1 Title:

2 **Influence of Lipopolysaccharide Outer-Core in The Intrinsic Resistance to**
3 **Antimicrobial Peptides and Virulence in *Edwardsiella ictaluri***
4

5 Authors:

6 Taylor Martin^{1,2}, Ignacia Diaz^{1,3}, Jacquelyn Kilbourne¹, Oscar Almarza³, Cristopher
7 Segovia^{3,4}, Roy Curtiss III^{1,2} and Javier Santander^{1,2,3*}
8

9 ¹Center for Infectious Diseases and Vaccinology, The Biodesign Institute, Arizona State
10 University, Tempe, AZ 85287; ²School of Life and Sciences, Arizona State University,
11 Tempe, Arizona 85287; ³Microbial Pathogenesis and Vaccinology Research Group,
12 Faculty of Sciences, Universidad Mayor, Huechuraba, Chile 8580745; ⁴PhD Program in
13 Integrative Genomics, Faculty of Sciences, Universidad Mayor, Huechuraba, Chile
14 8580745.
15
16

17 Running Title:

18 ***E. ictaluri* LPS outer-core and Virulence**
19

20 *Corresponding Author: Javier Santander. Universidad Mayor, Faculty of Sciences,
21 Microbial Pathogenesis and Vaccinology Laboratory, Camino la Piramide 5750,
22 Huechuraba, 8580745, Chile. Phone: 56-9-57537405; e-mail: jasantander@asu.edu
23
24

ABSTRACT

The genus *Edwardsiella* consists of bacteria with an intrinsic resistance to cyclic cationic antimicrobial peptides (CAMPs). *E. ictaluri*, a pathogen of the catfish (*Ictalurus punctatus*) and the causative agent of a systemic infection, is highly resistant to CAMPs. Previously, we determined that the oligo-polysaccharide (O-PS) of the lipopolysaccharide (LPS) does not play a role in the *E. ictaluri* CAMP resistance and an intact core-lipid A structure is necessary for CAMPs resistance. Here, we evaluated the influence of the outer-core in the CAMPs resistance and fish virulence. *E. ictaluri wabG*, a gene that encodes for the UDP-glucuronic acid transferase that links the lipid A-inner-core to the outer-core–oligopolysaccharides, was deleted. Deletion of $\Delta wabG$ caused a pleiotropic effect, influencing LPS synthesis, CAMPs resistance, growth, and biofilm formation. *E. ictaluri* $\Delta wabG$ was attenuated in zebrafish indicating the important role of LPS during fish pathogenesis. Also, we evaluated the inflammatory effects of *wabG* LPS in catfish ligated loop model, showing a decreased inflammatory effect at the gut level respects to the *E. ictaluri* wild type. We conclude that *E. ictaluri* CAMPs resistance is related to the molecules present in the LPS outer-core and that fish gut inflammation triggered by *E. ictaluri* is LPS dependent, reinforcing the hypothesis that fish gut recognizes LPS in an O-PS dependent fashion.

Keyword: *Edwardsiella*, glucuronic acid transferase, O-polysaccharide, fish virulence

1. Introduction

The genus *Edwardsiella* consists of five species *E. tarda*, *E. hoshinae*, *E. piscicida*, *E. anguillarum* and *E. ictaluri*. *Edwardsiella* species are intrinsically resistant to cationic antimicrobial peptides (CAMPs) [1, 2]. *E. ictaluri* is one of the most common pathogens of the channel catfish (*Ictalurus punctatus*) aquaculture [3], and it has the highest reported resistance to CAMPs [1].

The lipopolysaccharide (LPS) is the major constituent of the outer layer of the outer membrane of Gram-negative bacteria. The LPS is composed of the lipid A (the hydrophilic membrane anchor), the core region (a non-repeating oligosaccharide), and the O-polysaccharide (O-PS; an external, repeating oligosaccharide) [4, 5]. Recently, we determined that the LPS plays a critical role in *E. ictaluri* CAMP resistance. Specifically, an intact core-lipid A molecule of the *E. ictaluri* LPS is essential for the resistance to CAMPs [1]. The complete LPS structure of *E. ictaluri* is unknown. Nevertheless, the composition and structure of the *E. ictaluri* O-PS have been reported [6]. The *E. ictaluri* O-PS biosynthesis enzymes are encoded by four genes, *wibT* (NT01EI_1309), *galF* (NT01EI_1310), *gne* (NT01EI_1311) and *ugd* (NT01EI_1312), located in the O-PS gene cluster [1, 7]. As mentioned previously, we determined that the LPS O-PS does not play a role in the *E. ictaluri* CAMP resistance and an intact core-lipid A structure is necessary for CAMPs resistance. For instance, deletion of *wibT* and *gne* genes generated rough mutants, affecting the O-PS synthesis but not the CAMPs resistance [1]. In contrast, deletion of *ugd* gene caused *E. ictaluri* CAMPs sensitivity and core-lipid A alterations [1]. *E. ictaluri* *ugd* gene is predicted to encode for a UDP-glucose dehydrogenase that oxidizes UDP-glucose into UDP-glucuronic acid (UDP-GlcA) [7]. UDP-GlcA is the

precursor for the synthesis of molecules related to CAMP resistance, for instance, UDP-4-amino-4-deoxy-L-arabinose (L-Ara4N) [4, 8], which is a crucial element in bacterial resistance to CAMPs [9, 10].

The LPS core can be divided into two structural regions, the inner-core (lipid A proximal) and the outer-core. The inner-core typically contains residues of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and L-glycerol-D-mannose in contrast to the outer-core that is diverse [4, 11]. The glucuronic acid transferase (called WaaG, RfhH or WabG) links the outer-core with the inner-core of the LPS [11]. Here, we evaluated whether the LPS outer-core molecules play a role in the *E. ictaluri* in the CAMPs resistance, biofilm formation, and fish virulence mediated deletion of the glucuronic acid transferase (WabG; NT01EI_0064) encoding gene.

