Edwardsiella ictaluri Encodes an Acid-Activated Urease That Is Required for Intracellular Replication in Channel Catfish (*Ictalurus punctatus*) Macrophages[▽]

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Received 14 July 2009/Accepted 4 September 2009

Genomic analysis indicated that *Edwardsiella ictaluri* encodes a putative urease pathogenicity island containing the products of nine open reading frames, including urea and ammonium transporters. In vitro studies with wild-type *E. ictaluri* and a *ureG::kan* urease mutant strain indicated that *E. ictaluri* is significantly tolerant of acid conditions (pH 3.0) but that urease activity is not required for acid tolerance. Growth studies demonstrated that *E. ictaluri* is unable to grow at pH 5 in the absence of urea but is able to elevate the environmental pH from pH 5 to pH 7 and grow when exogenous urea is available. Substantial production of ammonia was observed for wild-type *E. ictaluri* in vitro in the presence of urea at low pH, and optimal activity occurred at pH 2 to 3. No ammonia production was detected for the urease mutant. Proteomic analysis with two-dimensional gel electrophoresis indicated that urease proteins are expressed at both pH 5 and pH 7, although urease activity is detectable only at pH 5. Urease was not required for initial invasion of catfish but was required for subsequent proliferation and virulence. Urease was not required for initial uptake or survival in head kidney-derived macrophages but was required for intracellular replication. Intracellular replication of wild-type *E. ictaluri* was significantly enhanced when urea was present, indicating that urease plays an important role in intracellular survival and replication, possibly through neutralization of the acidic environment of the phagosome.

Identification of virulence factors is vitally important to an understanding of the pathogenesis of *Edwardsiella ictaluri* and to the development of methods for controlling the spread of disease. Although the pathogenesis of *E. ictaluri* was reviewed in 1993 (28, 31), recent reports demonstrated that *E. ictaluri* is a facultative intracellular pathogen (3) and that a type III secretion system is required for intracellular survival and replication within channel catfish head kidney-derived macrophages (HKDM) (30). Using signature-tagged mutagenesis (STM) in an immersion challenge model for *E. ictaluri*, Thune et al. (30) identified 50 transconjugants carrying transposon insertions in genes required for survival and replication in the channel catfish host. Two of those mutants had insertions in genes encoding homologs of UreG and UreF, proteins that are essential for the production of an active urease enzyme in other bacteria (6, 10, 14, 26). UreG is a GTP-binding accessory protein that functions in energy-dependent assembly of the urease holoenzyme (19), while UreF is a urease accessory protein that functions in the generation or delivery of carbon dioxide to the urease metallocenter assembly site (19). Both the *ureG* and *ureF* mutant strains were further characterized in a competitive challenge with the wild-type (WT) parental strain and were confirmed to be significantly attenuated (30).

The identification of two mutants with insertions in urease-associated genes suggests an important role for urease activity in *E. ictaluri* pathogenesis, despite the fact that *E. ictaluri* is urease negative in standard biochemical tests. Consequently, the objectives of this study are to characterize the *E. ictaluri* urease pathogenicity island (PAI), to evaluate conditions for *E. ictaluri* urease activity, and to establish a possible role for urease in *E. ictaluri* pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, *Escherichia coli* was grown in Luria-Bertani broth (LB) at 37°C while WT *E. ictaluri* and the *E. ictaluri ureG::kan* (37) mutant were grown in brain heart infusion broth (BHIB) or BHIB with 6 mM United States Pharmacopoeia-grade urea (Amresco, Solon, OH) (BHIBU) at 28°C. When required, antibiotics were used at the following concentrations: kanamycin (Km) at 50 µg/ml, tetracycline at 65 µg/ml, and ampicillin at 200 µg/ml. All media that were prepared at pH 5.0 or below were buffered with 2-morpholinoethanesulfonic acid (Sigma Chemical Co.). When necessary, numbers of CFU 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 (BA) for colony counting.

SPF channel catfish. Egg masses were obtained from commercial channel catfish producers with no history of *E. ictaluri* outbreaks, disinfected with 100 mg/liter free iodine, and hatched in closed recirculating systems in the LSU specific-pathogen-free (SPF) aquatic laboratory. The holding systems consisted of four 350-liter fiberglass tanks connected to 45-liter biological bead filters (Aquaculture Systems Technologies, New Orleans, LA). Water temperature was maintained at 28 ± 2°C, and water quality parameters, consisting of total ammonia nitrogen, total nitrate, pH, hardness, and alkalinity, were determined three times per week by using a Hach aquaculture kit (Hach Company, Loveland, CO). Water quality was adjusted as necessary to maintain optimal conditions.

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[▽] Published ahead of print on 11 September 2009.

TABLE 1. Bacterial strains and plasmids used in this project

Strain or plasmid	Description or genotype	Source or reference
Strains		
<i>E. coli</i>		
XL1-Blue MRF	($\Delta mcrA$)183 $\Delta(mcrCB-hsdSMR-mrr)$ 173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^qZ</i> M15 Tn5 (Kan ^r)]	Stratagene, La Jolla, CA
CC118 λ pir	$\Delta(ara-leu)$ <i>araD</i> $\Delta lacX74 galE galK phoA20 thi-1 rpsE rpoB argE (Am) recA \lambdapir lysogen$	8
S17-1 λ pir	Tr Sm <i>thi pro recA hsdR hsdM⁺</i> RP4-2-Tc::Mu::Km Tn7 λ pir	8
XLOLR	($\Delta mcrA$)183 $\Delta(mcrCB-hsdSMR-mrr)$ 173 <i>endA1 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^qZ</i> M15 Tn10 (Tet ^r)] Su ⁻ (nonsuppressing) λ^r	Stratagene, La Jolla, CA
<i>E. ictaluri</i>		
93-146	WT <i>E. ictaluri</i> strain isolated in 1993 from moribund channel catfish in a natural outbreak on a commercial farm	Louisiana Aquatic Diagnostic Laboratory
<i>ureG::kan</i> mutant	Derived from the parental WT strain 93-146, having an insertion of STM tag L/M in the <i>ureG</i> gene	30
Plasmids		
pBluescript SK(-)	Phagemid cloning vector	Stratagene, La Jolla, CA
pBK-CMV	Phagemid cloning vector	Stratagene, La Jolla, CA

