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High-Throughput Bioluminescence-Based Mutant Screening Strategy for Identification of Bacterial Virulence Genes▽

Attila Karsi,[1,2]* Nagihan Gülsoy,[3] Erin Corb,[1] Pradeep R. Dumpala,[1] and Mark L. Lawrence[1,2]*

Department of Basic Sciences, College of Veterinary Medicine,[1] and Institute for Digital Biology,[2] Mississippi State University, Mississippi State, Mississippi 39762, and Department of Biology, Faculty of Art and Science, Marmara University, Göztepe, Istanbul 34722, Turkey[3]

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A high-throughput bioluminescence screening procedure for identification of virulence genes in bacteria was developed and applied to the fish pathogen *Edwardsiella ictaluri*. A random transposon mutant library expressing bioluminescence was constructed and robotically arrayed on 384-well plates. Mutants were cultivated and mixed with catfish serum and neutrophils in 96-well plates, and bioluminescence was used to detect mutants that are more susceptible to killing by these host factors. The virulence and vaccine efficacy of selected mutants were determined in channel catfish. Transposon insertion sites in 13 mutants attenuated in the natural host were mapped to the *E. ictaluri* genome. Ten unique genes were mutated, including genes encoding a negative regulator of sigmaE activity, a glycine cleavage system protein, tricarboxylic acid cycle enzymes, an O polysaccharide biosynthesis enzyme, proteins encoded on the native plasmid pEI1, and a fimbrial chaperon protein. Three of these mutants were found to have potential as live attenuated vaccines. This study demonstrates a novel application of bioluminescence to identify bacterial genes required for host resistance; as a result, efficacious and genetically defined live attenuated vaccine candidates were developed.

Identification of bacterial genes and proteins that are differentially expressed in response to a host defense can be an effective method for identifying putative bacterial virulence factors (4, 9, 48). In addition to microarrays and proteomics, other high-throughput methods have been used to identify bacterial genes upregulated in response to phagocytosis, including differential fluorescence induction (47), random luciferase transcriptional fusions (40), and selective capture of transcribed sequences (14, 17).

However, genes that are differentially regulated in response to a host defense are not necessarily the same as those that are required for survival. For example, not all of the genes that have increased expression after phagocytosis are required for survival in phagocytes (10, 20). It is also possible that not all of the genes required for survival have enough change in expression to allow detection. Therefore, mutagenesis studies complement gene and protein expression studies and are likely to detect a unique set of genes that are required for survival.

A major hurdle in identifying bacterial mutants susceptible to host defenses is that the screening methods tend to be labor intensive. Fields et al. (19) identified 83 *Salmonella enterica* serovar Typhimurium transposon mutants with impaired macrophage survival by screening individual transposon mutants with phagocytes in 96-well plates (19, 53). However, this assay required bacterial quantification from each well by plate counts. Zhao et al. (53) used the same method to identify 37 *Salmonella* mutants susceptible to chicken macrophages.

Improved high-throughput mutant screening methods have been reported, including the use of bioluminescence to identify mycobacterial genes required for survival in macrophages (27) and a microarray-based method for screening mutants (8, 51). However, none of these methods allow monitoring of bacterial mutant viability in real-time. In addition, the previously reported bioluminescence-based method requires a bacterial lysis step and the addition of extraneous luciferin substrate and ATP for determining luciferase activity, which increases handling requirements and cost while reducing the screening efficiency.

Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish, an important disease of farm-raised channel catfish. *E. ictaluri* is a member of the *Enterobacteriaceae*, and it shares similar aspects of pathogenesis with *Salmonella* and *Yersinia*. In particular, it causes a gastrointestinal septicemia, it is a facultative intracellular pathogen, and it has the ability to resist killing by professional phagocytes (1, 5). In particular, *E. ictaluri* is resistant to channel catfish neutrophils (1, 28, 49). This is an important aspect of pathogenesis because neutrophils are the predominant immune cell type in channel catfish intestine (21), which is an important site of entry for the pathogen (2, 37). *E. ictaluri* is also resistant to killing by the alternative complement pathway in channel catfish (28, 39).

