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Mechanisms of intrinsic resistance to antimicrobial peptides of *Edwardsiella ictaluri* and its influence on fish gut inflammation and virulence

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The genus *Edwardsiella* comprises a genetically distinct taxon related to other members of the family *Enterobacteriaceae*. It consists of bacteria differing strongly in their biochemical and physiological features, natural habitats, and pathogenic properties. Intrinsic resistance to cationic antimicrobial peptides (CAMPs) is a specific property of the genus *Edwardsiella.* In particular, *Edwardsiella ictaluri*, an important pathogen of the catfish (*Ictalurus punctatus*) aquaculture and the causative agent of a fatal systemic infection, is highly resistant to CAMPs. *E. ictaluri* mechanisms of resistance to CAMPs are unknown. We hypothesized that *E. ictaluri* lipopolysaccharide (LPS) plays a role in both virulence and resistance to CAMPs. The putative genes related to LPS oligo-polysaccharide (O-PS) synthesis were in-frame deleted. Individual deletions of *wibT*, *gne* and *ugd* eliminated synthesis of the O-PS, causing auto-agglutination, rough colonies, biofilm-like formation and motility defects. Deletion of *ugd*, the gene that encodes the UDP-glucose dehydrogenase enzyme responsible for synthesis of UDP-glucuronic acid, causes sensitivity to CAMPs, indicating that UDP-glucuronic acid and its derivatives are related to CAMP intrinsic resistance. *E. ictaluri* OP-S mutants showed different levels of attenuation, colonization of lymphoid tissues and immune protection in zebrafish (*Danio rerio*) and catfish. Orally inoculated catfish with O-PS mutant strains presented different degrees of gut inflammation and colonization of lymphoid tissues. Here we conclude that intrinsic resistance to CAMPs is mediated by Ugd enzyme, which has a pleiotropic effect in *E. ictaluri* influencing LPS synthesis, motility, agglutination, fish gut inflammation and virulence.

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INTRODUCTION

The genus *Edwardsiella*, which consists of the three species *Edwardsiella tarda*, *Edwardsiella hoshinae* and *Edwardsiella ictaluri*, belongs to the family *Enterobacteriaceae*. This genus is a genetically distinct taxon related to other members of the family *Enterobacteriaceae*. *Edwardsiella* bacteria differ strongly in their biochemical and physiological features, natural habitats, and pathogenic properties. High intrinsic resistance to cationic antimicrobial peptides (CAMPs) is one of the particular properties of the genus *Edwardsiella* (Muyembe *et al.*, 1973; Reinhardt *et al.*, 1985; Stock & Wiedemann, 2001).

E. ictaluri is one of the most important pathogens of commercially raised channel catfish (*Ictalurus punctatus*) (Shoemaker *et al.*, 2009), which account for more than 80 % of US aquaculture production, in spite of the recent production decrease (Hanson & Sites, 2012). In general, fish possess a strong innate immune system that acts as the first line of defence against a broad spectrum of pathogens. Fish continuously fight against pathogens by secreting a wide range of antimicrobial peptides as an innate defence mechanism (Bly & Clem, 1991; Tatner & Horne, 1983). CAMPs are short, amphipathic, positively charged peptides produced by organisms from bacteria to mammals

Abbreviations: Amp, ampicillin; CAMPs, cationic antimicrobial peptides; CCO, channel catfish ovary; Cm, chloramphenicol; Col, colistin; GlcA, glucuronic acid; Gm, gentamicin; Km, kanamycin; L-Ara4N, 4-amino-4-deoxy-L-arabinose; LPS, lipopolysaccharide; OMPs, outer-membrane proteins; O-PS, oligo-polysaccharide; Pmb, polymyxin B; TEM, transmission electron microscopy; Ugd, UDP-glucose dehydrogenase.

Four supplementary figures are available with the online version of this paper.

(Brogden, 2005). They have an important role in innate immunity responses (Ganz, 2003; Zanetti, 2004), killing bacteria by permeabilization of the cytoplasmic membrane (Brogden, 2005), and also through inhibition of essential microbial processes, such as protein, cell-wall and nucleic acid syntheses (Patrzykat *et al.*, 2002). Seven antimicrobial peptides have been described in channel catfish, including NK-lysin type 1, NK-lysin type 2, NK-lysin type 3 (Wang *et al.*, 2006), bactericidal permeability-increasing protein (BPI) (Xu *et al.*, 2005), cathepsin D (Dunham *et al.*, 2002), hepcidin (Bao *et al.*, 2005) and liver-expressed AMP 2 (LEAP2) (Bao *et al.*, 2006). Among these catfish described CAMPs, hepcidin, NK-lysin type 1, NK-lysin type 3 and cathepsin D seem to play a role during *E. ictaluri* infection (Pridgeon *et al.*, 2012).

In general, bacterial resistance to antimicrobial peptides is mediated by different mechanisms, including alteration of the bacterial surface charge, proteolytic degradation and export of peptides by efflux pumps (Peschel & Sahl, 2006). *Edwardsiella* species are particularly resistant to the CAMP polymyxin B and colistin (also called polymyxin E) (Muyembe *et al.*, 1973; Reinhardt *et al.*, 1985; Santander & Curtiss, 2010; Stock & Wiedemann, 2001). Several components of the lipopolysaccharide (LPS) are important for CAMP resistance. For instance, LPS plays an important role in the resistance to CAMPs in *Bordetella bronchiseptica* (Banemann *et al.*, 1998), and LPS outer and inner core regions contribute to resistance of *Yersinia enterocolitica* (Skurnik *et al.*, 1999) and *Escherichia coli* (Farnaud *et al.*, 2004), respectively. While some Gram-negative bacteria possess constitutive mechanisms of resistance, others such as *Salmonella enterica* serovar Typhimurium and *Y. pestis* possesses inducible resistances. Exposure to sublethal concentrations of CAMPs induces modifications to the LPS, including addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) (Guo *et al.*, 1997; Knirel *et al.*, 2007), palmitoylation and myristoylation (Guo *et al.*, 1998; Tran *et al.*, 2005) to resist CAMP action. These modifications increase the net charge of the membrane repelling CAMPs (Guo *et al.*, 1998; Raetz *et al.*, 2009; Tran *et al.*, 2005). Also, constitutive presence of L-Ara4N in lipid A has been documented in the CAMP-resistant bacterium *Burkholderia cenocepacia* (De Soyza *et al.*, 2004). Capsular polysaccharides have also been reported to contribute to antimicrobial peptide resistance (Llobet *et al.*, 2008).

The complete LPS structure of *E. ictaluri* has not been elucidated. Nevertheless, the composition and structure of the *E. ictaluri* oligo-polysaccharides (O-PS) have been reported (Vinogradov *et al.*, 2005). The *E. ictaluri* O-chain was found to be an unbranched linear polymer of a repeating tetrasaccharide unit composed of D-glucose, 2-acetamido-2-deoxy-D-galactose and D-galactose in a 1 : 2 : 1 ratio having the structure: [→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→]$_n$ (Vinogradov *et al.*, 2005). Serological analyses using monoclonal antibodies have indicated that *E. ictaluri* is serologically homogeneous (Bertolini *et al.*, 1990; Shotts & Waltman, 1990), suggesting that there is no variation in the O-PS between *E. ictaluri* strains.

The predicted *E. ictaluri* O-PS biosynthesis enzymes consist of four putative overlapping genes, *wibT*, *galF*, *gne* and *ugd*, located in the O-PS cluster under control of a jump-start promoter (Lawrence *et al.*, 2003). In this study, we evaluate the role of *E. ictaluri wibT*, *galF*, *gne* and *ugd* in the resistance to CAMPs. We describe the constitutive mechanism of *E. ictaluri* intrinsic resistance to CAMPs and its influence on virulence, tissue colonization, gut inflammation and immune protection in the fish host.

METHODS

Bacterial strains, plasmids, media and reagents. The bacterial strains and plasmids are listed in Tables S1 and S2, respectively (available in *Microbiology* Online). Bacteriological media and components are from Difco. Antibiotics and reagents are from Sigma. Luria-Bertani broth (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; double distilled H_2O, 1 litre) (Bertani, 1951) and Bacto brain heart infusion (BHI) were used routinely. When required, the media were supplemented with 1.5 % agar, 6 % sucrose, colistin (Col; 12.5 μg ml^{-1}), polymyxin B (Pmb; 20 μg ml^{-1}), ampicillin (Amp; 100 μg ml^{-1}), chloramphenicol (Cm; 25 μg ml^{-1}), gentamicin (Gm; 10 μg ml^{-1}) or kanamycin (Km; 50 μg ml^{-1}). Bacterial growth was monitored spectrophotometrically and/or by plating. Oligonucleotides were from IDT. Restriction endonucleases were from New England Biolabs. *Taq* DNA polymerase (New England Biolabs) was used in all PCR tests. Qiagen products were used to isolate plasmid DNA, gel-purify fragments or purify PCR products. T4 ligase, T4 DNA polymerase and shrimp alkaline phosphatase were from Promega.

Construction and characterization of *E. ictaluri* with defined deletions. The recombinant pEZ suicide vectors (Table S2) carrying the linked flanking regions to generate in-frame deletion of *wibT*, *galF*, *gne* and *ugd* genes were constructed as described previously (Santander *et al.*, 2007, 2010, 2011). The defined deletion mutations encompass a deletion including the ATG start codon, but do not include the TAG or TAA stop codon. The primers used to construct the suicide vectors are listed in Table S3. Primers 1 and 2 were designed to amplify the upstream gene-flanking regions. Primers 3 and 4 amplified the downstream gene-flanking regions. The flanking regions were ligated 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 (Roland *et al.*, 1999) 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 (Edwards *et al.*, 1998) adapted to *E. ictaluri* (Santander *et al.*, 2010, 2011). The colonies were selected for Amp^s and Col^r, and screened by PCR using primers 1 and 4. The Δ*wibT*, Δ*galF* and Δ*gne* mutants were screened for Amp^s, Col^r and PCR (Fig. 1). The Δ*ugd* mutant was screened for Amp^s, Col^s and PCR. Biochemical profiles of *E. ictaluri* strains were determined using the API 20E system (bioMérieux).