Sequencing of the urease operon. To sequence the region surrounding the transposon insertion in *ureG*, a 166-bp PCR product was amplified from the pBluescript plasmid carrying the subcloned *ureG* region by using two primers designed from the *ureG* sequence (30), 84LMLib1 (5'-GACTTCTTATTTACA CACAGCG3') and 84LMLib2 (5'-CTTTTTCCTCCACACAAC3'). The resulting product was labeled using the enhanced chemiluminescence (ECL) nucleic acid labeling system (GE Health Care, Piscataway, NJ) and was used as a probe to screen a previously described λ Zap Express *E. ictaluri* genomic library (29) in accordance with the manufacturer's instructions (Stratagene, Inc., La Jolla, CA). The pBK-CMV phagemid was excised from hybridization-positive plaques with the ExAssist helper phage and *E. coli* XLOLR in accordance with the Zap Express protocol and was sequenced by using primer walking on an ABI-Prism 377 automated sequencer (PE-Applied Biosystems, Foster City, CA). The phagemid sequence was subsequently used with the Basic Local Alignment Search Tool (BLAST) to analyze the partially completed *E. ictaluri* genome (http://www.microgen.ouhsc.edu/cgi-bin/blast_form.cgi), resulting in the identification of an 11,426-bp fragment carrying the seven genes of a urease operon, along with putative urea and ammonia transporters. The ORF Finder program on the National Center for Biotechnology Information (NCBI) webpage (<http://www.ncbi.nlm.nih.gov/>) was used to search the NCBI databases for gene identification and arrangement. The genetic structure of the urease operon was examined using the stem-loop and terminator programs of the Wisconsin package (Genetics Computer Group, Madison, WI) and the bacterial operon and gene-finding software program from SoftBerry, Inc.

Confirmation of a single insertion event in the *ureG::kan* mutant. A single chromosomal transposon insertion in the *ureG::kan* mutant was confirmed with genomic DNA prepared from the *ureG::kan* mutant by using the standard method of Ausubel et al. (2). A total of 10 μ g of this DNA was digested to completion with ClaI, which does not cut the STM transposon. Digested genomic DNA was separated on a 1% agarose gel and transferred to an ECL Hybond N+ nylon membrane (GE Health Care). A 373-bp STM PCR product was amplified using primers Tn5kan+ (ACACGTAGAAAGCCAGTCCG) and Tn5kan- (CCAGTCATAGCCGAATAG) and labeled using the ECL nucleic acid labeling system (GE Health Care). The probe was hybridized to the genomic DNA on the membrane and detected using the ECL reagents in accordance with the manufacturer's instructions.

Acid tolerance assay. The pH of PBS or PBS supplemented with 6 mM urea was adjusted to pH 7.0, 6.0, 5.0, 4.0, 3.0, and 2.0. Equal aliquots of an overnight culture of WT grown in BHI at pH 7 were transferred into each of 36 tubes and pelleted by centrifugation, and each of 3 tubes was resuspended in an equal volume of PBS or PBS supplemented with 6 mM urea at each pH. Cultures were incubated for 2 h at 28°C on a Cel-Grow rotator (Barnstead International, Dubuque, IA), after which numbers of CFU/ml were determined.

Effects of urea on *E. ictaluri* growth at acidic pH. The pH levels of BHIB and BHIBU were adjusted to pH 4.5, 5.0, or 6.0, after which triplicate 10-ml cultures of each broth type and each pH level were inoculated with 100 μ l of an overnight culture of the WT or the *ureG::kan* mutant. Cultures were grown on a rotator at

28°C for 24 h, and numbers of CFU/ml were determined. Numbers of CFU/ml were also determined for the overnight starter culture, and increase was determined by dividing the total number of CFU/ml recovered by the initial number of CFU/ml in the inoculum. The remaining culture was pelleted and the supernatant removed for measurement of pH with an Orion 520A digital pH meter (Orion Research, Inc., Beverly, MA).

Whole-cell urease assay. Urease activity was measured by determining the rate of ammonia production from the hydrolysis of urea, using an assay modified from the procedures of Young et al. (33). Briefly, broth cultures were pelleted by centrifugation, and cells were washed three times in double-deionized (DD) water. Final pellets were suspended in 250 μ l DD water, and 5.6- μ l aliquots of each cell suspension were transferred to each of 3 wells of a 96-well reaction plate containing 250 μ l of the appropriate PBS buffer with or without urea at various pH levels and prewarmed to 28°C. Viable cell counts for the concentrated suspension were determined as described above. After 1 min and 6 min, 50 μ l of each reaction was transferred to the wells of a Falcon 353947 Optiplux assay plate (Becton Dickinson, Franklin Lakes, NJ) containing 125 μ l of phenol nitroprusside (Sigma catalogue no. P6994) to stop the urease reaction, after which 125 μ l of alkaline hypochlorite (Sigma catalogue no. A1727) was added to induce the formation of the color indicator, indophenol. Plates were read after 30 min with a Spectromax M2 plate reader (Molecular Devices, Sunnyvale, CA) at optical density at 635 nm. Ammonia concentrations were calculated using an ammonium chloride standard curve, and the concentration of ammonia at 1 min was subtracted from the concentration at 6 min and then divided by 5 to determine the rate of ammonia production per minute. Rates were normalized by dividing by the number of viable CFU in the assay well.

Effect of growth and assay conditions on urease activity. In order to determine the effect of urea availability and growth pH on urease activity, WT *E. ictaluri* was grown in either BHIB or BHIBU at both pH 7 and pH 5. Cultures were harvested and assayed for urease activity in PBS at pH 5 and PBS at pH 2.5, both with and without urea. In order to determine the pH optimum for stimulation of *E. ictaluri* urease activity, an overnight culture grown in BHIB at pH 5 was assayed for urease activity in triplicate using PBS with and without urea at pH 6, 5, 4, 3, and 2.

To examine the effect of urea availability at low pH on survival, ammonia production, and final environmental pH, the WT and *ureG::kan* mutant *E. ictaluri* strains were grown at pH 5. Each cell suspension was standardized to an optical density at 600 nm of 1.6, and samples were removed to determine numbers of CFU/ml in the starter culture. Aliquots of each culture were transferred to PBS at pH 2, 2.5, and 3, with and without urea. A 50- μ l aliquot of each suspension was removed immediately, and the ammonia concentration at time zero was determined. After a 2-h incubation on a rotator at 28°C, the number of CFU/ml, the ammonia concentration, and the pH were determined. Percent survival was determined using the start and final CFU/ml data.

