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Influence of lipopolysaccharide outer-core in the intrinsic resistance to antimicrobial peptides and virulence in *Edwardsiella ictaluri*

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ABSTRACT

The genus *Edwardsiella* consists of bacteria with an intrinsic resistance to cyclic cationic antimicrobial peptides (CAMPs). *Edwardsiella 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 oligopolysaccharide (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 Δ*wabG* caused a pleiotropic effect, influencing LPS synthesis, CAMPs resistance, growth, and biofilm formation. *E. ictaluri* Δ*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.

1. Introduction

The genus *Edwardsiella* consists of five species *Edwardsiella 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-

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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 contras 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 plays 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; ddH_2O, 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 generate an in-frame deletion of the *wabG* gene (NT01EI_0064) (Fig. 1A) was constructed as described previously [13]. The defined deletion mutations encompass a deletion including the ATG start codon, but not including the TAG stop codon. Primers 1 (F1 (*Sph*I) 5′-ACATGCATGC GCTCTTTCTCAGGCGGGC-TACCCTA-′3) and 2 (R1 (*Xho*I-*Pst*I) 5′-CTCGAGCGGAAAACTG-CAGTTTTTCAAGTAACCTCGTTGCCGCCGCGA-′3) were designed to amplify the upstream gene-flanking region (443 bp). The downstream gene-flanking region (445 bp) was amplified by primers 3 (F2 (*Pst*I-*Xho*I) 5′-AAAACTGCAGTTTTCCGCTCGAGTAAATCATGC-GAATTTTAATGATTA-′3) and 4 (R2 (*Xba*I) 5′-TCGTCTAGA-CATCGGCTGAAGCCATGGCGATGTC-′3). The flanking regions were ligated (888 bp) and cloned into pMEG-375 digested with *Sph*I and *Xba*I. To construct *E. ictaluri* mutants, the suicide plasmid was conjugationally transferred from *Escherichia coli* χ7213 [14] to *E. ictaluri* strains. Strains containing single-crossover plasmid insertions were isolated on BHI agar plates containing Col and Amp. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity counter-selection system [15] adapted to *E. ictaluri* [13,16]. The colonies were selected for Amp^S, Col^r and screened by PCR using primers 1 and 4 (Fig. 1B). DNA sequencing analysis of the genome segment mutated was performed at the DNA sequencing core laboratory, Arizona State University. Biochemical profiles of *E. ictaluri* strains were determined using the API 20E system (bioMériux, Marcy I'Etoile, France). The Δ*wabG* 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 (bioMériux, Marcy I'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

Table 1
Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Source or reference
Escherichia coli		
χ289	K-12 wild type A- *ginV44* λ^- $T3^r$	Lab Collection
χ6212	φ*80d lacZ* Δ*M15 deoR* Δ(*lacZYA-argF*)-U169 *glnV44* λ^- *gyrA96 recA1 relA1 endA1* Δ*asdA4* Δ*zhf-2*::Tn*10 hsdR17* (r^- m^+); F^- $Rec^-(UV^s)$ DAP^- Lac^- Nal^r Tet^s	[48]
χ7213	*thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1* RP4-2-Tc::Mu [λ*pir*] Δ*asdA4* Δ(*zhf-2*::Tn10); Km^r Tet^s Amp^s DAP^-	[14]
χ7232	*endA1 hsdR17* (r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)-U169 λ*pir deoR* (φ*80dlac* Δ(*lacZ*)*M15*); Nal^r UV^s Thi^- Lac^-	[14]
Edwardsiella		
J100	Wild-type 2003/c; Isolated from Channel catfish, *Ictalurus punctatus;* $pEI1^+$; $pEI2^+$ API20E 400400057 100% *E. ictaluri;* smooth LPS; Col^r; Pmb^r; Pro^r; H_2S^-; $H_2O_2^+$	[16]
E. ictaluri		
J126	Δ*gne-31* J100 derivate; Isolated from Channel catfish, *Ictalurus punctatus;* $pEI1^+$; $pEI2^+$ API20E 400400057 100% *E. ictaluri;* rough LPS; Col^r Pmb^r Pro^r H_2S- $H_2O_2^+$	[1]
E. ictaluri		
J135	Δ*ugd-11* J100 derivate; Isolated from Channel catfish, *Ictalurus punctatus;* $pEI1^+$; $pEI2^+$ API20E 400400057 100% *E. ictaluri;* rough LPS; Col^S Pmb^S Pro^S H_2S- $H_2O_2^+$	[1]
E. ictaluri		
J148	Δ*wabG12* J100 derivate; Isolated from Channel catfish, *Ictalurus punctatus;* $pEI1^+$; $pEI2^+$ API20E 400400057 100% *E. ictaluri;* rough LPS; Col^r Pmb^r Pro^r H_2S- $H_2O_2^+$	This study
E. ictaluri		
J115 *E. tarda*	Wild-type PPD130/91; API20E 454400057 smooth LPS; Col^r Pmb^r Pro^r H_2S^+ $H_2O_2^+$	[49]
J145	Wild-type; smooth LPS; Col^r Pmb^r Pro^r H_2S^+ $H_2O_2^+$	ATCC 33379
E. hoshinae		
Plasmids		
pMEG-375	8142 bp, Cm, Amp, *lacZ*, R6K *ori, mob incP, sacR sacB*	[50]
pEZ151	4065 bp, Gm, pSC101 *ori*	[13]
pEZ193	Δ*wabG12*, pMEG-375	This study
pEZ251	P_{lac}-*wabG E. ictaluri*, Gm^r	This study