In the present study, we report a high-throughput bioluminescence mutant screening (BLMS) method that is not labor-intensive and that allows real-time monitoring of mutant viability. This method is efficient because it allows use of robotics to array mutants into 384-well plates and because it utilizes bacterial luciferase, which allays the need for extraneous addition of luciferin substrate. BLMS allows real-time monitoring of mutant viability and collection of data from multiple time points for real-time screening of bacterial mutants against host defense mechanisms. We utilized BLMS to identify *E. ictaluri* mutants that are susceptible to killing by channel catfish

* Corresponding author. Mailing address: College of Veterinary Medicine, 240 Wise Center Dr., Mississippi State, MS 39762-6100. Phone: (662) 325-1195. Fax: (662) 325-1031. E-mail for M. Lawrence: lawrence@cvm.msstate.edu. E-mail for A. Karsi: karsi@cvm.msstate.edu.

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neutrophils and serum. Thirteen of the mutants are attenuated in the natural host, and four of these are effective as live attenuated vaccines, demonstrating the utility of BLMS for vaccine development.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* SM10 λ*pir* (43) was used as the donor strain in conjugations for transfer of pAK*gfplux*2 (24) and p*MAR2xT7* (31) into *E. ictaluri* strain 93-146. *E. ictaluri* 93-146 and *E. coli* DH5α carrying pAK*gfplux*2 were used as resistant and susceptible controls, respectively, in neutrophil and serum screening experiments. *E. coli* strains were grown using Luria-Bertani broth and agar plates at 37°C, and *E. ictaluri* was grown using brain heart infusion (BHI) broth and agar plates at 30°C. Antibiotics were added to the following final concentrations: ampicillin (100 μg ml^{-1}), colistin (12.5 μg ml^{-1}), and gentamicin (12.5 μg ml^{-1}). IPTG (isopropyl-β-D-thiogalactopyranoside) at 2 mM was used in growth medium and screening assays to induce expression of bacterial luciferase operon (*luxCDABE*) from the *lacZ* promoter in pAK*gfplux*2. *E. ictaluri* minimal medium (12) was used to detect auxotrophic mutants.

Construction of *E. ictaluri* mutant library. *MAR2xT7* insertions were generated by introducing p*MAR2xT7* from *E. coli* SM10 λ*pir* into *E. ictaluri* carrying pAK*gfplux*2 using conjugal mating as previously described (24). Transposon mutants were selected on 20-by-20-cm Luria-Bertani agar bioassay plates (Fisher Scientific) containing 12.5 μg of colistin, 100 μg of ampicillin, and 12.5 μg of gentamicin ml^{-1}. Putative transposants were picked robotically using a Flexsys colony picker (Genomic Solutions) into 40 μl of BHI broth containing colistin, ampicillin, and gentamicin in 384-well microtiter plates (Fisher Scientific) and grown overnight in a HiGro shaker (Genomic Solutions). A duplicate library was prepared by the Flexsys colony picker before sterile glycerol was robotically added to the cultures at a final concentration of 20%. Plates were sealed with AluminaSeal aluminum foil (Diversified Biotech), lids were taped with PetriSeal (Diversified Biotech), and libraries were stored at −80°C.

Catfish serum and neutrophil preparation. Specific-pathogen-free (SPF) channel catfish were obtained from the SPF fish laboratory at the College of Veterinary Medicine, Mississippi State University. For serum preparation, 1- to 2-kg SPF catfish were anesthetized in water containing 200 mg of tricaine methane sulfonate (Argent Laboratories) $liter^{-1}$, and blood was collected from approximately 50 fish at 1% of body weight. Serum was obtained as described previously (24) and stored at −80°C as single-use aliquots. Neutrophils were isolated from single cell suspensions of anterior kidney cells from SPF catfish (38.63 ± 0.68 cm, 424.20 ± 23.34 g) by using a discontinuous Percoll (Amersham) gradient centrifugation procedure (38, 50). Purity of neutrophils collected from the 1.060-1.080 interface was determined by using a Becton-Dickinson FACSCalibur flow cytometer by dividing the number of gated neutrophils by the total number of events (i.e., 20,000).

