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Tissue persistence and vaccine efficacy of tricarboxylic acid cycle and one-carbon metabolism mutant strains of *Edwardsiella ictaluri*

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ABSTRACT

Edwardsiella ictaluri causes enteric septicemia in fish. Recently, we reported construction of *E. ictaluri* mutants with single and double gene deletions in tricarboxylic acid cycle (TCA) and one-carbon (C-1) metabolism. Here, we report the tissue persistence, virulence, and vaccine efficacy of TCA cycle (*Ei*Δ*sdhC*, *Ei*Δ*frdA*, and *Ei*Δ*mdh*), C-1 metabolism (*Ei*Δ*gcvP* and *Ei*Δ*glyA*), and combination mutants (*Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*glyA*) in channel catfish. The tissue persistence study showed that *Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*frdA*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*sdhC* were able to invade catfish and persist until 11 days post-infection. Vaccination of catfish fingerlings with all nine mutants provided significant ($P < 0.05$) protection against subsequent challenge with the virulent parental strain. Vaccinated catfish fingerlings had 100% survival when subsequently challenged by immersion with wild-type *E. ictaluri* except for *Ei*Δ*gcvP*Δ*glyA* and *Ei*Δ*gcvP*. Mutant *Ei*Δ*gcvP*Δ*sdhC* was found to be very good at protecting catfish fry, as evidenced by 10-fold higher survival compared to non-vaccinated fish.

1. Introduction

Enteric septicemia of catfish (ESC) continues to be an important disease of farm-raised channel catfish (*Ictalurus punctatus*). In 2010, catfish farmers operating in Alabama, Mississippi, Arkansas, and Louisiana reported 36.6% mortality rates due to ESC [1]. Although considerable progress has been made in developing live attenuated vaccines [2–8], and a commercial vaccine is available, ESC is still a major threat to the catfish industry.

Live attenuated strains of facultative intracellular bacterial pathogens are often effective in developing strong cell mediated immune responses [3]. We previously reported that genes encoding TCA cycle enzymes and one-carbon metabolism proteins are required for virulence of *Edwardsiella ictaluri* [9]. To better understand the role of *E. ictaluri* TCA cycle genes and one-carbon metabolism genes in pathogenesis and immunity, single gene deletion mutants and gene combination mutants were then constructed in our previous studies [5,14].

The *sdhC* gene encodes one of the four subunits of the succinate dehydrogenase (Sdh) complex and oxidizes succinate to fumurate while reducing ubiquinone to ubiquinol [10]. Fumrate reductase (Frd) catalyzes the reverse reaction in aerobic respiration [10]. Sdh is known to contribute pathogenicity in *Escherichia coli* and *Salmonella enterica* [10,11]. A *S. enterica* serovar Typhimurium Δ*sdhCDA* mutant had slight attenuation, and a Δ*frdABCD* mutant was fully virulent [11]. However, a *sdh* and *frd* combination mutant was completely attenuated [11]. Malate dehydrogenase (encoded by *mdh*) catalyzes the conversion of oxaloacetate and malate utilizing the NAD/NADH coenzyme system [12]. Using a mouse oral challenge model, *Salmonella mdh* mutants were avirulent [13]. The *gcvP* gene encodes a protein that is part of the glycine cleavage system, and *glyA* encodes serine hydroxymethyltransferase. To our knowledge, these two genes have not been linked with virulence in other bacterial species, but we showed that the GcvP protein is critical for both neutrophil and serum resistance in *E. ictaluri* [9].

Previously, we determined the degree of attenuation of TCA cycle and C-1 metabolism mutants in channel catfish fingerlings using IP injection route of exposure with bioluminescence imaging [14]. In the current study, vaccine efficacy of mutants was tested in catfish fingerlings and fry by the immersion route, which is a delivery method that can be used in commercial catfish production.

2. Materials and methods

2.1. Ethics statement

All fish experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University.

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2.2. Bacterial strains

E. ictaluri 93-146 and nine deletion mutant strains (*Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*mdh*, *Ei*Δ*gcvP*, *Ei*Δ*glyA*, *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*glyA*) were grown at 30 °C in brain–heart infusion (BHI) broth or agar plates (Difco, Sparks, MD). *E. ictaluri* selective medium (EIM) [15] was used for the tissue persistence study to quantify *E. ictaluri* from serially diluted tissue homogenate. For fish challenges, *E. ictaluri* 93-146 and mutants were grown in BHI broth for 18 h at 30 °C by shaking at 175 rpm. For the tissue persistence study, *E. ictaluri* 93-146, *Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*frdA*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*sdhC* with pAKgfplux1, which carries *luxCDABE* that constitutively expresses in *E. ictaluri* [16], were grown in BHI broth with ampicillin (100 μg/ml) for 18 h at 30 °C by shaking at 175 rpm.

2.3. Tissue persistence

Two hundred and forty catfish fingerlings (6-month-old) were stocked into ten 40-L tanks (24 fingerlings per tank) supplied with flow-through dechlorinated municipal water. Temperature was maintained at 26–28 °C throughout the trial. After a week of acclimation, duplicate tanks were exposed to each mutant (*Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*frdA*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*sdhC*) and wild-type *E. ictaluri* 93-146 (1×10^7 CFU/ml of water) by immersion [5]. Before exposure, one fish was removed from each tank to serve as negative control and establish the baseline.