2. Material and Methods

2.1. Ethics statement. All research involving animals was conducted as per protocol # 09-1042R, approved by the Arizona State University Institutional Animal Care and Use Committee.

2.2. Bacterial strains, plasmids, media, and reagents. The bacterial strains and plasmids are listed in Table 1. Bacteriological media and components are from Difco (Franklin Lakes, NJ). Antibiotics and reagents are from Sigma (St. Louis, MO). LB broth (tryptone, 10 g; yeast extract 5 g; NaCl, 10 g; ddH₂O, 1L) [12] and Bacto-Brain Heart Infusion (BHI) were used routinely. When required, the media was supplemented with 1.5% agar, 6% sucrose, colistin (Col; 12.5 µg/ml), polymyxin B (Pmb; 20 µg/ml), protamine (Pro; 20,000 µg/ml), ampicillin (Amp; 100 µg/ml), chloramphenicol (Cm; 25 µg/ml), gentamicin (Gm; 10 µg/ml) or kanamycin (Km; 50 µg/ml). Bacterial growth was monitored spectrophotometrically and by plating. Oligonucleotides were from IDT (Coralville, IA). Restriction endonucleases were from New England Biolabs (Ipswich, MA). Taq DNA polymerase (New England Biolabs) was used in all PCR tests. Qiagen products (Hilden, Germany) were used to isolate plasmid DNA, fragment purification or PCR cleanup. T4 ligase, T4 DNA polymerase, and shrimp alkaline phosphatase (SAP) were from Promega (Fitchburg, WI).

2.3. Construction and characterization of *E. ictaluri* with defined deletions. The recombinant pEZ193 suicide vector (Table 1) carrying the linked flanking regions to

120 generate an in-frame deletion of the *wabG* gene (NT01EI_0064) (Fig. 1A) was
 121 constructed as described previously [13]. The defined deletion mutations encompass a
 122 deletion including the ATG start codon, but not including the TAG stop codon. Primers 1
 123 (F1 (*SphI*) 5'-ACATGCATGC GCTCTTTCTCAGGCGGGCTACCCTA-3') and 2 (R1
 124 (*XhoI-PstI*) 5'-
 125 CTCGAGCGGAAACTGCAGTTTTTCAAGTAACCTCGTTGCCGCCGCGA-3')
 126 were designed to amplify the upstream gene-flanking region (443 bp). The downstream
 127 gene-flanking region (445 bp) was amplified by primers 3 (F2 (*PstI-XhoI*) 5'-
 128 AAAACTGCAGTTTTCCGCTCGAGTAAATCATGCGAATTTTAATGATTA-3') and
 129 4 (R2 (*XbaI*) 5'-TCGTCTAGACATCGGCTGAAGCCATGGCGATGTC-3'). The
 130 flanking regions were ligated (888 bp) and cloned into pMEG-375 digested with *SphI* and
 131 *XbaI*. To construct *E. ictaluri* mutants, the suicide plasmid was conjugationally
 132 transferred from *Escherichia coli* χ 7213 [14] to *E. ictaluri* strains. Strains containing
 133 single-crossover plasmid insertions were isolated on BHI agar plates containing Col and
 134 Amp. Loss of the suicide vector after the second recombination between homologous
 135 regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity
 136 counter-selection system [15] adapted to *E. ictaluri* [13, 16]. The colonies were selected
 137 for Amp^s, Col^r and screened by PCR using primers 1 and 4 (Fig. 1B). DNA sequencing
 138 analysis of the genome segment mutated was performed at the DNA sequencing core
 139 laboratory, Arizona State University. Biochemical profiles of *E. ictaluri* strains were
 140 determined using the API 20E system (bioMérieux, Marcy l'Etoile, France). The $\Delta wabG$
 141 mutants were screened for Amp^s, Col^s, and screening by PCR. Biochemical profiles of *E.*

ictaluri strains were determined using the API 20E system (bioMérieux, Marcy l'Etoile, France).

2.4. Sequence analysis. Nucleotide Basic Local Alignment Search Tool (BLAST) was performed based on the sequences of the putative O-PS genes present in the genome sequence of *E. ictaluri* 93-146 accessed from NCBI's Entrez Genome database (NC_012779). Amino acid sequence alignments were performed using the CLC Genomics Workbench software tool (v. 8.5 CLC bio A/S, Aarhus, Denmark). Protein structural-based alignments were performed by using the web-based interface for ESPript v.2.2 located at <http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi> [17]. The 3D structure of *E. ictaluri* proteins were predicted by using position specific iterative - BLAST (PSI-BLAST) alignment and HHpred [18]. Phylogenetic position of *E. ictaluri* WabG protein was performed with MEGA6 [19] mediated neighbor-joining method [20]. Bootstrap analysis was performed with 500 resamplings. The evolutionary distances were computed using the Poisson correction method [21] and are in the units of the number of amino acid substitutions per site.

2.5. Complementation of *E. ictaluri* mutants. The gene *wabG* was cloned independently into pEZ151 (Table 1) under P_{lac} control at the *AdhI* restriction site. The primers used to amplify the corresponding genes under P_{lac} control were F 5'-TTTACACTTTATGCTTCCGGCTCGTATGTTATGACCCAGTTCAGATTGGC-3' and R 5'-TTACTTCACCAGTTGTTCGGT-3'. The resulting plasmid pEZ251 was used to complement the *E. ictaluri* $\Delta wabG$ and *Salmonella enterica* $\Delta waaG$ mutant strains (Table 1). Expression of *wabG* in *Salmonella* was verified by RT-PCR.

166

167 **2.6. LPS purification and analysis.** Lipopolysaccharide (LPS) extraction was performed
168 by using TRI-reagent (Sigma) as described previously [22]. LPS profile was evaluated by
169 sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by silver
170 staining [23, 24].