In vitro mutant screening using catfish serum and neutrophils. For each 384-well plate, four 96-well plates containing 195 μl of BHI medium with colistin, ampicillin, and gentamicin and 2 mM IPTG were prepared. Portions (5 μl) of mutant bacteria from each well of the 384-well plate were used to inoculate each well in four 96-well plates. Each 96-well plate contained a serum-resistant (*E. ictaluri* 93-146 pAK*gfplux*2) and a serum-sensitive (*E. coli* DH5α pAK*gfplux*2) control well. Plates were covered with Breathe-Easy film (Diversified Biotech) and allowed to grow for 16 to 18 h with shaking at 250 rpm.

For serum screening, 10 μl of mutant culture containing ~10^6 CFU was mixed with 90 μl of catfish serum containing 2 mM IPTG in 96-well plates. Photon emissions were collected for 10 s at each time point by using an IVIS Imaging System 100 Series (Xenogen Corp.). Initial images were collected after a 5-min preincubation of samples at 30°C, and subsequent images were captured from the same plates at 15-min intervals for 90 min. Using the collected images, luminescence was quantified from each well by using Living Image Software v2.50 (Xenogen Corp.).

Neutrophil screening was accomplished using freshly isolated catfish neutrophils with ≥75% purity. Each well contained ~10^6 CFU mutant bacteria and 1.25×10^4 to 2.50×10^4 neutrophils (1:40 to 1:80 neutrophil/bacterium ratio) in a total volume of 100 μl. Neutrophil-bacterium suspensions also contained 15% SPF catfish serum for opsonization and 2 mM IPTG. Bioluminescence imaging was conducted as described above.

A total of 2,256 transposon mutants were screened separately for serum and neutrophil susceptibility as described above. The percent change in bioluminescence between the initial measurement and the 90-min measurement was determined for each mutant and compared to the mean percent change observed in serum-resistant *E. ictaluri* 93-146 pAK*gfplux*2 and serum-sensitive DH5α pAK*gfplux*2 controls. Mutants were sorted by the percent light change, and those falling below three standard deviations calculated from wild-type *E. ictaluri* 93-146 pAK*gfplux*2 were chosen for further analysis using quadruplicate samples. A total of 178 mutants with reduced bioluminescence were rescreened against serum and neutrophils in quadruplicate samples, and data were analyzed by using one-way analysis of variance using SAS v9.1 (SAS Institute, Inc.). Eighty-one mutants with significantly reduced resistance to serum and/or neutrophils ($P < 0.05$) were selected and screened for auxotrophy in minimal medium (12).

In vivo mutant screening. SPF channel catfish (5.20 ± 0.18 cm) were transferred from the MSU-CVM SPF fish facility to 40-liter flowthrough tanks (15 fish per tank) with dechlorinated municipal water. Fish were maintained in well-aerated tanks with a water temperature of 28°C throughout the experiments. Bacterial cultures were grown overnight, and bacterial concentrations were adjusted using the optical density at 600 nm. After 1 week of acclimation, fish were anesthetized in water containing 100 mg of MS222 $liter^{-1}$, and ~10^7 CFU of each mutant was injected intraperitoneally into 15 catfish in 100 μl of phosphate-buffered saline (PBS). Based on our experience in prior *E. ictaluri* challenges, this dose is the approximate 80% lethal dose for pathogenic isolates. One group of 15 fish was infected with parent strain 93-146, and one group was injected with PBS. Mortalities were recorded daily, and the percent mortality rates were calculated for each mutant. Thirteen mutants (designated EiAKMut01 to EiAKMut13) with lower mortality rates than strain 93-146 were selected for further characterization.