Complementation of *E. ictaluri* mutants. The *wibT*, *gne* and *ugd* genes were cloned independently into pEZ151 (Table S2) under P_{lac} control at the *Adh*I restriction site. The primers used to amplify the corresponding genes under P_{lac} control are listed in Table S3. The resulting plasmids were used to complement the *E. ictaluri* mutant strains (Table S1).

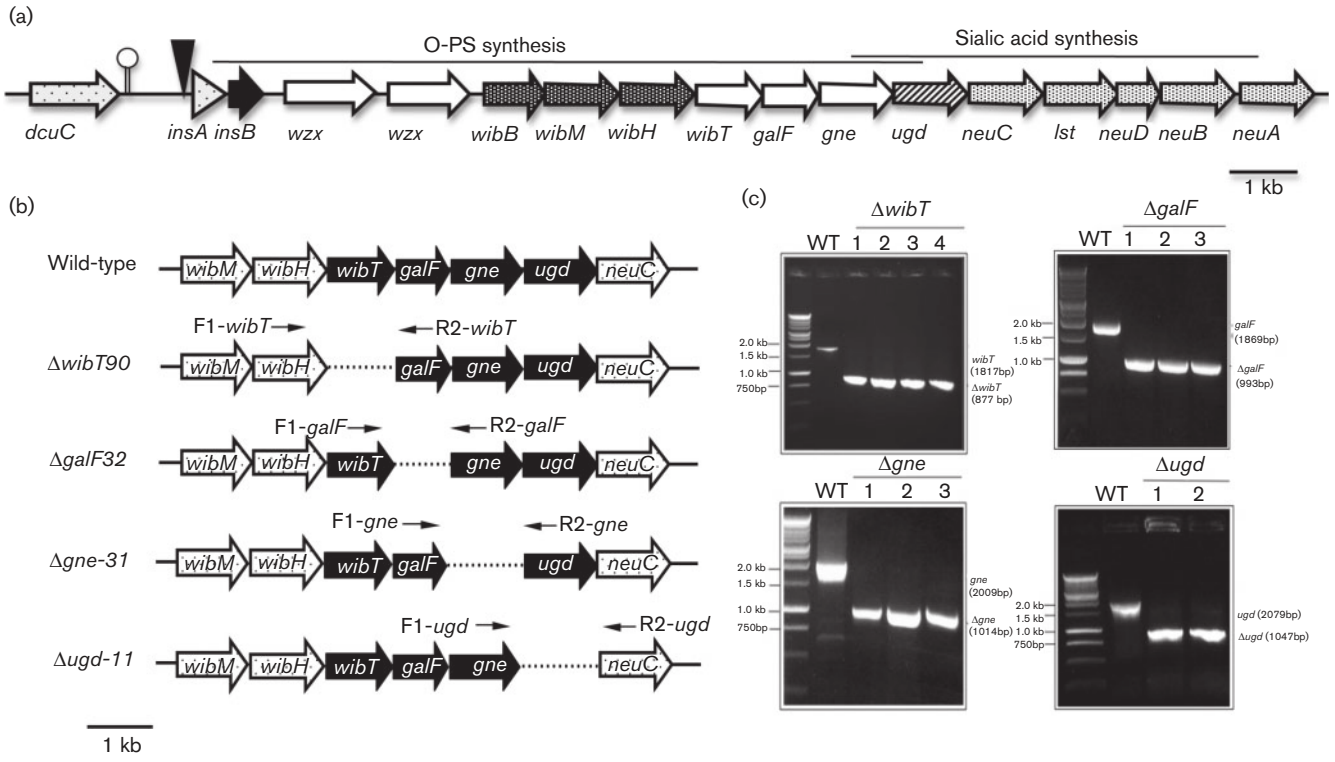

Fig. 1. *E. ictaluri* O-PS LPS synthesis operon. (a) O-PS operon. The symbols downstream of the *insA* gene indicate the O-PS operon jump-start promoter. (b) Map of the defined deletions in *E. ictaluri* O-PS operon. (c) PCR verification of the O-PS deletions.

LPS purification and analysis. LPS extraction was performed by using TRI-regent (Sigma) as described by Yi & Hackett (2000). LPS profiles were evaluated by SDS-PAGE and visualized by silver staining (Hitchcock & Brown, 1983; Tsai & Frasch, 1982).

Glycosyl composition. Glycosyl composition analysis was performed by combined GC/MS of the per-*o*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. Purified LPS aliquots of 300 μg were added to separate tubes with 20 μg inositiol as the internal standard. Methyl glycosides were then prepared from the dry sample following the mild acid treatment by methanolysis in 1 M HCl in methanol at 80 °C (16 h), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The sample was then per-*o*-trimethylsilyated by treatment with Tri-Sil (Pierce) at 80 °C (30 min). These procedures were carried out as described by Merkle & Poppe (1994). GC/MS analysis of the per-*o*-trimethylsilyl methyl glycosides was performed on an AT 6890N gas chromatograph interfaced to a 5975B MSD mass spectrometer, using a Supelco EC-1 fused silica capillary column (30 m × 0.25 mm ID).

Disc diffusion susceptibility and MIC assays. Disc diffusion susceptibility was determined in Mueller–Hinton (MH) medium supplemented with 5 % sheep blood agar (CLSI, 2005a). MICs were determined in MH agar supplemented with 5 % calf fetal bovine serum (HyClone) by microplate serial dilution assay (CLSI, 2005b). This assay was performed using flat-bottomed 96-well clear microtitre plates. Then, 20 000 μg polymyxin B (Pmb) ml^{-1} and 12 500 μg colistin sulfate (Col) ml^{-1} were serially diluted and then inoculated with mid-exponential-phase cultures of the *E. ictaluri* strains. The plates were incubated for 48 h at 28 °C.

Transmission electron microscopy (TEM). To increase flagella synthesis, the bacterial samples were collected from motility agar plates away from the point of inoculation (Panangala *et al.*, 2009). Negative staining was performed as described by Chandler & Roberson (2009).

Outer-membrane protein (OMP) profiles. Sarkosyl-insoluble OMPs were obtained as previously described (Santander *et al.*, 2012). The OMPs were normalized to 25 μg μl^{-1} by using an ND-1000 spectrophotometer (NanoDrop) and separated by 10 % (w/v) SDS-PAGE. Coomassie blue staining was performed to visualize proteins.

Protein identification. The upregulated OMP in *E. ictaluri* Δ*wibT*, Δ*gne* and Δ*ugd* mutants was excised from the SDS-PAGE gel for peptide sequencing analysis (ProtTech) by using NanoLC-MS/MS peptide sequencing technology. The protein gel band was destained, cleaned and digested in-gel with sequencing-grade modified trypsin (Promega). The resulting peptide mixture was analysed by an LC-MS/MS system, in which a high-performance liquid chromatograph with a 75 μm inner diameter reversed-phase C18 column coupled to an ion trap mass spectrometer (Thermo) was used. The MS data were utilized to search the non-redundant protein database at the National Center for Biotechnology Information (NCBI).

Quantitative bacterial adherence assay. To assess adherence, we inoculated 200 μl of BHI or LB broth in 96-well flat-bottomed microtitre polystyrene plates (Becton Dickinson) with 5 μl of an overnight BHI or LB culture. The plates were incubated statically over 7 days at 28 °C and visualized by staining with 0.5 % crystal violet for 5 min after washing with water. Bacterial adherence was quantified in

duplicate, after adding 200 µl of 95 % ethanol, by an ELISA plate reader at 570 nm.

Semi-quantitative Reverse Transcription - Polymerase Chain Reaction (RT-PCR). Expression of *ugd*, *phoP*, *arnT* and *neuC* genes was evaluated by RT-PCR. Total RNA extraction was performed using an RNeasy QIAgene kit from *E. ictaluri* wild-type grown in the presence and absence of Col. The cells were grown until the late-exponential phase (OD_{600} of 0.85, $\sim 1 \times 10^8$ c.f.u. ml^{-1}). cDNA synthesis was performed by SuperScript^{TM} III First-Strand Synthesis System (Invitrogen) using random hexamer primers. Semi-quantitative PCR (Ali *et al.*, 1997) was performed using the specific primers listed in Table S3. The number of PCR cycles was normalized to 28 for semi-quantitative RT-PCR. 16S (*rrn* gene) was used as expression control and *fadR* was used as single gene expression control (Santander *et al.*, 2012).

Survival in fish blood and resistance to complement. *E. ictaluri* strains were grown in BHI broth to a cell density of approximately 1×10^9 c.f.u. ml^{-1} (late-exponential phase). To evaluate the strain survival in whole catfish blood, the cells were diluted in PBS, pH 7.4, and inoculated into fresh catfish blood from naïve fish, either untreated or complement-inactivated by incubation at 55 °C for 1 h, at a density of 1.0×10^6 c.f.u. ml^{-1} and incubated at 28 °C for the duration of the assay. Bacterial viability was assessed by plating onto BHI agar at 0, 2 and 6 h after inoculation. The assay was performed in duplicate and was repeated 3–5 times using blood samples from different catfish batches (Pine Bluff, AR, or Louisiana State University, LA).

To evaluate resistance to the complement alternative pathway, serum from ten juvenile channel catfish (fingerlings) was collected and pooled. Half of the serum was heated at 55 °C for 30 min to inactivate complement. Normal serum and heat-inactivated serum were aliquoted and stored at −80 °C. Guinea pig serum (CalBiochem) was used as control. For survival of strains in serum, the cells were diluted in PBS, pH 7.4, and inoculated into serum, either untreated or complement-inactivated, at a density of 1.0×10^6 c.f.u. ml^{-1}. Control consisted of bacteria mixed with PBS. Bacterial viability was quantified in duplicate by serial dilution and plate counts. Four independent replicates from separate cultures were run for each strain and serum treatment. To evaluate resistance to the complement classical pathway, a method similar to that described above was performed, but the cells were inoculated into serum, either untreated or complement-inactivated plus anti-Hd rabbit antibodies (Difco) that react with all *E. ictaluri* strains (Fig. S3).