171

172 **2.7. Minimal inhibitory concentration (MIC) assays.** Assays were performed according
173 to the British Society for Antimicrobial Chemotherapy guidelines [25]. Minimal
174 inhibitory concentrations (MIC) were determined in Müller-Hinton broth supplemented
175 with 5% of calf fetal bovine serum (HyClone, Canada) by microplate serial dilution assay
176 [25]. This assay was performed using flat bottom 96-well clear microtitre plates. 20,000
177 µg/ml of polymyxin B (Pmb), 12,500 µg/ml of colistin sulfate (Col) and 20,000 µg/ml of
178 protamine (Pro) were serially diluted and then inoculated with mid-log-phase cultures of
179 the *E. ictaluri* strains. The plates were incubated for 48 h at 28°C. After incubation for 48
180 h at 28°C, the bacterial growth was spectrophotometrically measured at 600nm.

181

182 **2.8. Bile Sensitivity.** Sensitivity to bile was determined by the microplate serial dilution
183 assay. This assay was performed using flat bottom 96-well clear microtitre plates. Ox bile
184 and sodium deoxycholate were serially diluted in BHI broth and then inoculated with
185 mid-log-phase cultures of the *E. ictaluri* strains. The plates were incubated for 48 h at
186 28°C. After incubation for 48h at 28°C, the bacterial growth was spectrophotometrically
187 measured at 600nm [1].

188

2.9. Quantitative bacterial adherence assay. To assess adherence, we inoculated 200 µL of BHI or LB broth in 96-well flat-bottom microtiter polystyrene plates (Becton Dickinson, Franklin Lakes, NJ) with 5 µl of an overnight culture of *E. ictaluri* [1]. The plates were incubated statically for 7 days at 28°C and visualized by staining with 0.5% crystal violet for five minutes after washing with water. The bacterial adherence was quantified in duplicate, after adding 200 µl of 95% ethanol, by an enzyme-linked immunosorbent assay plate reader at 570 nm.

2.10. Outer membrane protein profiles. Sarkosyl-insoluble outer membrane proteins (OMPs) were obtained as previously described [13]. The outer membrane proteins were normalized to 25 µg/µl by using a Nanodrop spectrophotometer (ND-1000, NanoDrop) and separated by 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Coomassie blue staining was performed to visualize proteins.

2.11. Infection and immunization of zebrafish (*Danio rerio*). The zebrafish challenges were performed by the methodology described previously [13]. The water temperature was 26 ± 1 °C, the fish were acclimated during the 2 weeks before experimentation. Adult zebrafish Tab5 (average weight, 0.5 g; age 3-5 month) were sedated in 100 mg/L tricaine methanesulfonate (MS-222, Sigma), then injected (needle size 30G½) intramuscularly (i.m.) with the corresponding bacterial inoculate. Two sets of controls were used: fish that were injected with 10 µl of sterile phosphate-buffered saline (PBS) and fish that were non-injected. Moribund fish demonstrating clinical signs were euthanized and necropsied, and bacteria were isolated. Survivors of each dose at 6 weeks

post-injection (42 dpi) were challenged with 10^6 CFU of *E. ictaluri* wild type. The fish were fed daily with TetraMin Tropical Fish Flake Feed twice daily. During the experiments, the fish were observed daily, and every other day water quality was monitored for pH, NH_4 and NO_2 with standard kits (Lifeguard, Aquatics). Fish care and use was performed in agreement with the requirements of the Arizona State University, Institutional Animal Care and Use Committee.

2.12. Bacteria inoculate preparation. Bacterial strains were grown overnight in standing cultures (without aeration) and then diluted 1:20 in pre-warmed BHI broth and grown with mild aeration (180 rpm) at 28°C to an OD_{600} of 0.8 to 0.9 ($\sim 10^8$ CFU/ml). Bacteria were sedimented during 10 min by centrifugation (6,300 g) at room temperature and resuspended in PBS to densities appropriate for the inoculation. Plate counting was performed on BHI agar.

2.13. Intestinal loops rabbit surgery. The surgery was performed according to the literature [26]. Briefly, three rabbits were fasted (but provided *ad lib* water) overnight (maximum 16 h) and then pre-medicated with 30 mg/kg ketamine and 6 mg/kg xylazine intramuscular injection (i.m). The rabbits were monitored for depth of anesthesia using pulse oximetry, ventilatory rate, and the lack of a toe pinch reflex. An approximately 10 cm midline incision was made through the skin and body wall. The most distal region of the ileum was isolated, and a 4-6 cm section was double-ligated at each end. Approximately 6 to 9 intestinal loops were created, each at 1-2 cm apart. After creation of all loops, 1.0 ml of LPS (100 μg) or bacterial culture (about 10^7 CFU) or sterilized

saline (NaCl 0.85%), as negative control was injected into each loop. The body wall and skin were closed, and the rabbits were maintained under anesthesia for 6 h. After 6 h, the rabbits were euthanized. The intestinal loops were then removed, the length of each segment measured, and the intestinal fluid extracted with the volume-to-length ratio in milliliters per centimeter for each loop recorded. A piece of the intestinal tissue was fixed in 10% formalin and subjected to histopathological study.

