Construction and screening of a library of attenuated mutants in *Edwardsiella ictaluri*, identification of genes involved in bacterial invasion and potential as vaccines.

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Introduction

Edwardsiella ictaluri is the causative agent of enteric septicemia in channel catfish (Plumb, 1998).

It arguably is the most severe disease affecting the channel catfish industry causing losses estimated to be about ten million dollars annually.

E. ictaluri's resistance to neutrophils (Ainsworth and Chen, 1990; Karsi and Lawrence, 2007) as well as macrophages has been demonstrated (Thune, R.L., Stanley, L.A. and Cooper, R.K., 1993).

The bacterium can also invade non-professional phagocytic cells such as epithelial cells (Skirpstunas and Baldwin, 2002).

E. ictaluri can also colonize areas of abrasion on the host epithelium, leading to increased virulence (Menanteau-Ledouble, Karsi and Lawrence, under press).

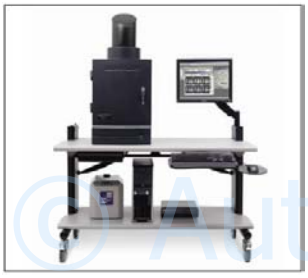
The present study was designed to investigate the genes involved in the attachment of *E. ictaluri* to sites of abrasion.

Furthermore, we investigated at which stage this colonization was impaired by studying the kinetics of infection.

Finally, because this study generated a number of attenuated strains, we decided to test these strains for potential as vaccine against *E. ictaluri.*

Material and Method – Mutant Library

E. ictaluri pathogenic isolate 93146 was transformed with pAKGFPLux, allowing for constitutive expression of the luciferase operon (Karsi, Menanteau-Ledouble and Lawrence, 2006; Karsi and Lawrence 2007).



This allowed for the detection of the bacteria by using the IVIS system (Figure 1).

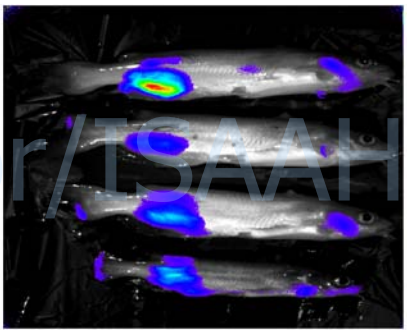
Figure 1: The IVIS system.

Bioluminescent *E. ictaluri* were transformed with a plasmid carrying the Mar2xT7 transposon, which led to random insertion of this transposon into the bacterial chromosome and random inactivation of bacterial genes.

A library of 1728 such mutants was constructed

Then these mutants were used in a series of nested challenged on catfish abrasion sites (Menanteau-Ledouble and Lawrence; submitted).

Figure 2: Example of bioluminescent *E. ictaluri* mutants on catfish skin abrasions.



At the end of the challenges, 18 mutants were identified that appeared to display a decrease in their ability to colonize the epithelium (Figure 2).

The abilities of the 18 mutants to colonize skin abrasions was compared to wild type 93-146 by quantifying bioluminescence at 72 hours post-infection following immersion exposures.

We also compared virulence of the mutants to wild type strain 93-146 in an immersion challenge using 2 tanks per mutant with 23 fish per tank.

In all but one case, luminescence was significantly lower in the mutants than in the wild type (Figure 3).

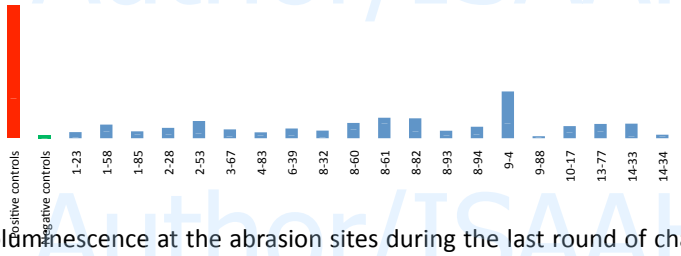


Figure 3: Bioluminescence at the abrasion sites during the last round of challenge as measured in photons/cm^2/second using an IVIS Imaging System (Caliper).

And so were percent mortalities (Figure 4).