Infection and immunization of zebrafish (*Dano rerio*). Zebrafish challenges were performed according to previously described methodology (Petrie-Hanson *et al.*, 2007) with modifications (Santander *et al.*, 2011). The water temperature was 26 ± 1 °C; the fish were acclimatized over 2 weeks before experimentation. Adult zebrafish (average weight, 0.5 g) were sedated in 100 mg tricaine methanesulfonate l^{-1} (MS-222; Sigma), then injected intramuscularly. Two sets of controls were used: fish that were injected with 10 µl of sterile PBS and fish that were not injected. Moribund fish demonstrating clinical signs were killed and necropsied, and bacteria isolated as previously described (Petrie-Hanson *et al.*, 2007). Survivors of each dose at 6 weeks post-injection were challenged with 10^6 c.f.u. of wild-type *E. ictaluri*. Fish care and use were performed in accordance with the requirements of the Arizona State University Institutional Animal Care and Use Committee.

Colonization of zebrafish tissues by *E. ictaluri*. Colonization of spleen, kidney and gills by *E. ictaluri* was evaluated as follows. Selected organs from infected and uninfected fish were removed by dissection with the aid of a stereomicroscope. Dissected organs were placed in a 1.5 ml microcentrifuge tube containing 200 µl PBS and homogenized with a pellet pestle (Pellet Pestle, catalogue no. K749520-0090; Fisher Scientific). Serial dilutions of homogenates were prepared in Buffer Saline Gelatin (BSG), and numbers of c.f.u. were determined by plating on BHI Col agar plates.

Tissue culture, attaching and invasion assay in channel catfish ovary (CCO) cells. The CCO cell line (Bowser & Plumb, 1980) was obtained from ATCC (CRL-2772). Cells growing in monolayers were cultured in tissue culture flasks in Dulbecco's modified Eagle medium (DMEM; 10-013; Cellgro) diluted to a catfish tonicity of 243 mOsm kg^{-1} by adding 1 part sterile deionized water (DMEM 9 : 1) and containing 0.05 mM β-mercaptoethanol (Sigma Chemicals), supplemented with 10 % fetal bovine serum (Cellgro). A standard gentamicin survival assay (Sizemore *et al.*, 1997) was used to evaluate the abilities of the wild-type and the O-PS mutants to attach to and enter CCO cells. Briefly, CCO cells were suspended to a final concentration of 1×10^7 cells ml^{-1}. One millilitre 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 the wells were washed three times with PBS to remove non-adherent cells and 1 ml of fresh DMEM 1 : 9 was added per well. To evaluate the efficiency of entry and replication, 1×10^7 cells ml^{-1} of either wild-type or O-PS mutants were added to triplicate wells of the 16 h CCO cultures, giving an m.o.i. of 1 (1 bacterium to 1 CCO cell). After infection, the plates were centrifuged at 200 ***g*** to synchronize contact of the bacteria with the adhered cell layer and allowed to incubate for 1 h at 28 °C. The medium was then removed from each well and the cells were washed three times with PBS. To determine the number of attaching *E. ictaluri* cells, 100 µl of a 1 % solution of Triton X-100 was added to lyse the CCO cells. The number of attaching *E. ictaluri* cells was determined by spreading serial dilutions on BHI agar plates. To determine the number of intracellular or invading *E. ictaluri* cells, DMEM 1 : 9 with 100 µg gentamicin ml^{-1} was added for 1 h to kill residual extracellular bacteria. Then the medium was removed from each well and the cells were washed three times with PBS and lysed with 100 µl of a 1 % solution of Triton X-100. The numbers of invading *E. ictaluri* cells were determined by spreading serial dilutions on BHI agar plates.

Survival assay in tissue-specific macrophages from channel catfish. Catfish macrophages (MØ) were isolated from intestinal mucosa as described by Clerton *et al.* (1998) with slight modifications, and from head kidney as reported by Secombes (1990) with modifications (Pohlenz *et al.*, 2012).

Catfish gut phagocytes were isolated by incubating pieces of anterior intestine in Hank's balanced salt solution (HBSS, phenol red and Ca^{2+}/Mg^{2+} free, pH 7.2; Sigma), containing EDTA and DTT (EDTA, 0.37 mg ml^{-1} + DTT 0.145 mg ml^{-1}; Sigma), for 15 min at 22 °C. The tissue pieces were then rinsed in rinsing medium [RM, HBSS + 5 % fetal calf serum (FCS), 1 % penicillin + streptomycin + 0.1 mg DNase I ml^{-1}; Sigma]. The fragments were transferred to a conical flask containing RM + collagenase (0.15 mg ml^{-1}, type II; Sigma) and incubated in a shaking water bath for 2 h at 22 °C. The supernatant was filtered and the resulting cell suspension was centrifuged twice at 400 ***g*** for 10 min at 4 °C. Finally the cells were resuspended in antibiotic-free L-15 medium + 0.1 % FCS.

Head-kidney tissue was filtered through a 100 µm nylon mesh. The resulting cell suspension was layered on a Percoll (Sigma) gradient (34 %/51 %, v/v) and centrifuged at 400 ***g*** for 30 min. The cell layer at the interface was collected and washed twice with ice-cold PBS at 200 ***g*** for 10 min. A final wash and resuspension was conducted with antibiotic-free L-15 + 0.1 % FCS.

Macrophages were enumerated using a haemocytometer and viability assessed by trypan blue staining (Sigma). Viability was >95 % in all cases. Head-kidney cell suspension was adjusted to 1×10^7 cells ml^{-1},

whereas gut MØ was adjusted to 1×10^6 cells ml^{-1}. Then, 100 µl of each MØ suspension was added per well in a sterile flat-bottomed 96-well microplate (Falcon).

The ability of MØ to kill *E. ictaluri* was evaluated using the method described by Secombes (1990) and modified by Shoemaker *et al.* (1997) and Pohlenz *et al.* (2012). Bacteria mutants were cultured in BHI broth for 18 h at 28 °C. Cultures were centrifuged at 2000 ***g*** for 10 min and the pellet washed once in HBSS; bacteria were then resuspended in antibiotic-free L-15 medium. Bacteria were enumerated using a bacterial counter chamber and the suspension was adjusted to 3×10^8 and 3×10^7 cells ml^{-1} for use in head-kidney and gut phagocyte cultures, respectively. The MØ primary cultures were incubated for 2 h, then washed twice with 200 µl L-15, and resuspended with L-15 + 2 % FCS medium. Primary MØ were infected with 20 µl of the respective bacterial strain in eight-well plates and centrifuged at 150 ***g*** for 5 min. The infections were incubated for 2.5 h at 28 °C. At 0 h and 2.5 h supernatants were removed and MØ lysed with 50 µl 0.2 % Tween 20 (Mallinckrodt) solution. Then, 100 µl of fresh BHI was added to each well and plates further incubated for 18 h at 27 °C in an orbital incubator. After the incubation period, 20 µl thiazolyl blue tetrazolium bromide (MTT, 10 mg mL^{-1}; Sigma) was added per well. The plates were read at 620 nm after 5 min of incubation. Bacterial concentrations were calculated by comparing the absorbance obtained for each well to a standard curve previously constructed (data not shown). Bactericidal capacity (% killing=bacteria 0 h − bacteria 2.5 h × 100/bacteria 0 h) was computed for each well and is presented as mean % killing. All the assays were performed four times.

Intracoelomic infection and immunization of catfish. Specific-pathogen-free channel catfish fingerlings from Louisianan State University were used with a mean weight of 18.5 ± 1.3 g (~ 6 months old). The animals were randomly assigned to treatment groups of 10–25 fish each in 100 l tanks. Each tank was equipped with a recirculating, biofiltered, mechanically filtered, and UV water-treated system with 12 h light cycle per day. The water temperature was set at 26 ± 1 °C during the first 2 weeks of acclimatization and during the course of the experiments. The fish were fed daily with commercial Aquamax grower 400 (Purina Mills Inc.). Catfish were infected by the intracoelomic (i.c.) route with 10^8–10^5 c.f.u. of *E. ictaluri* strains (fish were not fed until 1 h after infection). The fish were anaesthetized with buffered MS-222 (pH 7.5) (100 mg l^{-1}) prior to handling. Moribund animals were killed and then necropsied to evaluate the presence of *E. ictaluri* in kidney, spleen and liver.

Bath infection and immunization of catfish. Catfish fingerlings were immersed in a solution of *E. ictaluri* (wild-type or mutant strains) of 10^7 c.f.u. ml^{-1} for 30 min. Six weeks post-immunization, fish were challenged by bath immersion with 10^7 c.f.u. ml^{-1} of *E. ictaluri* (10 LD_{50}) for 30 min. The animals were fasted 24 h prior to the inoculation to 1 h post-inoculation. Non-immunized animals were used as controls. Fish were observed daily.

Oral infection of catfish. Catfish were fasted for 24 h before oral inoculation with the respective *E. ictaluri* strains. Fish were anaesthetized before handling. The animals were orally inoculated with 100 µl of the corresponding bacterial suspension (see below). PBS was used as a control. The fish were not fed until 1 h after inoculation.

Colonization of catfish tissues by *E. ictaluri* and histology. Colonization of spleen, kidney (posterior and head) and liver by *E. ictaluri* was evaluated as follows. Following death of the animals, selected organs from infected and uninfected fish were removed by dissection. Dissected organs were homogenized. Serial dilutions of homogenates were prepared in PBS, and numbers of c.f.u. were determined by plating on BHI Col agar plates. Parts of the organs were fixed in 10 % formalin and subjected to H & E staining for histopathological examination.

Bacteria inoculate preparation. Bacterial strains were grown overnight in standing cultures that were diluted 1 : 20 in prewarmed BHI broth and grown with mild aeration (180 r.p.m.) at 28 °C to an OD_{600} of 0.8–0.9 (~10^8 c.f.u. ml^{-1}). Bacteria were sedimented for 15 min by centrifugation (4000 r.p.m.) at room temperature and resuspended in PBS to densities appropriate for the inoculation.

Statistics. An ANOVA (SPSS Software), followed by a least significant difference method, was used to evaluate differences in bacterial titres discerned to 95 % confidence intervals. $P<0.05$ was considered statistically significant.