Effect of growth pH on protein expression. Overnight cultures of the WT were grown in BHIB at pH 7.0, BHIBU at pH 7.0, BHIB at pH 5.5, or BHIBU at pH 5.5. Cells were pelleted and washed three times in PBS, pH 7.3, and were then

FIG. 1. *Edwardsiella ictaluri* and *Yersinia enterocolitica* urease PAIs. Sequencing and subsequent BLAST search of the NCBI databases identified the STM transposon insertion in the 3' end of the putative *ureG* gene. Further sequencing of the region identified the presence of *ureEFGD* as well as a urea transporter gene and a putative ammonium transporter gene. Analysis of the recently sequenced *E. ictaluri* genome allowed the identification of the enzymatic subunit genes *ureABC*, located just upstream of the region encoding the accessory proteins. The *E. ictaluri* urease PAI sequence is available through GenBank (accession number AY607844).

resuspended in sterile DD water at a volume of 1.0 ml water for every 0.1-ml pellet. The cell suspension was sonicated at 40 W and a 50% duty cycle at 4°C until the suspension became clear. The lysate was held for 1 h at 4°C and then centrifuged at $12,000 \times g$ for 30 min at 4°C. A solution of 10% thimerosal (Sigma Chemical Co.) was added to give a final concentration of 0.01%. Protein concentrations were determined per the manufacturer's instructions with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as a standard. The lysates were aliquoted and stored at -80°C until used.

Protein samples were prepared for two-dimensional (2-D) polyacrylamide gel electrophoresis per the manufacturer's instructions by using a Bio-Rad Ready-Prep 2-D cleanup kit. The first dimension was run using a ReadyPrep 2-D starter kit and 11-cm ReadyPrep IPG strips, pI 4.7 to 5.9, per the manufacturer's instructions. The strips were run for 12 h at the following settings: rehydration, passive; rehydration time, 12 h; run temperature, 20°C ; maximum $\mu\text{A/gel}$, 50; step 1, 250 V with ramp L for 15 min; step 2, 8,000 V with ramp L for 2 h 30 min; step 3, 8,000 V with ramp L at 35,000 V · h; step 4: 500-V hold. The second dimension was run on Criterion 12.5% Tris-HCl with 1.0-mm Precast gels (Bio-Rad Laboratories) per the manufacturer's instructions at 200 V with cooling. The gels were fixed per the manufacturer's instructions and stained with SYPRO ruby protein gel stain (Bio-Rad Laboratories, Hercules, CA). Gels were scanned on a Bio-Rad Molecular Imager FX scanner (Bio-Rad Laboratories, Hercules, CA) and analyzed with PDQuest 2-D analysis software, version 7.3.1 (Bio-Rad Laboratories). Using theoretical molecular weights and isoelectric points based on bioinformatic analysis of the protein sequences, suspected UreA, -C, and -G spots were selected and excised from the gel. Each spot was digested in-gel with trypsin by using a Sigma ProteoProfile trypsin in-gel digest kit (Sigma Chemical Co., St. Louis, MO) in accordance with the manufacturer's instructions and submitted for peptide mass fingerprinting by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MS) to the Nevada Proteomics Center (University of Nevada, Reno, Reno, NV). The molecular mass peaks were compared with those generated by theoretical trypsin digests of *E. ictaluri* UreA, UreC, and UreG, using the FindMod program (<http://www.expasy.ch/tools/findmod/>), allowing a 5-Da margin of error, up to three missed cleavage sites, and two potential modifications within one peptide.

Invasion and persistence studies in vivo. To evaluate initial invasion of the channel catfish host by the *ureG* mutant, sixteen 14-g fish were stocked into each of six 20-liter tanks, and three tanks for each treatment were challenged by immersion with either the WT or the *ureG* mutant at a dose of 5×10^8 CFU/ml tank water. One fish was removed from each tank and euthanized in 1,000 mg/liter of tricaine methanesulfonate (MS-222) at 1, 2, 4, and 8 h postinfection. To evaluate long-term persistence, 75 fish, with an average weight of 141 g/fish, were stocked at a rate of 25 fish/tank into each of three tanks. One tank was inoculated for immersion challenge to give a final concentration of 5×10^8 CFU/ml of tank water each for the WT and the *ureG::kan* mutant. One tank inoculated with BHIB served as a negative control. Three fish were removed and euthanized from each tank daily over an 8-day period. For both the invasion and the persistence experiments, head kidney tissue was removed, weighed, homogenized, spread on BA plates, and incubated at 28°C for 48 h. Colonies were counted, and numbers of CFU recovered/g of tissue were determined.

Attenuation of the *ureG::kan* mutant in vivo. Two hundred twenty-five SPF channel catfish, with an average weight of 141 g/fish, were stocked at a rate of 25 per tank into nine 20-liter tanks, and three tanks each were randomly assigned to the WT, *ureG::kan* mutant, and broth-only treatments. Fish were challenged as described above except that the final challenge concentration was only 1×10^8 CFU/ml of tank water. Deaths were recorded for each 24-h period after experimental infection until three consecutive days passed without a death. Liver

samples from all dead fish were streaked on BA plates and incubated at 28°C for 48 h to confirm the presence of *E. ictaluri*.

Intracellular replication of the *ureG* mutant. A standard gentamicin survival assay (9) was used to evaluate the abilities of the WT and the *ureG::kan* mutant *E. ictaluri* strains to enter, survive, and replicate in channel catfish HKDM. Briefly, HKDM were isolated by the method of Booth et al. (3), and viable counts were determined using trypan blue dye exclusion (3, 24). Dissociated cells were suspended to give a final concentration of 1×10^7 cells/ml in channel catfish macrophage medium (CCMM) consisting of RPMI 1640 medium (GIBCO, Invitrogen Corporation, Carlsbad, CA) diluted to a catfish tonicity of 243 mosmol/kg H_2O by adding 1 part sterile deionized/distilled water (RPMI 9:1) and containing 15 mM HEPES buffer solution (GIBCO); 0.18% sodium bicarbonate solution (GIBCO); 0.05 mM 2-beta-mercaptoethanol (Sigma Chemical Co., St. Louis, MO); and 5% heat-inactivated, pooled channel catfish serum (18). One milliliter of the cell suspension was added to each well of a 24-well plate and allowed to adhere for 16 h at 28°C with 5% CO_2 , after which they were washed three times with RPMI 9:1 to remove nonadherent cells and 1 ml of fresh CCMM was added per well.