At each time point, three fish were randomly selected and removed from each tank and euthanatized by transferring to water containing 400–500 mg tricaine methanesulfonate (Sigma–Aldrich, St. Louis, MO) per liter of water. Sampling points were at 2 h, 6 h, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 17, and 21 d post-infection. Trunk kidney was aseptically removed from each fish and weighed. Bioluminescence was quantified from the tissue at each time point using an IVIS Imaging System (Caliper Corporation, Hopkinton, MA). After imaging, each tissue was suspended in 0.5 ml of sterile water and macerated. The resulting suspension was serially diluted depending upon the bioluminescent signal from IVIS imaging, and 15 μl aliquots were spread on EIM plates for quantification. Colony counts were determined at 3 d of incubation. The *E. ictaluri* colonies were identified as 0.5–1.0 mm green, translucent colonies [15]. The number of CFU/g of tissue was calculated for each fish.

For statistical analyses, the calculated CFU/g was transformed by taking the base 10 logarithm to improve normality. To compare mean bacterial concentrations between strains at each time point, a one-way ANOVA of the transformed data was conducted using SPSS 19 statistical software (IBM Corp., Armonk, NY). Pair wise comparison of the means was done using Tukey procedure at significance level of 0.05. Data was then retransformed for interpretation.

2.4. Vaccine trial

Four-month-old channel catfish (12.37 ± 0.5 cm, 46.31 ± 5.3 g) were stocked at a rate of 15 fish/tank into thirty three 40-L tanks and randomly divided into 11 treatment groups with three tanks per treatment. Nine of the 11 treatment groups were vaccinated with *Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*mdh*, *Ei*Δ*gcvP*, *Ei*Δ*glyA*, *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*glyA*, and one of the 11 treatment groups was exposed to wild type *E. ictaluri* 93-146. One treatment group served as sham-vaccinated control. Immersion challenge was done as previously described [5] using 3.25×10^7 CFU/ml of water. Mortalities were recorded for 21 days. On day 21 post-vaccination, vaccinated and non-vaccinated treatments were experimentally infected with wild-type *E. ictaluri* by immersion exposure with a final bacterial concentration of approximately 3.1×10^7 CFU/ml in water. Mortalities were recorded for 2 weeks.

For statistical analyses, the mean percent mortalities were calculated for each treatment, including mortalities on fish infected with mutants, wild-type strain, and sham. Percent mortality data were arcsine-transformed before analysis. Transformed mean percent mortality data were then compared by Student's *t*-test. For the first (vaccination) challenge, *t*-tests compared mean mortalities from each mutant treatment compared to the wild-type strain treatment. For the second (efficacy) challenge, *t*-tests compared mean mortalities from each vaccination treatment to the sham vaccination treatment.

2.5. Vaccine safety in catfish fry

Vaccine safety in catfish fry was determined for each of the nine mutant strains. Briefly, 14 day old specific pathogen free (SPF) catfish fry were transferred into 42 tanks (approximately 70 fry/tank, three replicates/treatment). Fry were immersion exposed to 10^7 CFU/ml for single mutants (*Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*mdh*, *Ei*Δ*gcvP*, and *Ei*Δ*glyA*) and either 10^7 CFU/ml or 10^6 CFU/ml for combination mutants (*Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*glyA*). Mortalities were recorded daily for 21 days. On day 21 post-vaccination, all treatments were immersion exposed to parent strain *E. ictaluri* 93-146 at approximately 10^7 CFU/ml.

For statistical analyses, the mean percent mortality data were calculated for each treatment. Percent mortality data were arcsine-transformed before analysis. For the first (vaccination) trial, transformed mean percent mortality data for the lower dose (10^6 CFU/ml) and the standard vaccine dose (10^7 CFU/ml) of combination mutants were compared by Student's *t*-test. Percent mortalities were also compared between each mutant and its corresponding combination mutant. For the second (efficacy) trial, transformed mean percent survival data for each vaccination treatment were compared with the sham vaccination treatment using Student's *t*-test. Percent survival was also compared between vaccination doses for each mutant.

3. Results

3.1. Tissue persistence

At each time point until 11 days post-infection, *Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*frdA*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*sdhC* mutants were isolated on EIM plates from trunk kidney of catfish. The mean CFU/g of posterior kidney tissue for mutants and wild-type *E. ictaluri* are shown in Fig. 1. The tissue concentration of wild-type *E. ictaluri* was significantly higher than the concentration of *Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*frdA*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*sdhC* at 9, 11, and 14 days post-infection ($P < 0.05$). However, there was no significant difference between the mean concentration of wild-type *E. ictaluri* and mutants *Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*frdA*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*sdhC* from 2 h until 7 days post-infection ($P > 0.05$).

No signal or very low bioluminescence was detected in the tissue of all the mutants at most of the time points except for 24–96 h post-infection for *Ei*Δ*sdhC*, *Ei*Δ*frdA*, and wild-type *E. ictaluri*. At these time points, bioluminescence in kidney tissue was high for these two mutants (data not shown).

3.2. Vaccine trial

To determine vaccine efficacy of the nine mutants (*Ei*Δ*sdhC*, *Ei*Δ*frdA*, *Ei*Δ*mdh*, *Ei*Δ*gcvP*, *Ei*Δ*glyA*, *Ei*Δ*frdA*Δ*sdhC*, *Ei*Δ*gcvP*Δ*sdhC*, *Ei*Δ*mdh*Δ*sdhC*, and *Ei*Δ*gcvP*Δ*glyA*), an

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