2.14. Channel catfish surgery. The channel catfish intestinal loop was performed according to Santander et al. [26]. Briefly, three outbreed channel catfish specific-pathogen-free with a mean weight of 20 kg $\pm$ 5 g were used. The animals were acclimatized during 1 week before to surgery in 100-liter tanks at 26 $\pm$ 1°C. Each tank was equipped with a re-circulating, biofiltered, mechanically filtered, and U.V. water treated system with 12 h light cycle per day. The water quality was monitored for pH, NO₂, and NO₃ with standard kits. Two days prior surgery the animals were fastened. The fish were anesthetized with buffered tricaine methanesulfonate (pH 7.5) [27]. The fish was moved to the surgery platform and connected to the surgical anesthesia dose [28, 29]. Once the fish was fully anesthetized, its body wall was cut, and the coelomic cavity entered via blunt dissection. The lower small intestine was isolated, and up to 6 sections of 3 cm each was prepared by double-ligation. One hundred μ g of purified LPS in 500 μ l of phosphate saline buffer (PBS) or 500 μ l of 10⁷ CFU/ml of *E. ictaluri* strains were injected into each intestinal section. The control consisted of loops inoculated with PBS, OMPs (100 μ g) or peptidoglycan (100 μ g; Sigma). After injection, the body wall was sutured closed, and the fish was moved to recovery anesthesia dose where it remained for

6 h. After this period, the fish was euthanized. The intestinal loops were then removed, the length of each segment measured, and the intestinal fluid extracted with the volume-to-length (V:L) ratio in milliliters per centimeter for each loop recorded. A piece of the intestinal tissue was fixed in 10% formalin and subjected to histopathological study. Each slide was graded by the degree of mucosal disruption, cellularity, and vascular congestion.

2.15. Statistics. An ANOVA (SPSS Software) analysis, followed by LSD (Least Significant Difference) method, was used to evaluate differences in bacterial titers discerned to 99% confidence intervals. $P<0.01$ was considered statistically significant.

3. Results

3.1. Mutant strain construction and lipopolysaccharide profile of *E. ictaluri* $\Delta wabG$.

The mutant was obtained by deletion of the gene, including the ATG start codon, but not including the TAG stop codon. The LPS profile of J148 $\Delta wabG$ mutant showed a lower molecular weight in contrast to *E. ictaluri* J100 wild type (Fig. 1A). The mutation was verified by sequencing the PCR fragment. The LPS profile was partially restored in the complemented mutant by P_{lac} *wabG* in pEZ251 (Fig. 1A).

3.2. Outer membrane protein analysis. *E. ictaluri* $\Delta wabG$ up-regulated an outer membrane protein of ~48 kDa identified as OmpN (Fig. 1B). Previously, we described this protein in *E. ictaluri* LPS mutants of $\Delta wibT$, Δgne and Δugd [1], suggesting that this protein is related to membrane stress. However, further studies are required to determine the role of OmpN in *E. ictaluri*.

3.3. Bacterial growth. *E. ictaluri* $\Delta wabG$ showed a shorter exponential growth phase and a lower total yield compared to the wild type (Fig. 1C). Agglutination was observed during growth in liquid media, similar to other *E. ictaluri* LPS mutants [1]. The growth rate and the lack of agglutination were recovered in the complemented mutant (Fig. 1C).

305

306 **3.4. Biofilm.** *E. ictaluri* wild type does not produce biofilm, either in presence or absence
307 of CAMPs [1]. *E. ictaluri* $\Delta wabG$ produced biofilm in rich media like BHI or LB in the
308 absence of CAMPs (Fig. 1D). The wild type phenotype was partially restored in the
309 complemented strain with pEZ251 (Fig. 1D).

310

311 **3.5. Antimicrobial peptide resistance.** As mentioned above, in general, bacterial
312 resistance to antimicrobial peptides is mediated by different mechanisms, including
313 export of peptides by efflux pumps, proteolytic degradation, and alteration of the
314 bacterial surface charge [30]. Previously, we determined that *Edwardsiella* intrinsic
315 resistance to CAMPs is not mediated by the O-PS, efflux-pumps or proteases but is
316 mediated by the core [1].

317 To determine if the *Edwardsiella* intrinsic constitutive resistance to CAMPs is
318 mediated by the outer-core, *E. ictaluri* $\Delta wabG$ was constructed. The resulting *E. ictaluri*
319 $\Delta wabG$ mutant strain J148 was extremely sensitive to CAMPs, in contrast to *E. ictaluri*
320 wild type, Δ gne O-PS mutant and *Edwardsiella* species control (Table 2). This phenotype
321 was partially reverted in the complemented strain (Table 2). This result indicates that the
322 outer-core plays an important role in the intrinsic resistance to CAMPs.

323

324 **3.6. Sequence Analysis.** *E. ictaluri* WabG has similar secondary structure to *S. enterica*
325 and *E. coli* WaaG (Fig. 2A). However, the glycotransferase C-terminal domain lack of the
326 residues Ala286, Ala287, Gly288, Ile289, at the catalytic active site (Fig. 2A). The
327 predicted 3D structure did not show significant differences between *Salmonella* WaaG

and *E. ictaluri* WabG (data not shown). The *E. ictaluri* WabG sequence presented “Rossmann-like” ($\beta/\alpha/\beta$) domains of a typical glycotransferase [31]. The N-terminal domain, from Ile5–Ala178, displays seven parallel β strands flanked by five α helices. The C-terminal domain (residues Ile179–Val373) displays six parallel β strands flanked by six α helices and terminates with the recurrent pattern of α kinked α helix that crosses over to the N-terminal domain, adding an extra α helix to the N-terminal Rossmann-like fold. The phylogenetic tree showed that *E. ictaluri* WabG diverged together with *E. tarda* WabG and *Klebsiella pneumoniae* WabG (Fig. 2B).

3.7. Complementation of *Salmonella enterica* $\Delta waaG$ with *E. ictaluri* *wabG* gene.

WabG is an ortholog of WaaG glucuronic acid transferase enzyme that links the outer core to the inner core in *Salmonella enterica*. The structure of the LPS core is relatively stable among the *Enterobacteriaceae* family. Here we evaluated whether *wabG* gene complement *S. enterica* $\Delta waaG$. Although, expression of *E. ictaluri* *wabG* in *S. enterica* was verified by RT-PCR, it does not complement *S. enterica* $\Delta waaG$ strain.