To evaluate the efficiency of entry and replication, 1×10^4 of either WT or *ureG::kan* mutant *E. ictaluri* bacteria that had been opsonized for 30 min in normal autologous serum were added to triplicate wells of the 16-h HKDM cultures, giving a multiplicity of infection of 1 bacterium to 10 HKDM. After infection, plates were centrifuged at $200 \times g$ to synchronize the contact of the bacteria with the adhered cell layer and allowed to incubate for 30 min. The medium was then removed from each well, and CCMM with 100 $\mu\text{g/ml}$ gentamicin was added for 1 h to kill residual extracellular bacteria. Cells were then washed three times with RPMI 9:1, and CCMM containing a 0.35- $\mu\text{g/ml}$ bacteriostatic dose of gentamicin was added to control the extracellular growth of any bacteria released from the cells. At 0 (90 min postinfection), 4, 8, and 12 h, the HKDM were lysed by the addition of 100 μl of a 1% solution of Triton X-100 (Fisher Scientific, Fair Lawn, NJ), and increase from time zero was determined as described above. In order to determine the effect of urea supplementation on intracellular survival, HKDM were infected with the WT in media with and without 6 mM urea. Cultures were incubated at 28°C and sampled after 0 (90 min postinfection), 4, and 8 h, and increase in CFU (n -fold) was calculated as described above.

Statistical analysis. The experimental designs for mortality, invasion, and persistence were completely randomized with a factorial arrangement of treatments. Data were analyzed by the general linear methods procedure by following a natural log transformation of the numbers of CFU recovered/well (Statistical Analysis Systems, version 9.1; SAS Institute, Inc., Cary, NC). When the overall model indicated significance at P values of ≤ 0.01 , Scheffe's test was used for pairwise comparison of main effects, and a least-square-means procedure was used for pairwise comparison of interaction effects. For the urease activity assays, survival studies, and growth experiments, the data were analyzed using two-way analysis of variance followed by Bonferroni's procedure for pairwise comparisons.

Nucleotide sequence accession number. The sequence of the *E. ictaluri* urease gene complex is available in GenBank under accession number AY607844.

RESULTS

Sequencing of the urease operon. Genomic analysis indicated that *E. ictaluri* encodes a putative urease PAI containing the products of nine open reading frames, with seven arranged in the same order and direction as the seven genes of the

FIG. 2. Growth of the WT and *ureG::kan* mutant strains of *E. ictaluri* in BHIB at various pH levels with and without 6 mM urea. Numbers above the bars indicate the final pH. Results are presented as means and standard errors of the means and are representative of three identical experiments. Growth of the WT strain at pH 5 with urea was significantly greater than that of either the WT grown without urea or the *ureG::kan* mutant grown with or without urea (***, $P \leq 0.001$), and the pH was increased from pH 5.0 to pH 7.2.

urease operon of *Yersinia enterocolitica*, but also including urea and ammonium transporters (Fig. 1). Further analysis revealed that the three genes encoding the urease enzymatic subunits *UreABC* and the four urease accessory proteins *UreEFGD* appear to be in a single operon, with an apparent sigma 70 transcriptional initiation site upstream from *ureA*. *UreA* appears to be translationally coupled to *UreBCDEFG* by hairpins associated with their translation initiation region, but the upstream regions of the urea and ammonium transporters carry individual sigma 70 promoters and translational initiation regions, suggesting that both are single-gene transcripts. Based on this genomic structure, the transposon insertion in the *ureG* mutant would be polar onto *ureD* but would not affect the expression of the transporters.

The *E. ictaluri* α subunit of the urease apoprotein (*UreC*) carries 8 of the 10 conserved histidine residues that comprise the essential nickel-binding metallocenter of the urease active site in other bacteria, including a histidine residue at position 320 that marks the active site (12, 19). The *Y. enterocolitica* urease is the prototype for a group of acid-activated ureases that also includes those produced by *Morganella morganii* and *Lactobacillus fermentum*. The *Y. enterocolitica* group of low-pH ureases carry a unique phenylalanine residue that occurs 7 residues upstream from the catalytic His-320 residue and a unique asparagine residue immediately following at position 321 (33). Both residues are associated with activity at low pH and are conserved in *E. ictaluri*, suggesting that *E. ictaluri* urease activity is also optimal at low pH.

Confirmation of single transposon insertion event in the *ureG::kan* mutant. Southern blot analysis of *Cla*I-digested DNA from the *ureG::kan* mutant detected a single band following hybridization with a Km probe, confirming a single STM transposon insertion in this strain (data not shown).

Acid tolerance assay. After a 2-h exposure, survival rates were high at all pH levels from pH 7 to pH 4, with a slight decline at pH 3, but there were no significant differences, regardless of the presence or absence of urea. At pH 2, however, neither the WT nor the *ureG::kan* mutant *E. ictaluri*

FIG. 3. Ammonia production from urea following growth of WT *E. ictaluri* at pH 5 and pH 7, with and without urea, when urease activity is assayed at both pH 2.5 and pH 5.0, with and without urea. Urease activity is significantly greater when *E. ictaluri* is grown at pH 5 and is also significantly greater when urea is available in the growth medium and urease activity is assayed at pH 2.5. Bars with different letter designations are significantly different ($P \leq 0.05$). Results are presented as means and standard errors of the means and are combined data from three experiments, with three replications per treatment per experiment.

strain was able to survive for 2 h. These results indicate that *E. ictaluri* is tolerant of acid conditions down to pH 3 but that urease activity is not required for acid tolerance (data not shown).

Effect of urea on growth of *E. ictaluri* at acidic pH. The growth results of the WT and *ureG* strains in BHIB or BHIBU at different pH levels are presented in Fig. 2. Neither the WT nor the *ureG::kan* mutant grew appreciably at pH 4.5, regardless of the presence or absence of urea. At pH 5.0, however, the availability of urea significantly enhanced the growth of the WT strain compared to that of the *ureG::kan* mutant strain ($P \leq 0.001$). In addition, the WT strain significantly elevated the medium from pH 5 to pH 7.2 after 24 h of growth in the presence of urea ($P \leq 0.001$), but the pH remained unchanged and there was no growth in the absence of urea. Urea had no effect on the pH of the culture supernatant for the *ureG::kan* mutant. At pH 6, the growths of the WT and the *ureG::kan* mutant were not significantly different, either with or without urea.