RESULTS

E. ictaluri O-PS mutant analysis

The *wibT* gene encodes a putative UDP-galactose 4 epimerase, which converts UDP-glucose to UDP-galactose. Deletion of *wibT* affects O-PS synthesis (Fig. 2a) and the LPS core, presenting only glucose and 3-deoxy-alpha-D-manno-oct-2-ulopyranosonic acid (KDO) traces in the LPS (Table 1). Galactose utilization was not affected in *E. ictaluri* Δ*wibT*. *E. ictaluri* Δ*wibT* colony morphology presented the typical rough phenotype of an LPS mutant strain (Fig. S1). Deletion of the *galF* gene, which encodes a putative modulator *galU*/UTP Glc-1-P uridylytransferase, did not affect LPS synthesis (Fig. 2a) or colony morphology (Fig. S1). The *gne* gene encodes a putative UDP-GlaNAc 4-epimerase enzyme (Lawrence *et al.*, 2003), which interconverts UDP-glucosamine (UDP-GlcNAc) to UDP-galactosamine (UDP-GlaNAc; 2-acetamido-2-deoxy-D-galactose), the major component of the *E. ictaluri* O-PS (Vinogradov *et al.*, 2005). Deletion of *gne* affects O-PS synthesis (Fig. 2a), but not the LPS core (Table 1). *E. ictaluri* Δ*gne* colony morphology presented the typical rough phenotype of an LPS mutant (Fig. S1). The *ugd* gene encodes a putative UDP-glucose dehydrogenase enzyme, which converts UDP-glucose to UDP-glucuronic acid. Glucuronic acid is not present in the O-PS of *E. ictaluri* (Vinogradov *et al.*, 2005) (Table 1), but deletion of *ugd* has profound effects on LPS synthesis (Fig. 2a). *E. ictaluri* Δ*ugd* presented only glucose in its O-PS, and KDO sugars were not detected (Table 1). *E. ictaluri* Δ*ugd* colony morphology presented a smooth phenotype, atypical for an LPS mutant (Fig. S1). The glycosyl analysis of the *ugd* mutant correlates with its LPS profile, which has a lower molecular mass in contrast with the wild-type or the Δ*wibT* and Δ*gne* mutants (Fig. 2a). LPS synthesis was re-established in all complemented mutants with their respective wild-type gene (Fig. S2).

In summary, these results indicate that *wibT*, *gne* and *ugd* participate directly in the synthesis of O-PS. Furthermore, *ugd* is related to core-lipid A synthesis in *E. ictaluri*.

Outer-membrane protein analysis

E. ictaluri Δ*wibT*, Δ*gne* and Δ*ugd* upregulate a similar OMP of ~48 kDa identified as OmpN (Fig. 2b). OmpN corresponds to a putative porin that may be related to

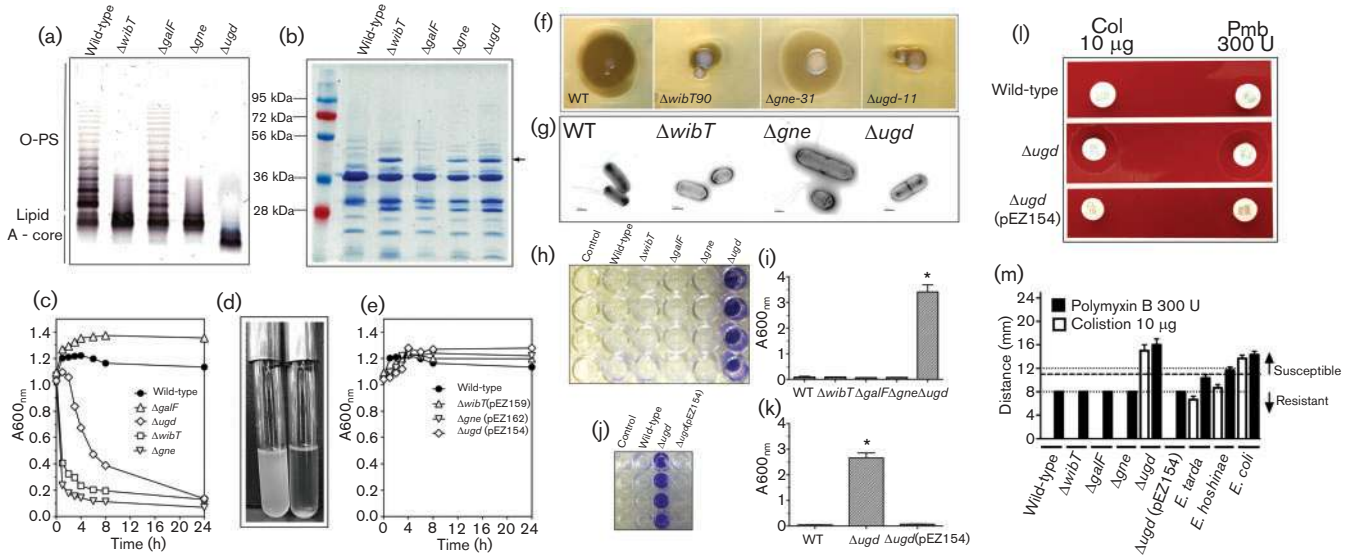

Fig. 2. *E. ictaluri* O-PS mutant phenotypes. (a) LPS profile of *E. ictaluri* O-PS mutant strains (16 % SDS-PAGE gel). (b) OMP profile of *E. ictaluri* O-PS mutant strains (10 % SDS-PAGE gel). The arrow indicates the upregulated OmpN porin (pI/Mw: 5.17/40 953.89). (c) *E. ictaluri* O-PS mutants aggregation and precipitation kinetics assay. (d) Precipitation by aggregation of *E. ictaluri* wild-type and *E. ictaluri* O-PS mutants. (e) Aggregation and precipitation assay of *E. ictaluri* O-PS mutants complemented in-trans. (f) Swarming motility assay in BHI 0.3 % agar. (g) TEM of *E. ictaluri* O-PS mutants. WT, Wild-type *E. ictaluri* J100. (h) Adhesion of *E. ictaluri* O-PS mutants after 7 days of incubation in BHI broth at 28 °C. (i) Surface adhesion quantification of *E. ictaluri* O-PS mutants after 7 days of incubation in BHI broth at 28 °C. The samples correspond to three independent experiments. **P*<0.001. (j) Adhesion of *E. ictaluri* Δ*ugd* mutant complemented in-trans. (k) Adhesion quantification of *E. ictaluri* Δ*ugd* mutant complemented in-trans. The samples correspond to three independent experiments. **P*<0.001. (l) Antibiogram assay in MH supplemented with 5 % sheep blood. (m) Antibiogram. Col^r, ⩾11 mm; Pol^r, ⩾12 mm.

the agglutination phenotype. However, further studies are required to determine the role of OmpN in *E. ictaluri*.

Agglutination

E. ictaluri Δ*wibT*, Δ*gne* and Δ*ugd* mutants presented auto-agglutination and precipitation in liquid culture (Fig. 2c–e). The Δ*gne* mutant showed the fastest agglutination and precipitation of all the mutants (~40 min), followed by Δ*wibT* and finally Δ*ugd*. This agglutination and precipitation phenotype could be attributed to membrane charge changes or to upregulation of OmpN. Further studies are required to determine the nature of this particular phenotype.

Table 1. LPS glycosyl composition analysis

Arabinose, fucose, mannose, glucoronic acid, galacturonic acid, *N*-acetylmannosamine, rhamnose and xylose were not detected in all samples. Gal, Galactose; Glu, glucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine. ND, Not detected.

Glycosyl residue	Wild-type		Δ*wibT*		Δ*gne*		Δ*ugd*	
	(Wt% CHO 20.04)		(Wt% CHO 56.3)		(Wt% CHO 28.3)		(Wt% CHO 89.3)	
	Mass (μg)	M%*	Mass (μg)	M%*	Mass (μg)	M%*	Mass (μg)	M%*
Gal	109.3	25.7	ND	–	ND	–	ND	–
Glu	234.3	55	101.9	⩽100	140.1	99.7	225.3	100
GalNAc	76.3	14.7	ND	–	ND	–	ND	–
GlcNAc	11.4	2.2	ND	–	ND	–	ND	–
KDO	15.3	2.6	Trace	–	10.7	0.7	ND	–

*Values are expressed as mol% of total carbohydrate.

Motility

E. ictaluri Δ*wibT* and Δ*ugd* presented defects in motility and absence of flagellar synthesis (Fig. 2f, g). The *E. ictaluri* Δ*gne* mutant did not have motility defects in contrast to the Δ*wibT* and Δ*ugd* LPS mutant strains (Fig. 2f, g). *E. ictaluri* Δ*galF* showed similar motility to the wild-type (data not shown). Motility and flagellar synthesis were restored upon complementation with the respective wild-type genes (data not shown).

Surface bacterial adhesion

E. ictaluri wild-type and Δ*wibT*, Δ*galF* and Δ*gne* mutants did not attach to the polystyrene plate surface, either in the presence or in the absence of CAMPs in BHI or LB broth. In contrast, *E. ictaluri* Δ*ugd* strongly adhered to the plate in either the presence or the absence of CAMPs (Fig. 2h–k). *E. ictaluri* Δ*ugd* did not adhere in minimal media or water derived from catfish tanks (data not shown). The Δ*ugd* mutant produced this biofilm-like formation only when it was grown in rich media such as BHI or LB broth. The nature of this bacterial adhesion and its biochemistry require further research.

Antimicrobial peptide resistance

Bacterial resistance to CAMPs is mediated by different mechanisms, including export of peptides by efflux pumps, proteolytic degradation and alteration of the bacterial surface charge (Peschel & Sahl, 2006). To determine if *Edwardsiella* intrinsic resistance to CAMPs is mediated by efflux pumps, cultures of *E. ictaluri*, *E. tarda* and *E. hoshinae* grown in the presence and absence of Col (12.5 μg ml^{-1}) or Pmb (25 μg ml^{-1}) were normalized to 1×10^6 c.f.u. ml^{-1} and treated with Cm (25 μg ml^{-1}) to preclude protein synthesis. The number of viable cells in the presence and absence of Col or Pmb was determined at 1, 2, 4, 8, 24 and 48 h. We did not detected significant differences in the number of c.f.u. between cells grown in the absence and presence of CAMPs. This result suggests that *Edwardsiella* intrinsic resistance to CAMPs is not mediated by efflux pumps.

To determine if *Edwardsiella* resistance to CAMPs is mediated by proteases, filter spent media from *Edwardsiella* wild-type cultures grown in the presence and absence of Col or Pmb were assayed on the CAMP-sensitive strains *Escherichia coli* K-12 and *E. ictaluri* Δ*ugd*. *Edwardsiella* wild-type species were utilized as resistant controls. *Escherichia coli* K-12 and *E. ictaluri* Δ*ugd* did not grow in filtered supernatants from the *Edwardsiella* wild-type species grown in the presence of Col or Pmb. In contrast, the *Edwardsiella* wild-type controls grew in all conditions. This result suggests that *Edwardsiella* intrinsic resistance to CAMPs is not mediated by proteases.