Determination of virulence and vaccine potential. Attenuation of mutants EiAKMut01 to EiAKMut13 was confirmed in catfish by using the intraperitoneal injection route of exposure (29, 30). Each 40-liter flowthrough tank contained 20 fish, and four tanks were used for each mutant. One group of four replicates was injected with wild-type *E. ictaluri* strain 93-146, and another group of four replicates were injected with PBS as positive and negative controls, respectively. Channel catfish (14.61 ± 0.33 cm, 32.70 ± 2.36 g) were anesthetized and infected by intraperitoneal injection with ~10^5 CFU. The percent mortalities were calculated for each tank, and the mean percent mortalities were analyzed by using one-way analysis of variance. Pairwise comparison of the means was done by using the Fisher least-significant-difference test procedure of SAS v9.1. A significance of 0.05 was used in all analyses.

The vaccine efficacy of eight mutants (EiAKMut01, EiAKMut02, EiAKMut04, EiAKMut05, EiAKMut06, EiAKMut08, EiAKMut12, and EiAKMut13) was compared to a commercial live attenuated vaccine (25) (AQUAVAC-ESC) marketed by Intervet/Schering-Plough Animal Health. Wild-type strain 93-146 and sham vaccination control treatments were included. Each 40-liter flowthrough tank contained 25 fish with four tanks per treatment. Fish were allowed to acclimate for 2 weeks before bacterial challenges. For vaccination, fish (11.62 ± 0.16 cm, 15.36 ± 0.65 g) were infected by immersion in water containing 2×10^7 CFU ml^{-1} for 1 h. After 21 days, vaccinated fish were infected with strain 93-146 by immersion in water with 10^7 CFU ml^{-1} for 1 h. Mortalities were recorded daily, and the mean percent mortalities for each treatment were calculated and analyzed as described above.

Identification of transposon insertion sites. Transposon insertion sites were identified in mutants EiAKMut01 to EiAKMut13 by using a single primer PCR protocol (23). Genomic DNA was prepared from overnight culture for each mutant by using a Wizard genomic DNA purification kit (Promega). Each 25-μl PCR contained 0.2 mM deoxynucleoside triphosphates, 0.2 μM transposon specific primer, 1.5 mM $MgCl_2$, and 1.25 U of *Taq* polymerase (Promega). ExoSAP-IT enzyme mix (USB Corp.) was used to clean 5 μl of the PCR. Sequencing was conducted using BigDye v3.1 with 2 μl of ExoSAP-IT-treated template and 10 μM nested transposon-specific primer. The resulting *E. ictaluri* sequences were first searched against the *E. ictaluri* genome (http://micro-gen.ouhsc.edu/cgi-bin/blast_form.cgi) and then against the nonredundant protein database of NCBI using BLAST algorithm to identify transposon insertion sites and gene names, respectively.

RESULTS

Identification of serum- and neutrophil-susceptible *E. ictaluri* mutants. A library of approximately 15,000 random transposon insertion mutations (~3.8-fold coverage of the *E. ictaluri* genome) was generated in bioluminescent *E. ictaluri* strain 93-146 carrying pAK*gfplux*2 using a derivative of the mariner transposon *Himar1* (42) carried on p*MAR2xT7* (31). Mutants were arrayed in duplicate in 39 384-well plates.