Effect of growth and assay conditions on urease activity. The effects of assay conditions and urea availability on the activity of the WT *E. ictaluri* urease as measured by conversion of urea to ammonia are presented in Fig. 3. No urease activity was detected when the WT was grown at pH 7, with or without urea and regardless of the pH of the assay buffer. Urease activity was detected when the WT was grown at pH 5 and urease activity was assayed at pH 5, but there was no significant difference in activity whether urea was present or not in the initial growth medium. Urease activity was significantly greater, however, when assayed at pH 2.5 and was greatest when urea was present in the growth medium. Together, these data suggest that urease activity is upregulated at pH 5, regardless of the availability of exogenous urea in the medium, but that the activity is significantly enhanced by low pH and the availability of urea. Examination of the data to further determine the

FIG. 4. Rate of ammonia production observed when WT *E. ictaluri* is grown at pH 5 and urease activity is assayed under various pH conditions with and without urea (U). No ammonia was produced in the urea-negative controls at any pH (data not shown). Results are presented as means and standard errors of the means and are representative of three identical experiments. Bars with different letter designations are significantly different ($P \leq 0.01$).

effect of pH on urease enzymatic activity is presented in Fig. 4, which clearly shows that the *E. ictaluri* urease has optimal activity from pH 2 to 3.

The relationship between urease activity, environmental pH, and *E. ictaluri* survival is further examined in the data in Fig. 5. In the absence of urea, neither the WT nor the *ureG::kan* mutant was able to withstand a 2-hour exposure to pH 2.0, and there were 87 and 50% reductions in CFU at pH 2.5 and 3, respectively. Minimal ammonia production was detected, and the pH of the medium increased only slightly. The same is true for the *ureG::kan* mutant in the presence of urea. In contrast, the WT had significantly greater survival at pH 2.5 and pH 3.0 when urea was available. In addition, there was substantial production of ammonia as well as an increase in environmental pH from pH 2.5 and 3.0 to pH 6.0 and 6.5, respectively. Although there was significant ammonia production at pH 2.0, it was insufficient to raise the pH enough to increase survival in the 2-h assay period. The data suggest that WT *E. ictaluri* can increase environmental pH in the presence of urea through the production of ammonia, resulting in enhanced survival and growth. The *ureG::kan* mutant, on the other hand, failed to produce ammonia, resulting in no increase in environmental pH.

Effect of growth pH on protein expression. Three proteins whose positions on the gels indicated matches for the molecular weights and pI levels of UreA, UreC, and UreG were excised from the 2-D gel images of lysates prepared from WT *E. ictaluri* grown in BHIB at pH 7.0 or 5.0, trypsin digested, and submitted for MS analysis. The peptide masses of two of the proteins matched with 100% coverage to those of the peptide fragments generated in a theoretical trypsin digest of the *E. ictaluri* UreG and UreA proteins. The peptide masses of the third protein matched 77.2% to those of the fragments expected from a theoretical trypsin digest of *E. ictaluri*'s UreC protein. The regions of the UreC sequence that did not match to the MS-generated mass peaks, however, were composed of two separate peptide sequences, one from amino acids 102 to 183 and one from amino acids 292 to 341. Neither peptide contained interior trypsin cut sites, and the predicted mass peaks of these two peptides were both greater than 5,800 Da,

FIG. 5. Survival rates, final ammonia concentrations, and final environmental pH levels of the WT and *ureG::kan* mutant *E. ictaluri* strains held at pH 2, 2.5, and 3, with and without urea. Numbers above the bars are final ammonia concentrations ($\mu\text{M/ml}$) shown above final pH levels. *** indicates that the survival rate, final ammonia concentration, and final pH of the WT are all significantly different from those of the mutant ($P \leq 0.001$). ***¹ indicates that only the ammonia concentration is significantly different ($P \leq 0.001$). Results are presented as means and standard errors of the means and are combined data from three identical experiments, with three replications per treatment per experiment.

which is beyond the detection limits of the MS analysis performed here. Because 100% of the peptides that were detected by MS matched to UreC, it is highly probable that the third protein assayed is UreC. Urease proteins UreB, UreE, UreF, and UreD all had pI values that excluded them from detection on gels with the pH range applied here and are therefore not represented in this analysis. The presence of all three expected proteins at both pH 5 and pH 7, however, indicates that urease expression is not transcriptionally or translationally regulated by pH. The lack of enzymatic activity at pH 7 (Fig. 4), regardless of the assay pH, indicates either that an inactive urease holoprotein is produced or that the holoprotein is not assembled.

Invasion and persistence studies in vivo. Numbers of WT and *ureG::kan* mutant *E. ictaluri* bacteria in the head kidney tissue were not significantly different over the first 12 h following immersion challenge (Fig. 6A), indicating that urease is not required for invasion of the catfish host from water. WT *E. ictaluri*, however, persisted and increased in numbers by more than 2.5 logs by day 7 (Fig. 6B). No data were available for day 8 for the WT-challenged fish, because all fish either were sampled or had died by day 7. The *ureG::kan* mutant, on the other hand, was significantly less abundant ($P \leq 0.001$) than the WT over the same time period and was cleared from head kidney tissue by day 8.

Attenuation of the *ureG::kan* mutant in vivo. Mortality results following immersion challenge are shown in Fig. 7. No mortalities were observed in fish challenged with the *ureG::kan* mutant or the BHIB control during the 16-day study. The

FIG. 6. Invasion (A) and persistence (B) results for WT and the *ureG* mutant in juvenile channel catfish. Results are presented as means and standard errors of the means observed for numbers of CFU/g of tissue from three individual fish per treatment per sample period following a $\log_{10}$ transformation of the CFU values. All fish from the WT group had died by day 7, so there are no values for day 8 in this treatment group. For the persistence results, all WT values are significantly greater than the *ureG::kan* mutant values at P values of ≤ 0.001 .

mortality rate for the WT was significantly greater than that for the mutant on every day after day 9, and the total average mortality rate for fish challenged with the WT was significantly greater, at 57%, than that for the mutant (0%).

Intracellular replication in channel catfish macrophages. Survival and replication of the WT and the *ureG::kan* mutant strain in channel catfish HKDM as determined using the gentamicin survival assay are presented in Fig. 8. The WT strain increased significantly after both 8 and 12 h postinfection, but the *ureG::kan* mutant had significantly reduced growth. Disruption of *ureG* did not have a significant effect on initial uptake of *E. ictaluri* by HKDM, but numbers of bacteria did not increase significantly at either 8 or 12 h compared to the level for time zero, indicating that the mutant survived conditions in the phagosome but was unable to replicate. Light microscopy showed only 1 or 2 bacteria in isolated macrophage cells at all time points for the *ureG::kan* strain (data not shown).