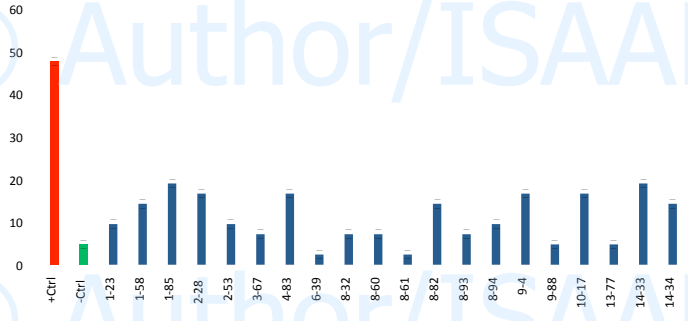


Figure 4: Percent mortalities during the last round of challenge.

Single primer PCR (Karlyshev, Pallen and Wren, 2000) was performed on genomic DNA from each mutant to amplify DNA flanking the transposon insertion sites (Figure 5).

Products were sequenced to identify the mutated genes.
Mutated genes were identified by BLAST against the *E. ictaluri* genome, and gene functions were predicted by BLAST search against the NCBI database (Table 1).

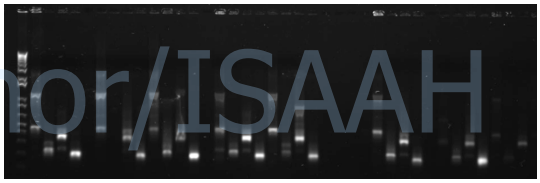


Figure 5: Gel electrophoresis of products from Single Primer PCR

Table 1. List of the genes mutated in this experiment.

Mutant identified:	Corresponding gene:
Plate 1; well 23	Sensor protein RstB
Plate 1; well 58	Periplasmic dipeptide transport protein (DBP)
Plate 1; well 85	Peptidase, U61 family
Plate 2; well 28	Septum site-determining protein MinC, putative
Plate 2; well 53	N-acetylmuramoyl-L-alanine amidase AmiD
Plate 3; well 67	Periplasmic dipeptide transport protein (DBP)
Plate 4; well 83	Glycerol-3-phosphate transporter
Plate 6; well 39	Dimethyladenosine transferase, putative
Plate 8; well 32	5-methyltetrahydropteroyltriglutamate-homocysteine, putative
Plate 8; well 60	Hypothetical protein (Type 2)
Plate 8; well 61	Valine-pyruvate aminotransferase
Plate 8; well 82	Hypothetical protein (Type 1)
Plate 9; well 4	Hypothetical protein (Type 3)
Plate 9; well 88	Hypothetical protein (Type 7)
Plate 10; well 17	Ribonuclease R, putative
Plate 13; well 77	Hypothetical protein (Type 4)
Plate 14; well 33	Hypothetical protein (Type 6)
Plate 14; well 34	Hypothetical protein (Type 5)

Most notable among the identified genes were:

RstA, a regulator linked to the regulation of the formation of curli fimbriae or amyloid fibers in *Escherichia coli*.

A heat-shock protein, presenting strong homology to one often associated with flagellar synthesis as well as its associated proton pump.

Also very interesting was 4-83, which is annotated as a transporter.

However, a very similar protein, termed SP41, was recently identified as playing a role in the adherence and invasion of *Brucella* (Castañeda-Roldan *et al*. 2006).

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Characterization of SP41, a surface protein of *Brucella* associated with adherence and invasion of host epithelial cells

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cells and this effect could be reversed by restoration of the *ugpB* on a plasmid. Lastly, we also show that most of the sera from individuals with acute brucellosis, but not sera obtained from healthy donors or patients with chronic brucellosis, mount antibody reactivity against SP41, suggesting that this protein is produced *in vivo* and that it elicits an antibody immune response. These data are novel findings that offer new insights into understanding the interplay between this bacterium and host target cells, and

Material and Method – Kinetics of infection

At this point, we had identified 18 attenuated strains.

Therefore, four strains were selected based on their predicted function and location on the surface of the bacterial cell:

- 1-58: A putative trans-membrane transporter involved in the transport of heme and fibronectin binding proteins.
- 1-85: A putative peptidase involved in the synthesis of the peptidoglycan cell wall .
- 2-53: A putative AmiD peptidase also involved in peptydolgycan metabolism .
- 4-83: A homolog to SP41, a new class of adhesin recently discovered in *Brucella*

These strains were used alongside a bioluminescent wild type *E. ictaluri* (intended as a positive control) and a bioluminescent *E. coli* (negative control).

Each treatment had two tanks, each containing four fish.

10 month old fish were abraded two times alongside the lateral line, and an overnight culture of *E. Ictaluri* was applied topically on the abrasion sites.