3.8. Virulence of *E. ictaluri* $\Delta wabG$ in zebrafish.

We found that *E. ictaluri* $\Delta wabG$ i.m administrated has attenuated virulence in zebrafish (Fig. 3). The survivors were challenged with *E. ictaluri* wild type (LD_{50} 100; 10^6 CFU/dose). We found that immunization with *E. ictaluri* $\Delta wabG$ did not confer protection to zebrafish (data not shown).

3.9. Intestinal loops model.

We evaluated the effect of *E. ictaluri* $\Delta wabG$ mutant strain

and its purified LPS in the catfish intestinal loop model and compared it to the rabbit intestinal loop model. We found that purified LPS from *E. ictaluri* $\Delta wabG$ mutant triggered similar fluid secretion in rabbit intestinal loops compared to LPS from *E. ictaluri* wild type (Fig. 4A). Purified LPS from *Salmonella enterica* triggered a very high fluid secretion in rabbit intestinal loops (Fig. 4A). In contrast, in catfish intestinal loops we found that *E. ictaluri* $\Delta wabG$ mutant and purified LPS triggered lower fluid secretion than *E. ictaluri* wild type and control Δugd (Figs. 4B and 4C). *Salmonella enterica* and its purified LPS triggered similar fluid secretion in catfish intestinal loops than *E. ictaluri* wild type (Figs. 4B and 4C).

The histopathology evaluation indicates that *E. ictaluri* $\Delta wabG$ mutant and its purified LPS did not cause tissue damage in both, rabbit and catfish intestinal loops. In contrast, purified *Salmonella* LPS caused a significant damage in rabbits, which correlates with the fluid secretion levels (Fig. 4A). *E. ictaluri* wild type trigger significant tissue damage in catfish gut, in contrast to the the *E. ictaluri* mutants and *Salmonella* (Fig. 4D). These results reinforce the notion that fish recognize different structures of the LPS than mammals.

4. Discussion

Previously, we determined that the LPS O-PS does not play a role in the *E. ictaluri* CAMP resistance and an intact core-lipid A structure is necessary for CAMPs resistance [1]. UDP-glucuronic acid dehydrogenase (*ugd*) mutants are CAMPs sensitive and show an altered core-lipid A profile [1]. Here, we evaluate whether the outer-core is related to CAMPs resistance and fish virulence. UDP-glucuronic acid transferase (WabG) links the lipid A–inner core to the outer core–oligopolysaccharides [34, 35]. We determined that deletion of $\Delta wabG$ triggered a pleiotropic effect in *E. ictaluri*, influencing LPS synthesis (Fig. 1A), OMP profile (Fig. 1B), growth (Fig. 1C), biofilm formation (Fig. 1D), CAMPs resistance (Table 2) and fish virulence (Fig. 3). *E. ictaluri* $\Delta wabG$ showed a similar phenotype than *E. ictaluri* Δugd [1], suggesting that the resistance to CAMPs is related to the molecules localized in the outer-core LPS.

Complementation of *Salmonella enterica* $\Delta waaG$ with *E. ictaluri* *wabG* gene was not successful. Although, *E. ictaluri* WabG and *S. enterica* WaaG have similar 2D and 3D structure, the active sites are not fully conserved (Fig. 2A). Perhaps, due to the lack of the residues Ala286, Ala287, Gly288 and Ile289, *E. ictaluri* WaaG cannot complement *S. enterica*. This might reflect differences in the LPS core structure between *E. ictaluri* and *Salmonella*. For instance, *E. ictaluri* WaaG has a similar phylogeny to *Klebsiella*

pneumoniae (Fig. 2B), which also has different LPS core structure than other Gram-negative bacteria [36].

E. ictaluri $\Delta wabG$ was attenuated in zebrafish (Fig. 3) indicating the important role of LPS and CAMP resistance during fish pathogenesis, as was previously demonstrated in others *E. ictaluri* mutants [1]. The survivors were challenged with *E. ictaluri* wild type (100 LD₅₀ or 10⁶ CFU/dose) four weeks post inoculation with no survivors post challenge. *E. ictaluri* $\Delta wabG$ did not confer immune protection, possibly due to its inability to resist fish CAMPs.

In mammals, the lipid A portion of the LPS acts as a toxin by over-stimulating the TLR-4 innate immune signaling, which induces inflammatory responses [37-41]. On the other hand, it is well established that fish are very resistant to the toxic effects of LPS [1, 42-44]. Several reports suggest that fish do not respond to LPS because they lack essential components for the TLR4 function, like PLB, CD14, MD2, and TCAM2 [43]. Indeed, more evolutionary advanced pufferfish lack TLR-4 orthologs [45, 46]. Also, it is suggested that LBP, CD14, MD2, and TCAM2 have recently arisen during vertebrate evolution, being limited only to higher vertebrates [43, 47]. Previously, we showed that modification of the O-PS leads to a different inflammatory response in catfish intestine [1, 26]. Here we evaluated the inflammatory intestinal effects of *E. ictaluri* *wabG* LPS in catfish ligated intestinal loops, compared to the rabbit ileal loops model. We determined that in rabbit, *E. ictaluri* $\Delta wabG$ LPS triggered similar fluid secretion than the loops injected with *E. ictaluri* wild type LPS, suggesting that the lipid A structure have not been modified (Figs. 4A). In contrast to *E. ictaluri* Δugd , which likely has modifications in the core and the lipid A [1], triggered lower fluid secretion (Fig. 4A). In catfish

intestinal loops, *E. ictaluri* $\Delta wabG$ showed similar results than *E. ictaluri* Δugd and a lower fluid secretion respect to *E. ictaluri* wild type (Figs. 4B and 4C). Both, *E. ictaluri* $\Delta wabG$ LPS, and *E. ictaluri* $\Delta wabG$ mutant did not cause tissue damage in contrast to *E. ictaluri* wild type LPS (Fig 4D). Previously, we showed that fish recognize LPS at the intestine level in an O-PS dependent fashion [1, 26]. The results obtained here reinforced the idea that fish recognize the O-PS portion of the LPS at the gut level. It is evident that aquatic vertebrates interact with a much higher bacterial load in their living environment than land vertebrates, especially trough the mucosal tissues. We believe that due to the selective pressure, imposed by the aquatic microbiome, fish evolution did not favor high sensitivity to LPS. However, the mechanisms of detection and response to high levels of LPS are still unknown.