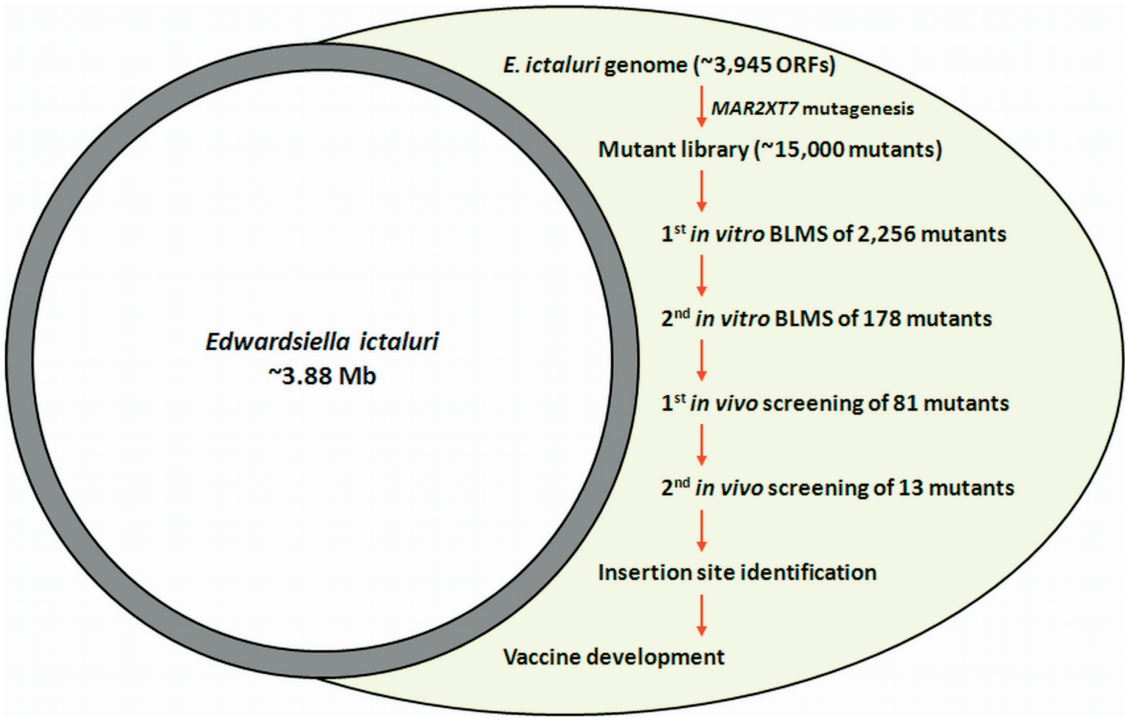

FIG. 1. Overview of the mutant screening process.

A high-throughput BLMS procedure was developed that allowed identification of virulence genes from gram-negative bacteria in vitro. A general outline of the integrated procedures including in vitro BLMS and in vivo fish screening is shown in Fig. 1. We screened 2,256 mutants against both catfish serum and neutrophils using BLMS. Although the BLMS method would have allowed efficient screening of additional plates for increased coverage of the *E. ictaluri* genome, we limited our experiment to 24 96-well plates due to the expense of isolating primary catfish neutrophils. After this initial screening, 178 mutants were identified that exhibited decreased luminescence during incubation with these host factors (Fig. 2).

A second screening of these 178 mutants against both serum and neutrophils in quadruplicate indicated that 152 of the mutants (79 neutrophil, 73 serum) had significantly reduced resistance compared to wild-type strain 93-146 ($P < 0.05$). Of these, 16 had reduced resistance to both neutrophils and serum. The parent *E. ictaluri* strain 93-146 pAK*gfplux*2 was resistant to both serum and neutrophils; thus, it was able to utilize the nutrients in catfish serum to increase in numbers over the 1.5 h of incubation. The mean percent light changes and standard deviations of 93-146 pAK*gfplux*2 in response to catfish serum and neutrophils were 21.62% ± 2.09% and 35.46% ± 0.53%, respectively. In contrast, *E. coli* DH5α pAK*gfplux*2 was inactivated by catfish serum and neutrophils. Thus, DH5α had a negative percent change in bioluminescence at 1.5 h compared to 0 h: −95.39% ± 0.17% and −77.67% ± 0.33%, respectively, in response to catfish serum and neutrophils.