To determine if *Edwardsiella* intrinsic constitutive resistance to CAMPs is mediated by O-PS, *E. ictaluri* O-PS putative related genes were deleted (Fig. 1a). *E. ictaluri* Δ*wibT*, Δ*galF* and Δ*gne* mutant strains are resistant to CAMP (Fig. 2l, m) (Table 2). *E. ictaluri* Δ*ugd* was extremely sensitive to CAMPs (Fig. 2l, m) (Table 2). This phenotype was reverted in the complemented strain (Fig. 2l, m) (Table 2 and Fig. S1). This result indicates that UDP-glucuronic acid and its derivatives are important for *Edwardsiella* intrinsic resistance to CAMPs.

As previously mentioned, Ugd (UDP-glucose dehydrogenase) converts UDP-glucose to UDP-glucuronic acid (UDP-GlcA). UDP-GlcA is used as substrate for colanic acid, type 4 capsules, sialic acid and L-Ara4N synthesis (Whitfield, 2006). Among these structural molecules, L-Ara4N is related to CAMP resistance in *S. enterica* (Bader *et al.*, 2005), *Y. pestis* (Rebeil *et al.*, 2004), *Pseudomonas aerugionosa* (McPhee *et al.*, 2003) and *Bulkholderia* species (Ortega *et al.*, 2007; Silipo *et al.*, 2005). *E. ictaluri* possesses the genes required for synthesis and transport of L-Ara4N, *arnB*, *arnC*, *arnA* and *arnT*. In *S. enterica* the *arnBCAT* gene cluster is controlled by the PmrA-PmrB two-component system, which is under control of the PhoP-PhoQ two-component system and iron environmental concentrations (Gunn *et al.*, 1996; Kox *et al.*, 2000). Also, in *S. enterica* and *Y. pestis*, the *ugd* gene is under indirect and direct control by PhoP, respectively (Aguirre *et al.*, 2000; Kato *et al.*, 2003; Winfield *et al.*, 2005; Wösten & Groisman, 1999).

Semiquantitative RT-PCR assay for *E. ictaluri ugd*, *phoP* and *arnT* genes indicates that these genes are constitutively expressed, regardless of the presence or absence of CAMPs (Fig. 3). This contrasts with the regulation described for *S. enterica* and *Y. pestis*. Recently, a subtle upregulation of *ugd* has been reported in *E. tarda* (Lv *et al.*, 2012), which coincides with our results (Fig. 4). In fact, all the genes evaluated and related to CAMP resistance were slightly upregulated in the presence of CAMPs (Fig. 3).

The *ugd* gene is part of the O-PS operon under control of a jump-start promoter and it overlaps with the *gne* gene at the 5′ end (Fig. 1a). This contrasts to the situation in *Salmonella* and *Yersinia* where the *ugd* gene does not overlap with other adjacent genes and has its own promoter under control of PhoP (Winfield *et al.*, 2005). The fact that the *E. ictaluri ugd* gene is controlled by a jump-start promoter correlates with the intrinsic constitutive resistance to CAMPs described in the early literature (Muyembe *et al.*, 1973; Reinhardt *et al.*, 1985; Stock & Wiedemann, 2001) and is coincident with our data (Table 2). However, the subtle regulation observed in *E. ictaluri ugd* and *arnT* genes (Fig. 3) may have implications during infection.

Survival in whole blood and resistance to complement

E. ictaluri wild-type and O-PS mutants survived in guinea and catfish serum, and whole catfish blood, indicating that

Table 2. Minimum inhibitory concentrations

Assays were performed in MH supplemented with 5 % fetal bovine serum. ND, Not determined.

Strain	Polymyxin B (μg ml^{-1})	Colistin (μg ml^{-1})	Ox-bile (mg ml^{-1})	Deoxicholate (mg ml^{-1})
J100 *E. ictaluri* 2003/c	400	200	>60	>60
J102 *E. ictaluri* ATCC 33202	300	200	ND	ND
J103 *E. ictaluri* ATCC 33829	300	200	ND	ND
J104 *E. ictaluri* ALO	300	200	ND	ND
J108 *E. ictaluri* S97-773	300	200	ND	ND
J115 *E. tarda* PPD130/91	50	50	>60	>60
J145 *E. hoshinae* ATCC 33379	200	200	>60	>60
χ289 *Escherichia coli* K-12	5	10	ND	ND
J124 *E. ictaluri* Δ*wibT90*	400	200	>60	>60
J124 *E. ictaluri* Δ*wibT90* (pEZ159)	300	200	>60	>60
J123 *E. ictaluri* Δ*galF91*	200	300	>60	>60
J126 *E. ictaluri* Δ*gne-31*	400	300	>60	>60
J135 *E. ictaluri* Δ*ugd-11*	1.25	2.5	>60	>60
J135 *E. ictaluri* Δ*ugd-11* (pEZ154)	300	100	>60	>60

LPS deletions do not affect resistance to complement (Figs S2–S4).

Zebrafish colonization and virulence

Zebrafish is not the natural host of *E. ictaluri*, but has been established as a reliable model system to evaluate *E. ictaluri* virulence (Petrie-Hanson *et al.*, 2007; Santander *et al.*, 2010, 2011). We found that *E. ictaluri* Δ*wibT* and Δ*gne* administered intramuscularly (i.m.) were not fully attenuated in zebrafish (Fig. 4a, b). The *E. ictaluri* Δ*ugd* was hyper-attenuated, in contrast to the wild-type (Fig. 4c).

Establishing the means by which *Edwardsiella* infect and colonize fish tissues provides a strategy not only to develop effective live attenuated vaccines but also to determine the effects of the different O-PS mutated genes on pathogenesis. We evaluated the colonization of spleen, kidney and gills 3 days post i.m. infection with *E. ictaluri* O-PS mutant strains in comparison with the wild-type. *E. ictaluri* Δ*wibT* colonized spleen, kidney and gills at similar levels to the wild-type (Fig. 4d–f). In contrast, *E. ictaluri* Δ*ugd* presented significantly lower levels of colonization (Fig. 4f). The *E. ictaluri* Δ*gne* showed intermediate levels of colonization with respect to the wild-type and Δ*ugd* mutant. This indicated that *E. ictaluri* O-PS mutant strains reached the lymphoid tissues after i.m. immunization, thus trigging a protective immune response (Fig. 4g–i). However, *E. ictaluri* Δ*wibT* conferred only 60 % protection, in contrast to Δ*gne* and Δ*ugd* that conferred 100 % protection at the lowest dose of immunization by the i.m. route (Fig. 4g–i).

Attachment and invasion in catfish ovary cells

CCO cells and the gentamicin exclusion method were used to evaluate attachment and invasion of the *E. ictaluri* O-PS mutants. *E. ictaluri* Δ*wibT* and Δ*gne* showed a slight increase in attachment and invasion in comparison with the wild-type (Fig. 5a, b). *E. ictaluri* Δ*ugd* presented a significant decrease in attachment and colonization (Fig. 5a, b).

Survival in catfish primary macrophages

The role of *E. ictaluri* LPS in macrophage phagocytosis resistance was evaluated in intestinal and head-kidney primary macrophages. *E. ictaluri* wild-type proliferated ~20 % in intestinal macrophages (Fig. 5c) and killed ~20 % in head-kidney macrophages (Fig. 5d). All *E. ictaluri* O-PS mutants were killed ~35 % in intestinal primary macrophages (Fig. 5c). *E. ictaluri* Δ*wibT* was killed ~20 % in head-kidney macrophages, similar to *E. ictaluri* wild-type (Fig. 5d). This is coincident with the residual virulence observed in *E. ictaluri* Δ*wibT* (Fig. 4). *E. ictaluri* Δ*gne* was killed in around 50 % and *E. ictaluri* Δ*ugd* in around 60 % by the head-kidney macrophages (Fig. 5d).

Catfish virulence and colonization

Fingerlings were orally inoculated with 100 μl of the respective *E. ictaluri* O-PS mutant and wild-type. Colonization of lymphoid tissues was evaluated at 4 and 8 days post-infection. At 4 days post-infection all the *E. ictaluri* O-PS mutants were present in the lymphoid tissues tested, but at lower levels than the wild-type (Fig. 5e–h). *E. ictaluri* Δ*ugd* presented significantly lower levels of colonization in the intestine, liver and spleen 4 days post-oral infection. At 8 days post-infection the *E. ictaluri* O-PS mutants persisted in the intestine with Δ*ugd* at low levels (Fig. 5f). *E. ictaluri* Δ*wibT* and Δ*ugd* were found in low levels in the kidney 8 days post-infection (Fig. 5h). The *E. ictaluri* Δ*gne* was not detected in the kidney 8 days