443

444

445

446

447

448 **Acknowledgments**

449

450 This work was supported by USDA grant CRIS-ARZR-2009-01801, SoLARA
451 Fellowship (School of Life Sciences, Arizona State University), Comisión Nacional de
452 Investigación Científica y Tecnológica (CONICYT), Gestión Propia Fellowship
453 12864830-5, grant FONDECY Regular competition 1140330, grant FIC BIP30170275-0,
454 Chile and grant COPEC-UC 2014.J0.71. We thank to Erika Arch, Paul Hartig, Tina
455 Hartig (Arizona State University) and Francisca Rodriguez (Universidad Mayor) for their
456 logistic support.

457 .

458 **Conflict of interests**

459

460 The authors declare that they have no competing interests.

461

462

463

464

465

466

467

468

REFERENCES

- [1] Santander J, Martin T, Loh A, Pohlenz C, Gatlin DM, Curtiss R. Mechanisms of intrinsic resistance to antimicrobial peptides of *Edwardsiella ictaluri* and its influence on fish gut inflammation and virulence. Microbiol 2013; 159:1471-86
- [2] Stock I, Wiedemann B. Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae*. Antimicrob Agents Chemother 2001; 45:2245-55.
- [3] Harvey D. Aquaculture Outlook. USDA, ERS, LDP – AQS-23. 2006.
- [4] Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem. 2002; 71:635-700.
- [5] Heinrichs DE, Yethon JA, Amor PA, Whitfield C. The assembly system for the outer core portion of R1- and R4-type lipopolysaccharides of *Escherichia coli*. The R1 core-specific beta-glucosyltransferase provides a novel attachment site for O-polysaccharides. J Biol Chem 1998; 273:29497-505.
- [6] Vinogradov E, Nossova L, Perry MB, Kay WW. The structure of the antigenic O-polysaccharide of the lipopolysaccharide of *Edwardsiella ictaluri* strain MT104. Carbohydr Res 2005; 340:1509-13.
- [7] Lawrence ML, Banes MM, Azadi P, Reeks BY. The *Edwardsiella ictaluri* O polysaccharide biosynthesis gene cluster and the role of O polysaccharide in resistance to normal catfish serum and catfish neutrophils. Microbiol 2003; 149:1409-21.
- [8] Breazeale SD, Ribeiro AA, Raetz CR. Oxidative decarboxylation of UDP-glucuronic acid in extracts of polymyxin-resistant *Escherichia coli*. Origin of

- lipid a species modified with 4-amino-4-deoxy-L-arabinose. J Biol Chem 2002; 277:2886-96.
- [9] Guo L, Lim KB, Poduje CM, Daniel M, Gunn JS, Hackett M, et al. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 1998; 95:189-98.
 - [10] Trent MS, Ribeiro AA, Lin S, Cotter RJ, Raetz CR. An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. J Biol Chem 2001; 276:43122-31.
 - [11] Heinrichs DE, Yethon JA, Whitfield C. Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. Mol Microbiol 1998; 30:221-32.
 - [12] Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 1951; 62:293-300.
 - [13] Santander J, Mitra A, Curtiss R, 3rd. Phenotype, virulence and immunogenicity of *Edwardsiella ictaluri* cyclic adenosine 3',5'-monophosphate receptor protein (Crp) mutants in catfish host. Fish Shellfish Immunol 2011; 31:1142-1153.
 - [14] Roland K, Curtiss R, 3rd, Sizemore D. Construction and evaluation of a delta cya delta crp *Salmonella typhimurium* strain expressing avian pathogenic *Escherichia coli* O78 LPS as a vaccine to prevent airsacculitis in chickens. Avian Dis 1999; 43:429-41.
 - [15] Edwards RA, L. H. Keller, and D. M. Schifferli. Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene 1998; 207:149-57.

- [16] Santander J, Xin W, Yang Z, Curtiss RIII. The aspartate-semialdehyde dehydrogenase of *Edwardsiella ictaluri* and its use as balanced-lethal system in fish vaccinology. Plos One 2010; 5:e15944.
- [17] Gouet P, Courcelle E, Stuart DI, Metoz F. ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics 1999; 15:305-8.
- [18] Soding J, Biegert A, Lupas AN. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res 2005; 33:W244-8.
- [19] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013; 30:2725-9.
- [20] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-25.
- [21] Zuckerkandl E. Remarks on the evolution of polynucleotides compared to that of polypeptides. Bull Soc Chim Biol (Paris) 1965; 47:1729-30.
- [22] Yi EC, Hackett M. Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. Analyst 2000; 125:651-6.
- [23] Hitchcock PJ, Brown TM. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J Bacteriol 1983; 154:269-77.
- [24] Tsai CM, Frasch CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 1982; 119:115-9.
- [25] CLSI, Clinical and Laboratory Standards Institute. (2005b). Methods for broth dilution susceptibility testing of bacteria isolated from aquatic animals; Proposed guideline. CLSI document M49-P [ISBN 1-56238- 577-1]. Clinical and

laboratory standards institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.

