase function, and urea production are important for *E. ictaluri* intracellular pathogenesis. In all 3, the null hypothesis is rejected in part or in whole. *E. ictaluri* can alter HKDM phagosome pH using urease and AdiA, and HKDM arginase is required as a source for urea.

What does all this mean in a practical sense? Currently, ESC affects over a third of all channel catfish production operations in the United States of America, causing mortality events in all ranges of severity in fry and foodsize fish (USDA 2009b). More than 20% of catfish fry are lost to ESC yearly, making it an important disease for farm management. However, approximately only 12% of all fry are vaccinated for ESC, and the overall impression of efficacy by producers is equivocal (USDA 2009a). As candidate vaccine strains are still desirable for ESC, arguably the ideal bacterium would be an infectious, nonvirulent strain that retains the phenotypic characteristics of highly virulent strains with regards to persistence and sustained interaction with the adaptive immune response, but lacks the capacity to exploit the niche it normally inhabits during disease. By directed and investigative research such as the work presented here, a highly specific approach to vaccine strain development based on pathogenesis at the molecular level can be achieved. It is to these ends that the most fruitful inroads into the control of ESC will be made.

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APPENDIX

ABBREVIATIONS COMMONLY USED IN THIS DISSERTATION

ADI - Agmatine Deiminase

AdiA - *E. ictaluri* arginine decarboxylase

AQP - Aquaporin

AR - Acid resistance

ARG - Arginase

CAT - Cationic amino acid transporters

CD - Cluster of differentiation

CFU - Colony forming units

ESC - Enteric Septicemia of catfish

HKDM - Head kidney derived macrophages

IFN- γ - Interferon gamma

IL - Interleukin

LB - Luria-Bertani Broth

LPS - Lipopolysaccharide

LTR - LysoTracker Red DND-99

NO - Nitric oxide

NOS - Nitric oxide synthase

ODC - Ornithine decarboxylase

OG - Oregon green 514 succinimidyl ester

OU-C - Ornithine urea cycle

RNI - Reactive nitrogen intermediates

ROS - Reactive oxygen species

SOD - Superoxide dismutase

SpeA - *E. ictaluri* arginine decarboxylase

SpeB - *E. ictaluri* agmatinase

STM - Signature tagged mutagenesis

TGF- β - Transforming growth factor beta

Th1(2) - T helper cell type 1(2)

TLR - Toll-like receptor

TNF- α - Tumor necrosis factor alpha

WT - Wild type *E. ictaluri*

VITA

Wes Arend Baumgartner was born December 16, 1975, to a family who were and are proud farmers. He has an older sister Keena, and a first cousin Wade, whom he considers as a brother. He grew up on a swine farm in the heart of flatland Central Illinois- so flat one can watch one's dog run away for 3 days. After an interesting stint in rural schooling and many unsavory farm chores, he attended the University of Illinois at Urbana-Champaign from 1994-2002, earning a bachelor's degree in animal sciences and a doctorate in veterinary medicine. During this time he managed an interesting and sometimes harrowing semester abroad at the University of Queensland in Brisbane, Australia. After graduation, he left the Midwest for sunny California in 2002, surviving as a small animal emergency/internal medicine intern in San Diego until he decided to get some real culture...in the Mississippi delta. In Leland Mississippi, on the same creek where Kermit the Frog was hatched, Wes spent a year as an intern for the diagnostic service of the Thad Cochran National Warmwater Aquaculture Center. After enough boiled peanuts and fried catfish were had, he began his veterinary anatomic pathology residency and doctoral combined program at Louisiana State University in 2005. During that time, Wes learned much about good cooking and hurricanes, got married, passed the pathology board exam, lost the best cat ever, gained a sweet little baby, and learned to be even more pedantic about a thing or two. In October of 2011, Wes defended and finished this very dissertation, earning a doctorate in philosophy. He now resides near Starkville Mississippi, where he enjoys a faculty position at the Mississippi State University College of Veterinary Medicine in the Department of Pathobiology and Population Medicine. He understands that corporate rock, like a cake doughnut, is an inferior product. He also prefers his monkey brains scooped hot from the freshly trepanned skull and his french fries, extra crispy.