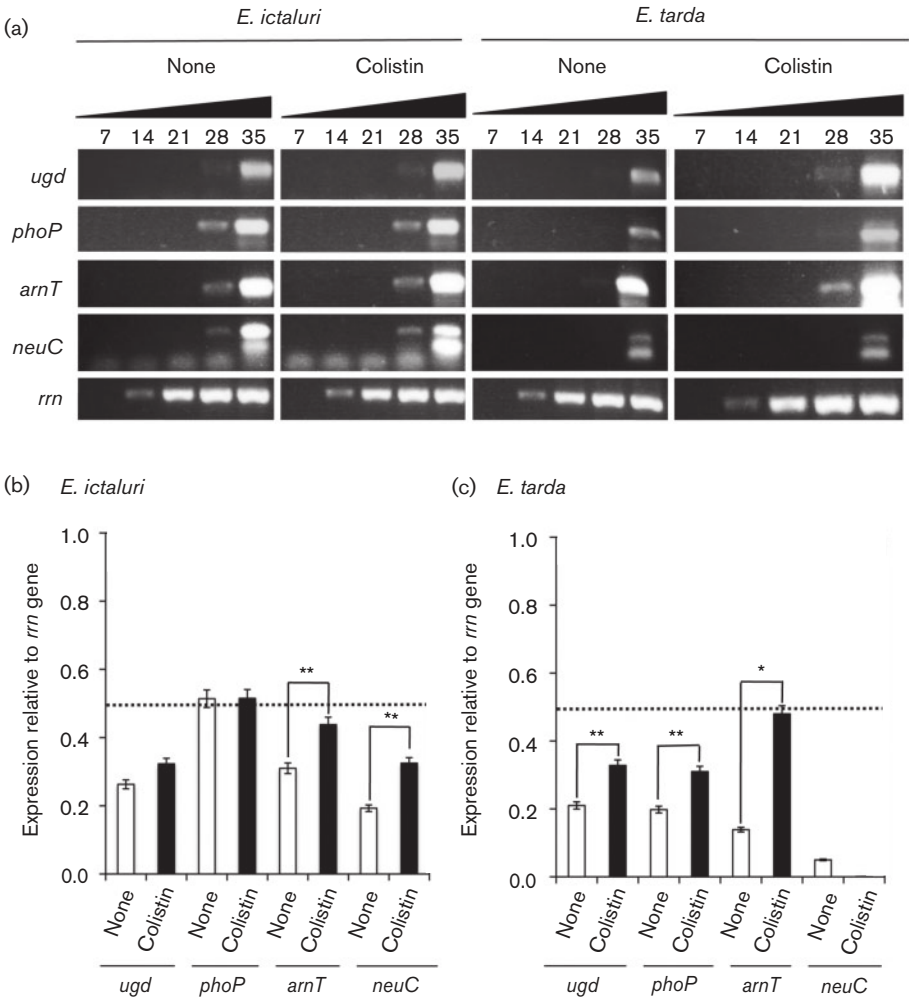

Fig. 3. Semiquantitative RT-PCR for the *ugd*, *phoP*, *arnT* and *neuC* genes in *E. ictaluri* and *E. tarda* grown in the absence and presence of Col (12.5 μg ml^{-1}). The dashed line represents the expression of the *fadR* housekeeping gene. The *neuC* gene was used as upstream gene control for *ugd* (see Fig. 1). (a) RT-PCR; (b, c) semiquantitative RT-PCR. The samples correspond to two independent experiments. *$P<0.001$; **$P<0.05$.

post-infection (Fig. 5h). *E. ictaluri* Δ*ugd* was not detected in the liver 8 days post-infection (Fig. 5j). At 8 days post-infection, *E. ictaluri* O-PS mutants were not detected in the spleen, in contrast to the wild-type (Fig. 5l).

Gut inflammation and histopathology

Orally inoculated catfish with the wild-type strain presented significant fluid secretion 5 days post-infection (Fig. 6b, c) as well as gut inflammation (Fig. 6d). This was corroborated by histopathology analysis where *E. ictaluri* wild-type caused significant damage to the intestinal epithelial and liver tissue (Fig. 6d). *E. ictaluri* Δ*wibT* caused damage to the intestinal tissue and liver (Fig. 6d). This result correlates with the remaining virulence of Δ*wibT* in zebrafish and catfish (Fig. 4a and Table 3). *E. ictaluri* Δ*gne* and Δ*ugd* caused moderate to low damage to the intestinal epithelia (Fig. 6d). Liver damage was not detected in catfish orally inoculated with *E. ictaluri* Δ*gne* and Δ*ugd* (Fig. 6d). Kidney damage was not detected in any fish inoculated with O-PS mutants (data not shown).

Virulence and immune protection in catfish

The *E. ictaluri* O-PS Δ*gne* and Δ*ugd* mutants are totally attenuated when they are administered i.c., orally or by immersion to the fish. The Δ*wibT* mutany administered i.c., orally and by immersion to the fish caused mortality at lower levels than the wild-type (Table 3).

E. ictaluri Δ*wibT*, Δ*gne* and Δ*ugd* administered i.c. conferred full protection to the immunized fish against the immersion challenge, but did not confer protection against the i.c. challenge. Δ*wibT* and Δ*ugd* administered orally or by immersion conferred poor immune protection

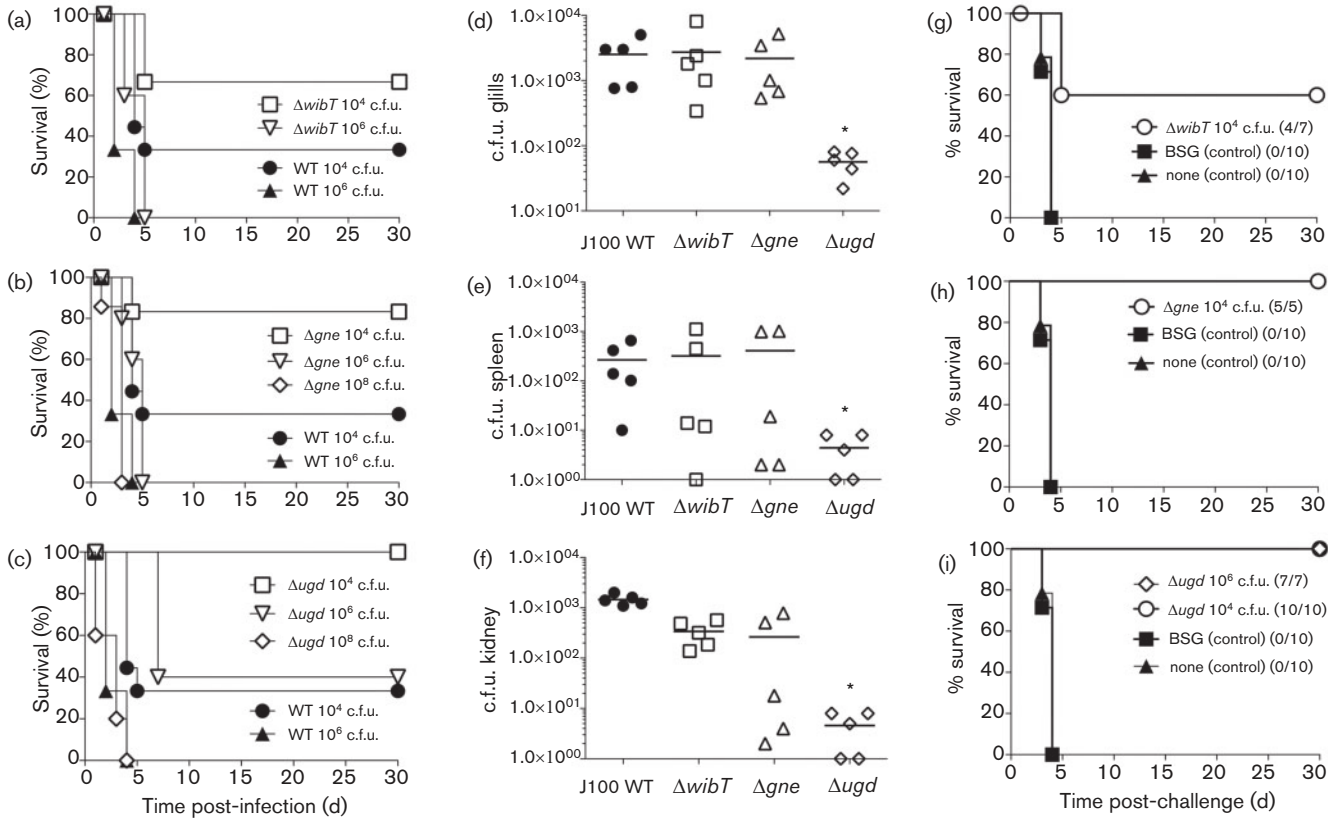

Fig. 4. Evaluation of *E. ictaluri* O-PS mutant strain virulence, colonization and immunogenicity in zebrafish (*D. rerio*) host. (a–c) Virulence of *E. ictaluri* O-PS mutants in zebrafish i.m. infected. (d–f) Colonization of zebrafish tissues 3 days post i.m. infection. The samples correspond to five independent animals. *$P<0.001$. (g–i) Zebrafish challenged with *E. ictaluri* wild-type 4 weeks post-immunization with *E. ictaluri* O-PS.

(<60 %) against the immersion challenge (Table 3). In contrast, Δ*gne* administered orally or by immersion conferred a protective immune response against the immersion challenge (>70 %) (Table 3). These results indicate that *E. ictaluri* Δ*gne* could be considered a component in the design of oral–immersion live attenuated vaccines for the catfish industry.

DISCUSSION

Here, we have determined the genetic basis for O-PS synthesis and CAMP resistance in *E. ictaluri*. We propose a hypothetical pathway related to O-PS synthesis and CAMP resistance in *E. ictaluri* (Fig. 7). Also, the influence of the O-PS on fish gut inflammation, internal tissue colonization, virulence and immune protection was determined.

LPS profile and glycosyl composition analysis indicates that *wibT*, *gne* and *ugd* have different effects on O-PS synthesis. The *E. ictaluri* Δ*wibT* O-PS mutant showed agglutination (Fig. 2c–e), lack of motility (Fig. 2f, g) and residual virulence (Fig. 4a) (Table 3). These results are similar to a previous report describing the *E. ictaluri wibT*::Tn5Km mutant (Lawrence *et al.*, 2003). Additionally, we determined that *E. ictaluri* Δ*wibT* utilizes galactose and synthesizes a rough LPS even in the presence of galactose in the culture growth medium. Furthermore, the *E. ictaluri wibT* gene does not complement the *S. enterica galE* mutant strain (data not shown). Based on previous bioinformatics analysis (Lawrence *et al.*, 2003) and our results, we believe that *wibT* encodes a one-way UDP-4 galactose epimerase. In theory deletion of *wibT* should only affect the external part of the O-PS subunit, leaving an O-PS with three-sugar subunits. However, deletion of *wibT* results in an O-PS subunit containing only glucose (Table 1) (Fig. 7). We think that this three-sugar subunit could be unstable or not assembled by the cell. In terms of vaccinology, *E. ictaluri* Δ*wibT* is not fully attenuated, causing mortalities and internal tissue damage, and conferring low immune protection (<60 %) (Figs 4b, h and 6i) (Table 3).