The 81 *E. ictaluri* mutants showing greatest sensitivity to serum and/or neutrophils were selected for further screening. Thirty-four of these were neutrophil sensitive, thirty-one were serum sensitive, and sixteen were both serum and neutrophil sensitive. When the mutants were grown in minimal medium, no auxotrophs were detected. Next, the mutants were tested for attenuation in the natural host by intraperitoneal injection. Of the 81 BLMS selected mutants, 13 mutants (designated EiAKMut01 to EiAKMut13) were identified as putatively attenuated (7 neutrophil sensitive, 1 serum sensitive, and 5 mutants sensitive to both neutrophils and serum).

The relative serum and neutrophil resistance for some of these 13 mutants is shown in Fig. 3. Bioluminescence of wild-type *E. ictaluri* increased over the 90-min incubation with both serum and neutrophils. Interestingly, although mutants demonstrated significantly reduced resistance, they were not killed like the *E. coli* strain; rather, the ability of mutants to resist host serum or neutrophils was weakened. Twelve mutants had significantly reduced resistance to catfish neutrophils; nine of these (EiAKMut09, EiAKMut10, EiAKMut07, EiAKMut02, EiAKMut03, EiAKMut08, EiAKMut06, EiAKMut01, and EiAK11) had significantly reduced resistance compared to the remaining three (EiAKMut12, EiAKMut05, and EiAK Mut04). Six mutants had significantly reduced resistance to catfish serum (EiAKMut02, EiAKMut03, EiAKMut08, EiAK Mut04, EiAKMut05, and EiAKMut13), five of which had significantly reduced resistance to both neutrophils and serum (all except EiAKMut13).

Virulence and vaccine potential of *E. ictaluri* mutants. To confirm that they are attenuated, mutants EiAKMut01 to EiAKMut13 were tested in channel catfish by intraperitoneal injection using quadruplicate tanks. All 13 mutants were significantly attenuated compared to wild-type strain 93-146, and 11 of these were highly attenuated (<15% mortalities). EiAK