- [26] Santander J, Kilbourne J, Park JY, Martin T, Loh A, Diaz I, et al. Inflammatory effects of *Edwardsiella ictaluri* lipopolysaccharide modifications in catfish gut. *Infect Immun* 2014; 82:3394-404.
- [27] Popovic NT, I. Strunjak-Perovic, R. Coz-Rakovac, J. Barisic, M. Jadan, A. Persin, Berakovic, R. Sauerborn Klobucar. Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. *J Appl Ichthyol* 2012; 28:553-64.
- [28] Brown LA. Recirculation anaesthesia for laboratory fish. *Lab Anim* 1987; 21:210-5.
- [29] Harms CA. Surgery in fish research: common procedures and postoperative care. *Lab Anim (NY)* 2005; 34:28-34.
- [30] Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol* 2006; 4:529-36.
- [31] Martinez-Fleites C, Proctor M, Roberts S, Bolam DN, Gilbert HJ, Davies GJ. Insights into the synthesis of lipopolysaccharide and antibiotics through the structures of two retaining glycosyltransferases from family GT4. *Chem Biol* 2006; 13:1143-52.
- [32] Petrie-Hanson L, Romano CL, Mackey RB, Khosravi P, Hohn CM, Boyle CR. Evaluation of zebrafish *Danio rerio* as a model for enteric septicemia of catfish (ESC). *J Aquat Anim Health* 2007; 19: 151-8.
- [33] Santander J, Golden G, Wanda SY, and Curtiss RIII. The Fur regulated iron uptake system of *Edwardsiella ictaluri* and its influence on pathogenesis and immunogenicity in the catfish host. *Infect Immun* 2012; 80: 2689-2703.

- [34] Qian J, Garrett TA, Raetz CR. In vitro assembly of the outer core of the lipopolysaccharide from *Escherichia coli* K-12 and *Salmonella typhimurium*. *Biochemistry* 2014; 53:1250-62.
- [35] Yethon JA, Vinogradov E, Perry MB, Whitfield C. Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation. *J Bacteriol* 2000; 182:5620-3.
- [36] Izquierdo L, Coderch N, Pique N, Bedini E, Corsaro MM, Merino S, et al. The *Klebsiella pneumoniae wabG* gene: role in biosynthesis of the core lipopolysaccharide and virulence. *J Bacteriol* 2003; 185:7213-21.
- [37] Gioannini TL, Teghanemt A, Zhang D, Levis EN, Weiss JP. Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation. *J Endotoxin Res* 2005; 11:117-23.
- [38] Mathison JC, Tobias PS, Wolfson E, Ulevitch RJ. Plasma lipopolysaccharide (LPS)-binding protein. A key component in macrophage recognition of gram-negative LPS. *J Immunol* 1992; 149:200-6.
- [39] O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 2007; 7:353-64.
- [40] Tobias PS, Soldau K, Gegner JA, Mintz D, Ulevitch RJ. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. *J Biol Chem* 1995; 270:10482-8.
- [41] Vogel SN, Fenton M. Toll-like receptor 4 signalling: new perspectives on a complex signal-transduction problem. *Biochem Soc Trans* 2003; 31:664-8.

- [42] Berczi I, Bertok L, Bereznoi T. Comparative studies on the toxicity of *Escherichia coli* lipopolysaccharide endotoxin in various animal species. *Can J Microbiol.* 1966; 12:1070-1.
- [43] Iliev DB, Roach JC, Mackenzie S, Planas JV, Goetz FW. Endotoxin recognition: in fish or not in fish? *FEBS Lett* 2005; 579:6519-28.
- [44] Swain P, Nayak SK, Nanda PK, Dash S. Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: a review. *Fish Shellfish Immunol* 2008; 25:191-201.
- [45] Oshiumi H, Tsujita T, Shida K, Matsumoto M, Ikeo K, Seya T. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. *Immunogenetics* 2003; 54:791-800.
- [46] Jault C, Pichon L, Chluba J. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. *Mol Immunol* 2004; 40:759-71.
- [47] Sepulcre MP, Alcaraz-Perez F, Lopez-Munoz A, Roca FJ, Meseguer J, Cayuela ML, et al. Evolution of lipopolysaccharide (LPS) recognition and signaling: fish TLR4 does not recognize LPS and negatively regulates NF-kappaB activation. *J Immunol* 2009; 182:1836-45.
- [48] Curtiss RIII. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. *J Bacteriol* 1965; 89:28-40.
- [49] Chakraborty S, Sivaraman J, Leung KY, Mok YK. The two-component PhoB-PhoR regulatory system and ferric uptake regulator sense phosphate and iron to control virulence genes in type III and VI secretion systems of *Edwardsiella tarda*. *J Biol Chem* 2011; 285:38876-88.

- [50] Santander J, Wanda SY, Nickerson CA, Curtiss R, 3rd. Role of RpoS in fine-tuning the synthesis of Vi capsular polysaccharide in *Salmonella enterica* serotype Typhi. Infect Immun 2007; 75:1382-92.

FIGURE LEGENDS

Figure 1. *E. ictaluri* $\Delta wabG12$ phenotype and complementation. **A.** LPS profile of *E. ictaluri* $\Delta wabG12$ pEZ251 complemented; **B.** Outer membrane profile of *E. ictaluri* $\Delta wabG12$ pEZ251 complemented; **C.** in vitro growth in BHI at 28°C with aeration (180 rpm); **D.** Quantification of surface adhesion after 7 days of incubation in BHI broth at 28°C. The samples correspond to three independent experiments. * $P < 0.001$.

Figure 2. *E. ictaluri* WabG. **A.** Protein alignment and secondary structure; the top secondary structure correspond to *E. ictaluri* WabG; the bottom secondary structure correspond to *S. enterica* WaaG; **B.** *E. ictaluri* WabG phylogeny. The evolutionary history was inferred using the Neighbor-Joining method. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The analysis involved 50 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 288 positions in the final dataset. The * represent the donor site interactions of the WaaG glycosyltransferase. Bottom *: residues missing in *E. ictaluri* WabG.

Figure 3. *E. ictaluri* $\Delta wabG12$ virulence in zebrafish (*D. rerio*). **A.** *E. ictaluri* J100 wild type; **B.** *E. ictaluri* $\Delta wabG12$.