Effect of urea supplementation on survival and replication in channel catfish macrophages. Addition of urea to the

CCMM in the HKDM cultures resulted in a significant increase in replication of WT *E. ictaluri* (Fig. 9). As expected, the WT increased significantly at both 4 and 8 h postinfection, but the addition of urea to the culture medium resulted in significantly increased numbers of intracellular bacteria at both 4 ($P \leq 0.05$) and 8 ($P \leq 0.01$) h. After 4 h, there was a 2.2-fold increase without urea, compared to a 6.0-fold increase with urea present. After 8 h, the urea-supplemented cultures had increased 10.4-fold, compared to only 3.9-fold in the non-supplemented cultures.

DISCUSSION

Bacterial ureases are known to hydrolyze urea to produce ammonia and carbamate. In solution, carbamate spontaneously decomposes to form more ammonia and carbonic acid. The carbonic acid equilibrates in water, as do the two molecules of ammonia, which become protonated to yield ammonium, NH_4^+ . The net result of these reactions is an increase in

FIG. 7. Comparative mortality results for the WT and *ureG::kan* mutant strains of *E. ictaluri* observed following an immersion challenge. Results are presented as means and standard errors of the means for three replicate tanks of 25 fish for each treatment. There were no mortalities in any of the tanks infected with the *ureG* mutant or in the BHI control broth. The level for day 9 is significantly greater at P values of ≤ 0.01 , and the levels for days 10 to 16 are significantly different at P values of ≤ 0.001 .

FIG. 8. Intracellular survival and replication of the WT and *ureG::kan* mutant strains in channel catfish HKDM. While WT *E. ictaluri* numbers increased 18-fold by 12 hours postinfection, the *ureG* strain was significantly attenuated for replication within macrophages, increasing in numbers less than 3-fold at the same time ($P \leq 0.001$). The mutant strain was, however, able to survive within these cells, and there was no significant difference in initial uptake between the two strains. Results are presented as means and standard errors of the means for three wells per treatment and are representative of three replicate studies.

FIG. 9. Effects of exogenous urea on the intracellular survival and replication of the WT strain (multiplicity of infection, 1:10) in channel catfish HKDM. Results are presented as means and standard errors of the means for combined data from two replicate studies, with three replicates for each treatment in each study (*, $P \leq 0.05$; **, $P \leq 0.01$).

the pH of the reaction environment (4). A number of enteric bacterial pathogens use ammonia produced through urease activity to maintain intracellular pH and/or to provide a protective microenvironment during passage through or residence in the low-pH environment in the stomach (17). Examples include *Helicobacter pylori* (17), *Y. enterocolitica* serotype O8 (7), *Morganella morganii* (33), *Klebsiella pneumoniae* (16, 27), and enterohemorrhagic *Escherichia coli* (11).

Traditional in vitro biochemical tests indicate that *E. ictaluri* is a urease-negative organism (32), so it was surprising to find urease-associated genes represented twice among attenuated mutants identified by STM (30). Sequencing and bioinformatic analysis, however, confirmed that the *E. ictaluri* genome contains a PAI that carries nine genes associated with urease activity. Based on amino acid sequence analysis, the *E. ictaluri* urease enzyme has close homology to the urease produced by *Y. enterocolitica*, which has optimal activity at low pH. Data presented here indicate that the *E. ictaluri* urease is also acid activated and is relatively inactive at pH levels greater than 4. The standard in vitro biochemical test for urease in bacteria uses phenol red as a pH indicator, which has a pK of 7.9 and detects pH changes only in the range of 6.8 to 8.1 (1). Because the *E. ictaluri* urease is not active at neutral-to-basic pH levels, urease activity is not detected under standard conditions.

As suggested above, the *E. ictaluri* urease enzyme is most similar to members of the *Y. enterocolitica* class of acid activated ureases, which includes those produced by *M. morganii* and *K. pneumoniae* (33). The urease enzymes of this group are unique among bacterial ureases because of the unusually low pH required for activity, with optimal activity at pH 1.5 to 2. The members of the *Y. enterocolitica* class of acid-activated ureases are all required for acid resistance of the respective pathogen (33), and resistance to acid is required for bacterial survival in the low-pH environment of the stomach (7). These acid-activated ureases carry a set of conserved amino acid residues associated with the acid activation motif (33), and the presence of the same residues in *E. ictaluri* suggests that the *E. ictaluri* urease is also acid activated. Data presented here indicate that *E. ictaluri* is indeed highly tolerant of acid conditions and that *E. ictaluri* urease activity is optimal at low pH but that, unlike the *Y. enterocolitica*-like acid ureases, the *E. ictaluri* urease is not required for tolerance of acidic conditions.

Growth studies, however, indicate that *E. ictaluri* cannot replicate at a pH lower than 6 even though *E. ictaluri* survives very well at low pH. WT *E. ictaluri*, however, is able to increase the extracellular pH from 5 to over 7 when exogenous urea is provided in the growth medium, and the increase in pH subsequently allowed replication to take place. In addition, the low-pH survival study, with and without urea, indicated that the WT, but not the *ureG::kan* mutant, produced significant amounts of ammonia and increased the environmental pH levels from 2.5 and 3.0 to 6.0 and 6.5, respectively, in just 2 hours when urea was present. As a result, the WT had significantly greater survival rates after 2 hours than the *ureG::kan* mutant. The survival rates of the WT and urease mutant *E. ictaluri* strains were not significantly different when urea was not added.

As determined by the urease activity assays, *E. ictaluri* is capable of utilizing exogenous urea and subsequently releasing ammonia, suggesting that the ammonia and urea transporters in the urease PAI are functional. The activity of a urea transporter in the urease complex of this fish pathogen is interesting, because fish excrete nitrogenous waste as ammonia and because urea transporters are relatively rare in bacteria (23). The ammonia transporter, *amtB*, is ubiquitous among bacteria (13) and generally is transcriptionally linked to *glnK*, which encodes a signal transduction protein that regulates *amtB* activity, but neither is linked to a urease gene cluster or urease activity. Analysis of the *E. ictaluri* genome indicates that *E. ictaluri* carries an *amtB-glnK* complex that encodes proteins with about 72% amino acid identity and 86% similarity to AmtB and 76% identity and 89% similarity to GlnK when the two are compared to other members of the *Enterobacteriaceae*. The *E. ictaluri* urease-associated ammonia transporter, however, is only 230 amino acids, compared to 428 amino acids, for the *E. coli* AmtB protein and has only 42% identity over amino acids 28 to 241 of AmtB. The presence of a second ammonia transporter in association with the urease complex of *E. ictaluri* is unique among bacterial pathogens.