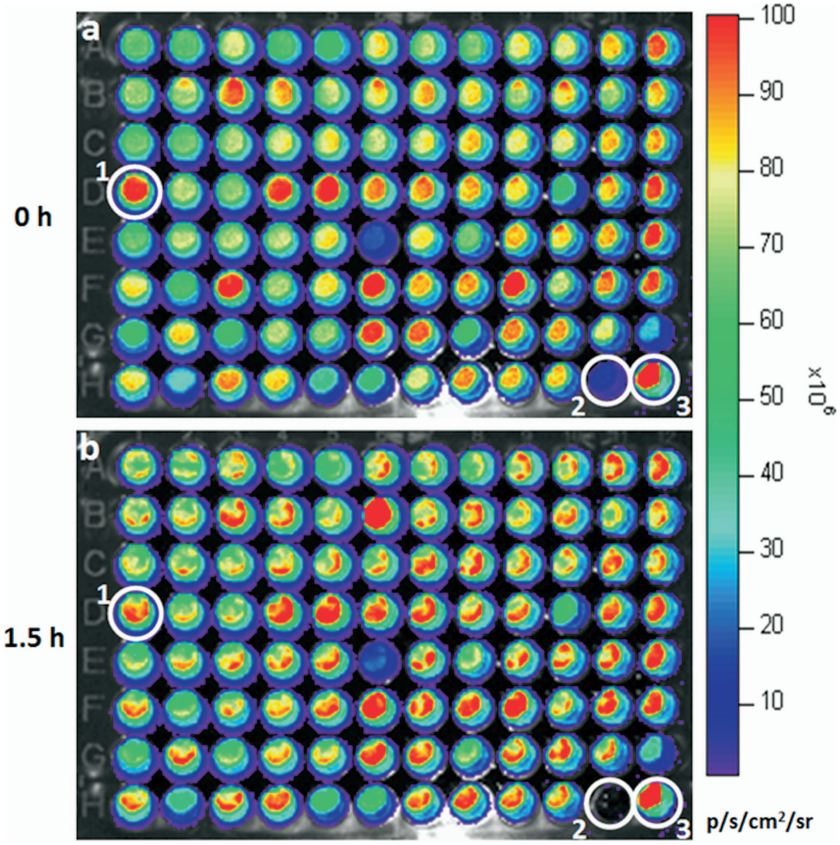

FIG. 2. Representative image of bioluminescence mutant screening. The 96-well plate shown is from the serum screening experiment and contained 94 mutants, a serum-susceptible control (circle 2, *E. coli* DH5α), and a serum-resistant control (circle 3, *E. ictaluri* 93-146). Using this image, the amount of bioluminescence was quantified from each well at 0 h and at 1.5 h, and the percent change in bioluminescence for each individual mutant was used to determine serum sensitivity relative to the serum-resistant control. On the plate shown, one mutant (circle 1) demonstrated reduced bioluminescence at the end of serum incubation (1.5 h) compared to the initial value (0 h). Parent strain 93-146 and all of the other mutants on this plate had slightly increased bioluminescence over the 1.5 h of incubation. Bioluminescence scale is shown in the bar on the right.

Mut07 and EiAKMut09 were the least attenuated mutants, causing 58.33 and 48.33% fish mortalities, respectively (Fig. 4).

Eight of the highly attenuated *E. ictaluri* mutants were compared to the only commercially available live attenuated vaccine, AQUAVAC-ESC (marketed by Intervet/Schering-Plough Animal Health), to assess their vaccine efficacy. Immersion vaccination with AQUAVAC-ESC, EiAKMut02, EiAKMut05, EiAKMut08, and EiAKMut13 caused no mortalities, and although vaccination with EiAKMut01, EiAKMut04, EiAKMut06, and EiAKMut12 caused mortalities, they were significantly lower than mortalities caused by parent strain 93-146 (Fig. 5a). EiAKMut05 and EiAKMut12 provided the best protection against subsequent challenge with the virulent parent strain. Five other mutants also exhibited significantly improved protection compared to AQUAVAC-ESC (Fig. 5b). Mutants EiAKMut05, EiAKMut02, and EiAKMut08 demonstrated the best potential as live attenuated vaccines because vaccination caused no mortalities, and they provided significantly better protection than AQUAVAC-ESC. Vaccination with EiAKMut13 also caused no mortalities, but it was inferior to AQUAVAC-ESC in providing protection.

Identification of *MAR2xT7* insertions in *E. ictaluri* genome. *MAR2xT7* insertion locations for mutants EiAKMut01 to EiAKMut13 were determined by using single primer PCR amplification of transposon ends and nested primer sequencing (Table 1). The *gcvP* gene (encoding glycine cleavage system protein P) was disrupted in three of the mutants (EiAKMut02, EiAKMut03, and EiAKMut08) but at different locations. Similarly, the *rseB* gene (encoding a negative regulator of sigmaE) was mutated at the same location in EiAKMut01 and EiAK Mut07. Interestingly, two genes located on one of the native plasmids of *E. ictaluri* (pEI1) were also mutated. One of these genes encodes a putative RNA one modulator protein, while the other encodes a hypothetical protein.

DISCUSSION

We describe here a new high-throughput method (BLMS) that utilizes a measurable phenotype (luminescence) to screen gene mutations in bacteria and allows application of forward genetics. BLMS involves random transposon mutagenesis of a bacterial strain expressing bacterial luciferase (*luxCDABE*). Using this approach, we produced a random *E. ictaluri* mutant library that expresses *luxCDABE* genes from a stable plasmid, pAK*gfplux*2 (24). The use of a colony picking robot to array mutants in 384-well plates and bioluminescence to measure