Figure 4. Fluid secretion in intestinal loops (volume : length (ml/cm)). **A.** Rabbit loops 6 h post-inoculation with 100 μ g of LPS; **B.** Catfish loops 6 h post-inoculation with 100 μ g of LPS; **C.** Catfish fluid secretion from the intestinal loops 6 h post-inoculation with 10^7 CFU/ ml of *E. ictaluri*; *S. Typhimurium* was used as control 10^8 CFU/ml. The number of animals per group is 3. The experiment was repeated 3 times independently. The error bars indicate the standard deviation. * $P < 0.001$. **D.** Comparative intestinal loop histology 6 h post inoculation with 100 μ g of LPS or 10^7 CFU/dose of bacteria. Mock intestinal loops were injected with saline (NaCl 0.85%). The ileal loops were injected with 1 ml of the respective sample. *S. enterica* serovar Typhimurium UK1 was used as control. These experiments were repeated 3 times independently.

A **B** **C** **D**

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
χ 289	K-12 wild type A- <i>ginV44</i> λ^- T3 ^r	Lab Collection
χ 6212	ϕ 80d <i>lacZ</i> Δ M15 <i>deoR</i> Δ (<i>lacZYA-argF</i>)-U169 <i>glnV44</i> λ^- <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> Δ <i>asdA4</i> Δ <i>zhf-2::Tn10</i> <i>hsdR17</i> (r^- m^+); F ⁻ Rec ⁻ (UV ^s) DAP ⁻ Lac ⁻ Nal ^r Tet ^s	[48]
χ 7213	<i>thi-1</i> <i>thr-1</i> <i>leuB6</i> <i>glnV44</i> <i>fhuA21</i> <i>lacY1</i> <i>recA1</i> RP4-2-Tc::Mu [<i>λpir</i>] Δ <i>asdA4</i> Δ (<i>zhf-2::Tn10</i>); Km ^r Tet ^s Amp ^s DAP ⁻	[14]
χ 7232	<i>endA1</i> <i>hsdR17</i> (r_K^- m_K^+) <i>glnV44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA</i> <i>relA1</i> Δ (<i>lacZYA-argF</i>)-U169 <i>λpir</i> <i>deoR</i> (ϕ 80dlac Δ (<i>lacZ</i>)M15); Nal ^r UV ^s Thi ⁻ Lac ⁻	[14]
<i>Edwardsiella</i>		
J100 <i>E. ictaluri</i>	Wild-type 2003/c; Isolated from Channel catfish, <i>Ictalurus punctatus</i> ; pEI1 ⁺ ; pEI2 ⁺ API20E 400400057 100% <i>E. ictaluri</i> ; smooth LPS; Col ^r ; Pmb ^r ; Pro ^r ; H ₂ S ⁻ ; H ₂ O ₂ ⁺	[16]
J126 <i>E. ictaluri</i>	Δ <i>gne-31</i> J100 derivate; Isolated from Channel catfish, <i>Ictalurus punctatus</i> ; pEI1 ⁺ ; pEI2 ⁺ API20E 400400057 100% <i>E. ictaluri</i> ; rough LPS; Col ^r Pmb ^r Pro ^r H ₂ S ⁻ H ₂ O ₂ ⁺	[1]
J135 <i>E. ictaluri</i>	Δ <i>ugd-11</i> J100 derivate; Isolated from Channel catfish, <i>Ictalurus punctatus</i> ; pEI1 ⁺ ; pEI2 ⁺ API20E 400400057 100% <i>E. ictaluri</i> ; rough LPS; Col ^s Pmb ^s Pro ^s H ₂ S ⁻ H ₂ O ₂ ⁺	[1]
J148 <i>E. ictaluri</i>	Δ <i>wabG12</i> J100 derivate; Isolated from Channel catfish, <i>Ictalurus punctatus</i> ; pEI1 ⁺ ; pEI2 ⁺ API20E 400400057 100% <i>E. ictaluri</i> ; rough LPS; Col ^r Pmb ^r Pro ^r H ₂ S ⁻ H ₂ O ₂ ⁺	This study
J115 <i>E. tarda</i>	Wild-type PPD130/91; API20E 454400057 smooth LPS; Col ^r Pmb ^r Pro ^r H ₂ S ⁺ H ₂ O ₂ ⁺	[49]
J145 <i>E. hoshinae</i>	Wild-type; smooth LPS; Col ^r Pmb ^r Pro ^r H ₂ S ⁺ H ₂ O ₂ ⁺	ATCC 33379
Plasmids		
pMEG-375	8,142 bp, Cm, Amp, <i>lacZ</i> , R6K <i>ori</i> , <i>mob</i> <i>incP</i> , <i>sacR</i> <i>sacB</i>	[50]
pEZ151	4,065 bp, Gm, pSC101 <i>ori</i>	[13]
pEZ193	Δ <i>wabG12</i> , pMEG-375	This study
pEZ251	P _{lac-wabG} <i>E. ictaluri</i> , Gm ^r	This study

Table 2. Minimal inhibitory concentration (MIC)

Strain	Polymixyn B	Colistin	Protamine	Ox-bile	Deoxicholate
	μg/ml	μg/ml	μg/ml	mg/ml	mg/ml
J100 <i>E. ictaluri</i> 2003/c	700	700	2000	>60	>60
J115 <i>E. tarda</i> PPD	100	25	3000	>60	>60
J124 <i>E. ictaluri</i> Δ <i>gne-31</i>	700	700	2000	>60	>60
J145 <i>E. hoshinae</i> ATCC 33379	200	200	3000	>60	>60
χ289 <i>Escherichia coli</i> K-12	<0.15	3.35	900	>60	>60
J148 <i>E. ictaluri</i> Δ <i>wabG</i>	2.5	25	200	>60	>60
J148 <i>E. ictaluri</i> Δ <i>wabG</i> (pEZ251)	400	200	2000	>60	>60