After infection, the fish were imaged every 24 hours using the IVIS to monitor bacterial quantities.

Results – Kinetics of infection

Bioluminescence indicated that the bacteria were able to colonize the abrasion sites (Figure 6).

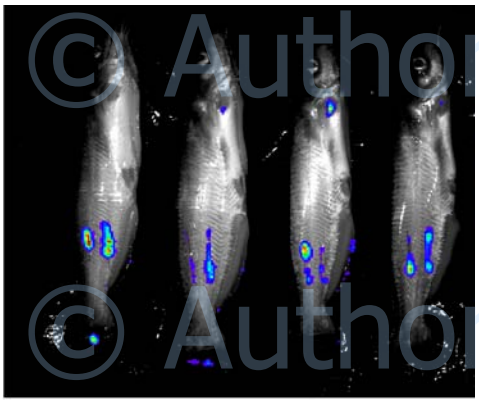

Figure 6: Image collected during the abrasion study (strain 2-53 at 72 hours P.I). Bioluminescent bacteria are visible on abrasion sites on the lateral abdomen.

Results – Kinetics of infection

As expected, bacterial loads were low in every treatment group (3,86E+04 p/s/cm^2/sr average across treatment and time points) and no better than the *E. coli* negative control (Figure 7).

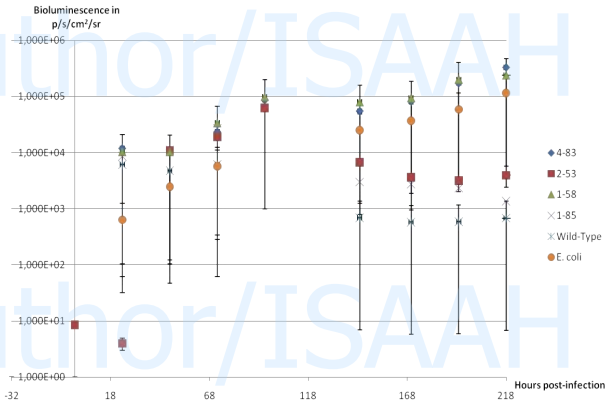

Figure 7:
Mean bioluminescence over time in the abrasion experiment.

Bacterial loads for all four mutants increased until 96 h post-infection.

Bacterial quantities at 72 h post-infection were consistent with the earlier study (Fig. 3)

After 96 h post-infection, two of the mutants (4-83 and 1-58) increased to $>10^5$ p/s/cm^2/sr, while two (1-85 and 2-53) decreased to $<10^4$ p/s/cm^2/sr

Unfortunately, wild type *E. ictaluri* control infection results were not consistent with previous experiments.

Material and Method – Vaccine Trial

Finally, we used these four strains in a vaccine trial.

Trial was designed to mimic vaccination methods used in commercial catfish production:

- Fourteen day old specific pathogen free catfish fry were used.
- Two vaccine doses were used: 10^5 and 10^6 CFU per ml of tank water.
- Fry were immersed in this suspension for 5 minutes; water was then added to dilute it 4 times.
- Fry were maintained in this vaccine dose for 1 hour.
- Mortalities following vaccination were recorded daily.

Unfortunately, results were disappointing.

Despite being strongly attenuated in older fish, all vaccines caused mortalities in catfish fry.

Among these strains, 2-53 was the least virulent but still induced 29.67% mortality at its lowest dose.

All these strains provided some varying levels of protection, but the best protection was provided by the least attenuated strains.

Discussion

Using bioluminescence in a series of nested challenges, high throughput screening of a large number of *E. ictaluri* mutants was accomplished to identify mutants that fail to bind on catfish skin abrasions.

18 mutants were identified that have decreased abilities to colonize abraded epithelium and induce mortalities.

Among the corresponding genes were a *rtsA* homolog and gene encoding a flagellar regulator as well as a new adhesion protein that were not previously known as virulence factors in *E. ictaluri*.

Kinetics of abrasion colonization revealed that the attenuated mutants were able to attach but were slow colonizers.

Bacterial numbers stabilized at 72 hours post-infection indicating that the bacteria were unable to cause a systemic infection.

The two mutants involved in peptidoglycan metabolism (1-85 and 2-53) had a more defective colonization phenotype; after peaking in numbers at 96h post-infection, quantities slowly decreased.

Finally, while promising on older fish, the strains tested during the vaccine challenge were still pathogenic and only elicited limited protection.

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