Results from 2-D gel analysis indicate that the *E. ictaluri* urease proteins are constitutively expressed at both pH 5 and pH 7, but in vitro growth, acid tolerance, and acid adaptation studies all indicate that the *E. ictaluri* urease is acid activated. This is also consistent with the *Y. enterocolitica* class of acid-activated urease enzymes, in which de novo urease synthesis does not take place following acid exposure but, rather, pre-existing proteins are conformationally activated by the drop in pH (33). This suggests that *E. ictaluri*'s urease activity is neither transcriptionally nor translationally activated but that increased urea hydrolysis at low pH is due to conformational changes in preexisting urease, as it is in *Y. enterocolitica* (33). Although not evaluated in the *Y. enterocolitica* studies, the *E. ictaluri* urease activity study indicated that the enzyme was activated only under acid conditions when the bacteria were pregrown under acid conditions. Following growth at pH 7, urease proteins were detected on 2-D gels, but there was no detectable urease activity when cells grown at pH 7 were assayed at pH 2.5 or 5. This indicates that either the holoenzyme or the metallocenter or both were not assembled. Synthesis of the urease metallocenter is a complex process that requires nickel, carbon dioxide, and GTP, and proper assembly presumably requires low-pH conditions (21).

In vivo data show that initial invasion and uptake of the WT and the urease mutant by catfish were not significantly different but that the mutant was unable to increase in numbers after the initial invasion. The gentamicin survival assay with HKDM also indicated that initial uptake was not significantly different but that the urease mutant was unable to replicate. The failure of a urease mutant to proliferate in the catfish host or to replicate in HKDM indicates that ammonia production is essential to *E. ictaluri* pathogenesis. Given that in vitro studies demonstrated the ability of *E. ictaluri* to utilize ammonia produced by urease activity to increase environmental pH, it is tempting to hypothesize that the urease PAI is involved in pH modulation of the *E. ictaluri*-containing vacuole and that phagosomal pH is increased to a level that is permissive for *E. ictaluri* replication.

Helicobacter pylori is the only other pathogen reported to involve urease activity in survival in macrophages (25). *Helicobacter pylori* appears to modulate internal and external pH via cytoplasmic and surface-localized urease, respectively (31), and the activity is involved in intracellular survival induced by an unknown mechanism. Although urease plays a role in intracellular replication in *H. pylori* (25), genomic analysis indicates that ammonia and urea transporters are not associated with the *H. pylori* urease operon, suggesting a mechanism different from that proposed for *E. ictaluri*.

Edwardsiella ictaluri is capable of robust replication in macrophages without urea supplementation. As reported here, however, urea supplementation of macrophages infected with WT *E. ictaluri* resulted in intracellular-replication rates that were twofold greater than those observed in macrophages when urea was unavailable, again indicating a functional urea transporter. The ability of WT *E. ictaluri* to replicate in vitro without urea supplementation, however, suggests de novo synthesis of urea in the HKDM. The report that two arginine decarboxylase (AdiA) mutants were also identified as attenuated by using STM suggests a role for AdiA in the virulence of *E. ictaluri* (30). In the arginine degradation pathway, AdiA and agmatinase break arginine down to urea and putrescine. There are no published reports, however, that document the shuttling of urea from the arginine-degradative pathways for hydrolysis by urease during acid resistance. De novo synthesis of urea for use in acid resistance has been theorized (22) but not yet demonstrated. It is also possible, however, that the arginase activity of the catfish macrophage provides a source of urea. Macrophage-encoded arginase degrades arginine to ornithine and urea and is involved in the regulation of nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) by modulating the supply of arginine (5, 20). In fact, a recent report indicates that arginase activity in the macrophage is upregulated by *Salmonella* bacteria during an infection, which results in reduced NO production because of reduced arginine availability (15).

Results presented here demonstrate the role of urease in regulation of environmental pH and the importance of urease in intracellular replication of *E. ictaluri*. *Edwardsiella ictaluri* is tolerant of acid stress even in the absence of urease activity, suggesting alternative acid tolerance mechanisms. Although urease is not essential for tolerance of acid stress by *E. ictaluri*, urease activity enables growth under acid conditions through the neutralization of low pH levels. The hypothesized ability of

E. ictaluri to neutralize the acidic pH of the phagosome may also function to inhibit activation of lytic enzymes, further enhancing intracellular replication. The hypothesized degradation of arginine by AdiA for production of urea may have additional ramifications on *E. ictaluri* resistance to killing by HKDM. Because arginine is the primary substrate for NO production by iNOS (26), arginine depletion could serve to inhibit iNOS activity, similar to the function of arginase discussed above. AdiA activity in *E. ictaluri* could also provide urea for ammonia production by urease. Further studies for evaluation of the possible role of arginine cycling in *E. ictaluri* pathogenesis are in progress.

In conclusion, the *E. ictaluri* genome contains a PAI composed of seven genes carrying an operon involved in the production of a urease enzyme as well as associated urea and ammonia transporters. In vitro studies indicate that the *E. ictaluri* urease enzyme is activated by an acidic environment and that optimal activity occurs at low pH. Although *E. ictaluri* is acid tolerant to pH levels down to 3, acid tolerance is independent of urease activity. Urease activity assays demonstrated that *E. ictaluri* releases ammonia to the external environment when exogenous urea is available, resulting in an increase in the external pH from 5 or less to 6 or greater and suggesting that the urea and ammonia transporters play an important role. Because *E. ictaluri* is unable to grow unless pH levels are greater than pH 5 to 5.5, the shift to neutral pH levels enabled replication. Further, because urease activity is required for intracellular replication in channel catfish HKDM, it is hypothesized that urease functions to increase the pH of the *E. ictaluri*-containing phagosome, making the environment conducive to replication and to the development of the bacterium-filled, spacious phagosomes that are characteristic of *E. ictaluri*-infected HKDM (3).

ACKNOWLEDGMENTS

This research was supported by the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service, grant number 2002-35204-12605 (to Ronald L. Thune). MS was supported by NIH grant number P20 RR-016464 from the INBRE Program of the National Center for Research Resources.