The *E. ictaluri gne* gene is predicted to encode a UDP-GlcNAc-4-epimerase (Lawrence *et al.*, 2003) that interconverts UDP-GlcNAc and UDP-GalNAc (Fig. 7), the central piece of the unbranched O-PS (Fig. 7). *E. ictaluri* Δ*gne* is attenuated, colonizes deep lymphoid tissues after

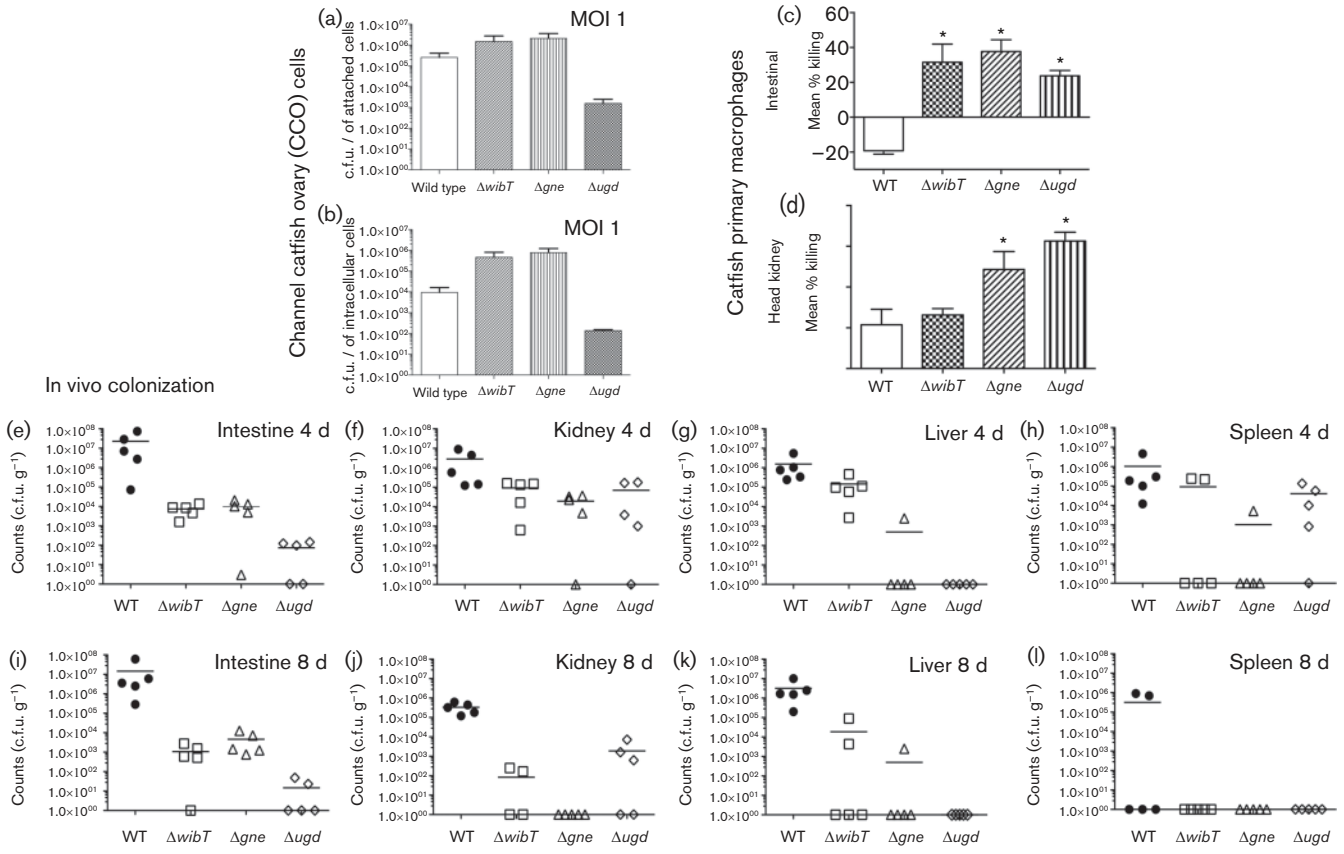

Fig. 5. Colonization of CCO cell line and catfish tissues by *E. ictaluri* O-PS. (a) Attachment. (b) Invasion. (c) Intestinal macrophage *E. ictaluri* killing assay. (d) Head-kidney macrophage *E. ictaluri* killing assay. (e–h) Four days post-oral infection. (i–k) Eight days post-oral infection. The samples correspond to five independent animals. *$P<0.001$.

oral administration and confers immune protection against the wild-type challenge (Fig. 4h and Table 3). The *E. ictaluri* Δ*gne* mutant has a good balance between attenuation and colonization, offering potent immune protection to the fish (>70 % protection). This could explain in part the ability of Δ*gne* to have increased colonization of epithelial cells, motility and mild survival in fish macrophages, in contrast to Δ*wibT* and Δ*ugd*. *E. ictaluri* Δ*gne* possesses motility, in contrast to Δ*wibT* and Δ*ugd*, which might have a major impact on tissue colonization and protective immune response. *E. ictaluri* Δ*gne* provides protective immune stimulatory antigens to the animal during bacterial colonization. Therefore, deletion of *gne* can be used as a means to attenuate *E. ictaluri* for oral–immersion vaccine development.

The *E. ictaluri ugd* gene is predicted to encode a UDP-glucose dehydrogenase (Lawrence *et al.*, 2003) that oxidizes UDP-glucose to UDP-GlcA. UDP-GlcA is found in several capsular polysaccharides (K-antigens) and in colanic acid (M-antigen), an extracellular polysaccharide produced by many *Escherichia coli* strains (Whitfield, 2006). Also, UDP-GlcA participates in the synthesis of UDP-L-Ara4N (Breazeale *et al.*, 2002; Raetz & Whitfield, 2002; Strominger, 1957), which is a crucial element in bacterial resistance to antibiotics such as polymyxin B and cationic peptides of the innate immune system (Guo *et al.*, 1998; Raetzsch *et al.*, 2009; Trent *et al.*, 2001).

A widely recognized mechanism of resistance to CAMPs in Gram-negative bacteria is the decoration of lipid A phosphate residues with the positively charged sugar L-Ara4N, which requires synthesis of a UDP-L-Ara4N precursor from UDP-GlcA (Ernst *et al.*, 1999; Helander *et al.*, 1994; Nummila *et al.*, 1995; Vaara *et al.*, 1981). L-Ara4N substitution reduces the net negative charge of the lipid A molecule and hampers the ability of CAMPs to bind to the outer membrane (Vaara *et al.*, 1981). In *Pseudomonas aeruginosa* and *S. enterica*, these substitutions are induced upon treatment with CAMPs (Bader *et al.*, 2005; McPhee *et al.*, 2003) and are dispensable for growth under normal laboratory conditions. In *Burkholderia cepacia*, L-Ara4N is constitutively incorporated into both lipid A and LPS core oligosaccharide (Silipo *et al.*, 2005). Some evidence suggests that this is also true in *B. cenocepacia*, where UDP-L-Ara4N synthesis is essential for viability (Ortega *et al.*, 2007). These observations highlight the importance of L-Ara4N in membrane

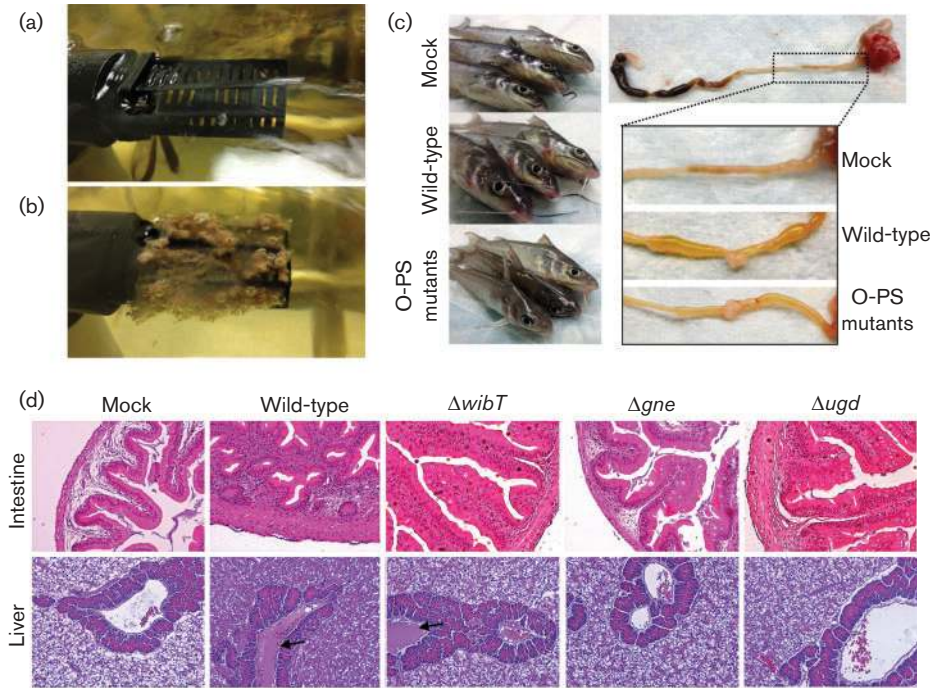

Fig. 6. External and intestinal symptoms after oral infection with *E. ictaluri* wild-type and O-PS mutants. (a) Return filter-pipe at the O-PS mutants (Δ*wibT*, Δ*gne* and Δ*ugd*) infected fish tank. No mucus secretions were detected. (b) Return filter-pipe at the wild-type infected fish tank. (c) External signs and intestinal inflammation of orally infected catfish. Catfish orally infected with *E. ictaluri* wild-type presented redness in the lateral fin, caudal fin and mouth. (d) Intestinal and liver histopathology of orally infected catfish 4 and 8 days post-oral inoculation. Mock intestine was inoculated with sterile PBS. Magnification 10×.

integrity in organisms that present high intrinsic resistance to CAMPs such as *B. cepacia*, *B. cenocepacia* and *Edwardsiella*.

Edwardsiella resistances to CAMPs are far higher (Table 2) compared with other resistances described in the family *Enterobacteriaceae*, such as *Salmonella* or *Yersinia* resistances (Aguirre *et al.*, 2000; Gunn *et al.*, 1996; Kato *et al.*, 2003; Kox *et al.*, 2000; Winfield *et al.*, 2005; Wösten & Groisman, 1999). Since its first description, *Edwardsiella* has been characterized by a constitutive resistance to CAMPs (Muyembe *et al.*, 1973; Reinhardt *et al.*, 1985; Stock & Wiedemann, 2001) in contrast to the regulated resistance described in *Salmonella* and *Yesinia* (Aguirre *et al.*, 2000; Gunn *et al.*, 1996; Kato *et al.*, 2003; Kox *et al.*, 2000; Winfield *et al.*, 2005; Wösten & Groisman, 1999). As previously mentioned, in *Salmonella* resistance to CAMPs is induced after exposure to sublethal concentrations of CAMPs or by inducing the activation of PhoP and RscB response regulators that upregulate the expression of the *ugd* gene (Gunn *et al.*, 1996; Kox *et al.*, 2000). The *Salmonella ugd* promoter region has several PhoP binding boxes that allow *ugd* upregulation upon PhoP binding (Gunn *et al.*, 1996; Kox *et al.*, 2000). In *Edwardsiella*, including *E. ictaluri* and *E. tarda*, the *ugd* gene does not present a promoter region or characteristic PhoP or RscB binding boxes downstream of the *ugd* gene. In fact, *ugd* is part of an operon that has a jump-start promoter (Lawrence *et al.*, 2003). A recent report suggests that *E. tarda ugd* is regulated by PhoP in a similar fashion to the *Salmonella ugd* gene (Lv *et al.* 2012). However, if PhoP regulates *ugd* gene expression, deletion of PhoP must have severe effects on LPS synthesis and CAMP resistance. To our knowledge, *E. ictaluri* does not show changes in LPS synthesis or significant changes in expression levels of the *ugd* and *phoP* genes in the presence or absence of CAMPs (Fig. 3). Also, the presence and absence of CAMPs do not affect cell viability, supporting the fact that *ugd* is constitutively expressed. Furthermore, it has been reported that deletion of PhoP in *E. tarda* does not affect LPS synthesis or CAMP resistance (Chakraborty *et al.* 2010). Thus, according to our data and the current literature, the reported regulation of *ugd* by PhoP in *E. tarda* is nothing more than an artefact that does not have implications for CAMP resistance.

E. ictaluri Δ*wibT* and Δ*ugd* lack motility and flagella synthesis, in contrast to the wild-type and Δ*gne* (Fig. 2g). This phenotype may be due to the inability of Δ*wibT* and Δ*ugd* mutants to assemble the flagella. However, further studies are required to understand this particular phenotype.

E. ictaluri has the ability to enter, survive and replicate in catfish macrophages (Booth *et al.*, 2009). Here, we evaluate the role of *E. ictaluri* LPS in intestinal and head-kidney macrophage survival. We determined that LPS plays an

Table 3. Infection and challenge of channel catfish with *E. ictaluri* O-PS mutants

Strain	Infection-immunization route	Infection-immunization dose (c.f.u. per dose)	Survival/total	Challenge route	Wild-type challenge dose (c.f.u. per dose)	Survival/total
Wild-type	i.c.	1.2×10^4	5/10			
	i.c.	1.2×10^6	0/13			
	i.c.	1.2×10^8	0/10			
Δ*wibT*	i.c.	1.4×10^5	10/10	i.c.	1.4×10^5	0/10
	i.c.	1.4×10^7	3/10	i.c.	1.4×10^5	0/3
	i.c.	1.4×10^8	0/10	i.c.	1.4×10^5	
Δ*gne*	i.c.	1.7×10^5	10/10	i.c.	1.4×10^5	0/10
	i.c.	1.7×10^7	10/10	i.c.	1.4×10^5	0/10
	i.c.	1.7×10^8	7/10	i.c.	1.4×10^5	0/7
Δ*ugd*	i.c.	2.4×10^5	10/10	i.c.	1.4×10^5	0/10
	i.c.	2.4×10^7	10/10	i.c.	1.4×10^5	0/10
	i.c.	2.4×10^8	8/10	i.c.	1.4×10^5	0/8
Wild-type	Oral	1.1×10^5	2/5			
	Oral	1.1×10^7	0/5			
	Oral	1.1×10^8	0/5			
Δ*wibT*	Oral	1.2×10^5	5/5	Immersion	2.1×10^7	0/5
	Oral	1.2×10^7	4/5	Immersion	2.1×10^7	1/4
	Oral	1.2×10^8	3/5	Immersion	2.1×10^7	1/3
Δ*gne*	Oral	1.6×10^5	5/5	Immersion	1.4×10^7	5/5
	Oral	1.6×10^7	5/5	Immersion	1.4×10^7	5/5
	Oral	1.6×10^8	7/7	Immersion	1.4×10^7	7/7
Δ*ugd*	Oral	1.0×10^5	6/6	Immersion	1.3×10^7	0/6
	Oral	1.0×10^7	6/6	Immersion	1.3×10^7	0/6
	Oral	1.0×10^8	5/5	Immersion	1.3×10^7	1/5
Wild-type	Immersion	1.4×10^7	1/5			
Δ*wibT*	Immersion	1.8×10^7	4/5	Immersion	2.3×10^7	2/5
Δ*gne*	Immersion	1.9×10^7	5/5	Immersion	2.3×10^7	5/5
Δ*ugd*	Immersion	1.6×10^7	6/6	Immersion	2.3×10^7	1/6

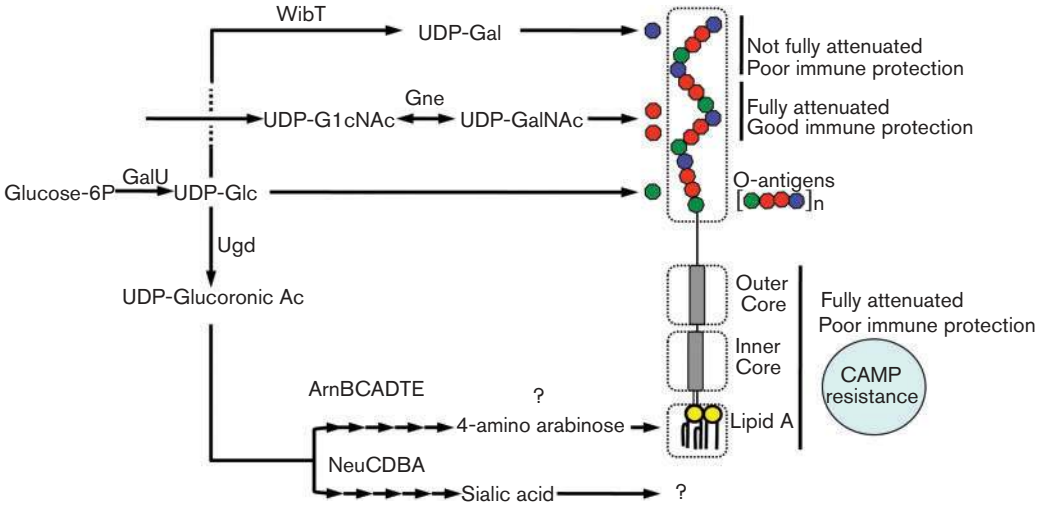

Fig. 7. Hypothetical pathways of LPS synthesis in *E. ictaluri.* The coloured circles represent the *E. ictaluri* O-chain unbranched linear polymer composed of D-glucose (green circle), 2-acetamido-2-deoxy-D-galactose (red circle) and D-galactose (blue circle) in a 1 : 2 : 1 ratio having the structure: [→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→]$_n$ (Vinogradov *et al.*, 2005).

important role in *E. ictaluri* intracellular macrophage survival that influences bacterial tissue colonization and immune protection. *E. ictaluri* O-PS LPS mutants were killed to the same level by intestinally derived macrophages, in contrast to the wild-type that was able to replicate within the gut derivative macrophages (Fig. 5c). *E. ictaluri* O-PS LPS mutants were killed by the head-kidney macrophages according to the gene deletion, wild-type=Δ*wibT*<Δ*gne*<Δ*ugd* (Fig. 5d). These results are coincident with the virulence data, where Δ*wibT* remains virulent and retains its ability to survive in head-kidney macrophages in contrast to Δ*gne* and Δ*ugd* that have their ability to survive within macrophages compromised (Figs 4 and 5d) (Table 3). We observed a clear difference in the rate of macrophage *E. ictaluri* killing between intestinal and head-kidney macrophages. This difference may be related to the macrophage differentiation process. In summary, *E. ictaluri* LPS plays an important role in macrophage survival influencing virulence, colonization and immune protection.

Fish have no or a poor inflammatory immune response to LPS (Berczi *et al.*, 1966; Iliev *et al.*, 2005; Swain *et al.*, 2008). However, there are, to our knowledge, no studies about fish intestinal gut inflammation related to *Edwardsiella* LPS. We observed that after 5 days post-oral infection with *E. ictaluri* wild-type, catfish begin to excrete faeces with mucus secretion and high *E. ictaluri* titres (10^4–10^5 c.f.u. per millilitre of faeces) (Fig. 6a, b). In contrast, catfish non-infected or orally inoculated with *E. ictaluri* Δ*wibT*, Δ*gne* or Δ*ugd*, did not present mucus secretion in their faeces (Fig. 6a, b). However, all *E. ictaluri* O-PS mutants were able to colonize intestinal tissues (Fig. 5). These results suggest that LPS O-PS has effects on fish intestinal gut inflammation and mucus secretion. The intestinal necropsy and histopathology showed that *E. ictaluri* LPS O-PS induces intestinal inflammation and fluid secretion (Fig. 6h, i). Several reports suggest that fish do not respond to LPS mainly because of the lack of PLB, CD14 and TIRF1, essential components for the function of Toll-like receptor 4 (Iliev *et al.*, 2005). Adult fish or zebrafish i.c. injected with high doses of LPS from different bacterial species, including *Edwardsiella* (100 μg), do not present mortalities or toxic shock syndrome symptoms (Swain *et al.*, 2008; our data not shown). However, at the intestinal level we observed that *E. ictaluri* LPS deletions have a profound effect on fish intestinal inflammation depending on the type of effect that the deletion has on the bacterial LPS (Fig. 6i).

In terms of vaccinology, we believe that the ideal vaccine for the aquaculture industry should be immersion–oral. Although, *E. ictaluri* Δ*gne* and Δ*ugd* conferred immune protection to the i.m. injected zebrafish (Fig. 4), only *E. ictaluri* Δ*gne* conferred protection to the immersion bath-vaccinated catfish (Table 3). Also, *E. ictaluri* Δ*gne* does not produce intestinal mucus secretion, making massive shedding to the water after oral vaccination impossible. We conclude that deletion of *gne* could be used as a component to develop effective immersion *E. ictaluri* live attenuated vaccines.

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