REFERENCES

1. Atlas, R. M. 1993. Handbook of microbiological media. CRC Press, Boca Raton, FL.
2. Ausubel, F. M., R. E. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, NY.
3. Booth, N. J., A. ElKamel, and R. L. Thune. 2006. Intracellular replication of *Edwardsiella ictaluri* in channel catfish macrophages. *J. Aquat. Anim. Health* 18:101–108.
4. Burne, R. A., and Y. Y. Chen. 2000. Bacterial ureases in infectious diseases. *Microbes Infect.* 2:533–542.
5. Chang, C. I., J. C. Liao, and L. Kuo. 1998. Arginase modulates nitric oxide production in activated macrophages. *Am. J. Physiol.* 274:H342–H348.
6. Chang, Z., J. Kuchar, and R. P. Hausinger. 2004. Chemical cross-linking and mass spectrometric identification of sites of interaction for UreD, UreF, and urease. *J. Biol. Chem.* 279:15305–15313.
7. de Koning-Ward, T. F., and R. M. Robins-Browne. 1995. Contribution of urease to acid tolerance in *Yersinia enterocolitica*. *Infect. Immun.* 63:3790–3795.
8. de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* 172:6568–6572.
9. Elsinghorst, E. A. 1997. Measurement of invasion by gentamicin resistance, p. 667–681. In V. L. Clark and P. M. Bavoil (ed.), *Bacterial pathogenesis*. Academic Press, San Diego, CA.

10. Heimer, S. R., and H. L. Mobley. 2001. Interaction of *Proteus mirabilis* urease apoenzyme and accessory proteins identified with yeast two-hybrid technology. *J. Bacteriol.* **183**:1423–1433.
11. Heimer, S. R., R. A. Welch, N. T. Perna, G. Posfai, P. S. Evans, J. B. Kaper, F. R. Blattner, and H. L. Mobley. 2002. Urease of enterohemorrhagic *Escherichia coli*: evidence for regulation by fur and a *trans*-acting factor. *Infect. Immun.* **70**:1027–1031.
12. Jabri, E., M. B. Carr, R. P. Hausinger, and P. A. Karplus. 1995. The crystal structure of urease from *Klebsiella aerogenes*. *Science* **268**:998–1004.
13. Javelle, A., E. Severi, J. Thornton, and M. Merrick. 2004. Ammonium sensing in *Escherichia coli*. Role of the ammonium transporter AmtB and AmtB-GlnK complex formation. *J. Biol. Chem.* **279**:8530–8538.
14. Kim, K. Y., C. H. Yang, and M. H. Lee. 1999. Expression of the recombinant *Klebsiella aerogenes* UreF protein as a MalE fusion. *Arch. Pharm. Res.* **22**:274–278.
15. Lahiri, A., P. Das, and D. Chakravorty. 2008. Arginase modulates Salmonella induced nitric oxide production in RAW264.7 macrophages and is required for Salmonella pathogenesis in mice model of infection. *Microbes Infect.* **10**:1166–1174.
16. Maroncle, N., C. Rich, and C. Forestier. 2006. The role of *Klebsiella pneumoniae* urease in intestinal colonization and resistance to gastrointestinal stress. *Res. Microbiol.* **157**:184–193.
17. Marshall, B. J., L. J. Barrett, C. Prakash, R. W. McCallum, and R. L. Guerrant. 1990. Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effect of acid. *Gastroenterology* **99**:697–702.
18. Miller, N. W., and E. C. McKinney. 1994. *In vitro* culture of fish leukocytes, p. 339–351. In P. W. Hochachka and T. P. Mommsen (ed.), *Biochemistry and molecular biology of fishes*, vol. 3. Analytical techniques. Elsevier Science Publishers, New York, NY.
19. Mobley, H. L., M. D. Island, and R. P. Hausinger. 1995. Molecular biology of microbial ureases. *Microbiol. Rev.* **59**:451–480.
20. Mori, M. 2007. Regulation of nitric oxide synthesis and apoptosis by arginase and arginine recycling. *J. Nutr.* **137**:1616S–1620S.
21. Mulrooney, S. B., and R. P. Hausinger. 2003. Nickel uptake and utilization by microorganisms. *FEMS Microbiol. Rev.* **27**:239–261.
22. Nakamura, H., H. Yoshiyama, H. Takeuchi, T. Mizote, K. Okita, and T. Nakazawa. 1998. Urease plays an important role in the chemotactic motility of *Helicobacter pylori* in a viscous environment. *Infect. Immun.* **66**:4832–4837.
23. Sachs, G., J. A. Kraut, Y. Wen, J. Feng, and D. R. Scott. 2006. Urea transport in bacteria: acid acclimation by gastric *Helicobacter* spp. *J. Membr. Biol.* **212**:71–82.
24. Sambrook, J., and D. W. Russel. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. Schwartz, J. T., and L. A. Allen. 2006. Role of urease in megasome formation and *Helicobacter pylori* survival in macrophages. *J. Leukoc. Biol.* **79**:1214–1225.
26. Soriano, A., G. J. Colpas, and R. P. Hausinger. 2000. UreE stimulation of GTP-dependent urease activation in the UreD-UreF-UreG-urease apoprotein complex. *Biochemistry* **39**:12435–12440.
27. Stingl, K., E. M. Uhlemann, R. Schmid, K. Altendorf, and E. P. Bakker. 2002. Energetics of *Helicobacter pylori* and its implications for the mechanism of urease-dependent acid tolerance at pH 1. *J. Bacteriol.* **184**:3053–3060.
28. Thune, R. L. 1993. Bacterial diseases of catfishes, p. 511–520. In M. K. Stoskopf (ed.), *Fish medicine*. W. B. Saunders Company, Philadelphia, PA.
29. Thune, R. L., D. H. Fernandez, and J. R. Battista. 1999. An *aroA* mutant of *Edwardsiella ictaluri* is safe and efficacious as a live, attenuated vaccine. *J. Aquat. Anim. Health* **11**:358–372.
30. Thune, R. L., D. H. Fernandez, J. L. Benoit, M. Kelly-Smith, M. L. Rogge, N. J. Booth, C. A. Landry, and R. A. Bologna. 2007. Signature-tagged mutagenesis of *Edwardsiella ictaluri* identifies virulence-related genes, including a salmonella pathogenicity island 2 class of type III secretion systems. *Appl. Environ. Microbiol.* **73**:7934–7946.
31. Thune, R. L., L. A. Stanley, and R. K. Cooper. 1993. Pathogenesis of gram-negative bacterial infections in warmwater fish. *Annu. Rev. Fish Dis.* **3**:37–68.
32. Waltman, W. D., E. B. Shotts, and T. C. Hsu. 1986. Biochemical characteristics of *Edwardsiella ictaluri*. *Appl. Environ. Microbiol.* **51**:101–104.
33. Young, G. M., D. Amid, and V. L. Miller. 1996. A bifunctional urease enhances survival of pathogenic *Yersinia enterocolitica* and *Morganella morganii* at low pH. *J. Bacteriol.* **178**:6487–6495.