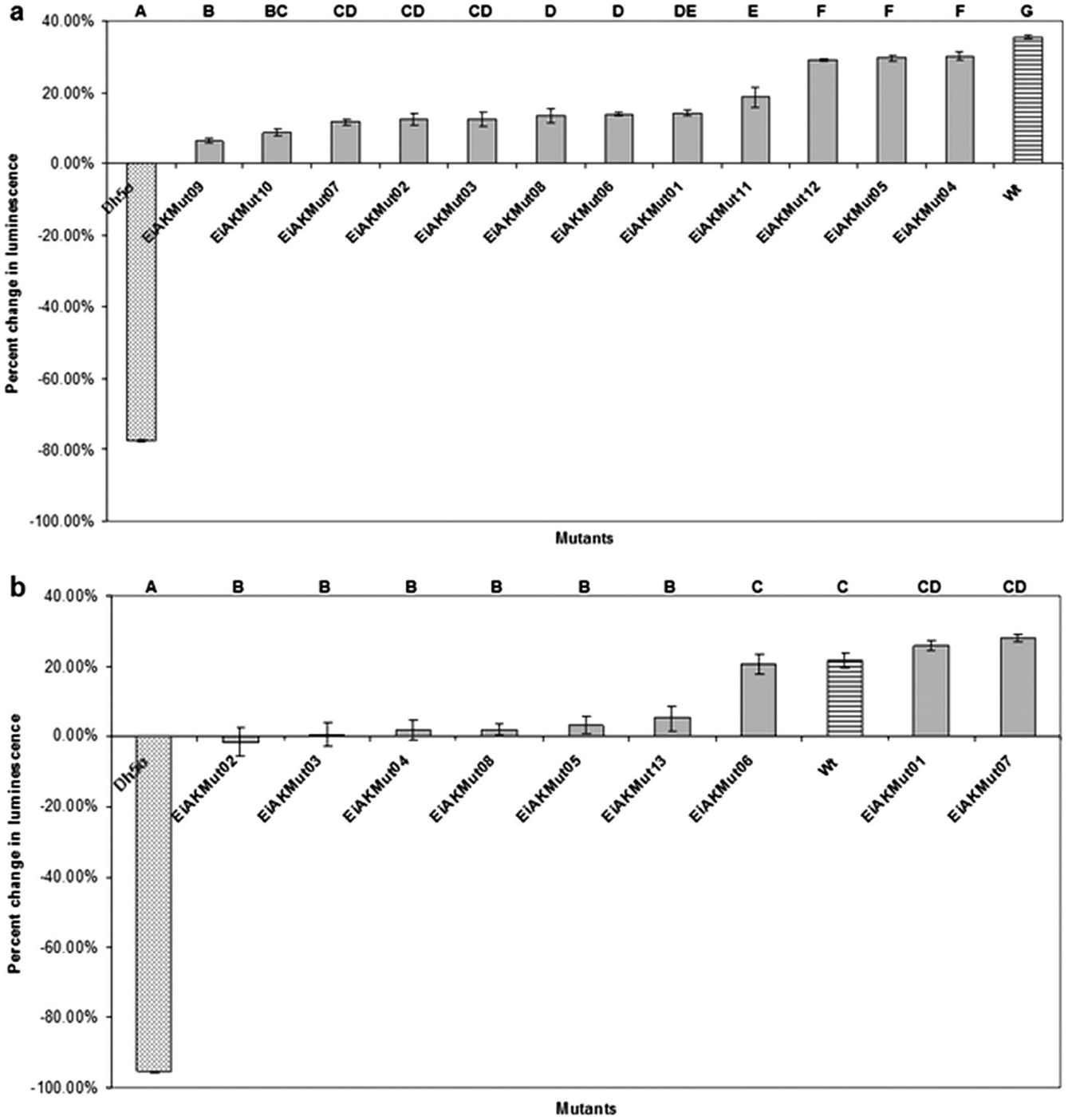

FIG. 3. Response of selected *E. ictaluri* mutants to catfish neutrophils (a) and serum (b). The percent change in luminescence was calculated by dividing the measured bioluminescence at 90 min by the measurement at 0 min. Capital letters indicate statistical groupings. Groups marked with the same capital letter do not show statistically significant differences ($P < 0.05$).

bacterial viability (as opposed to serial dilutions and plate counts) made the screening of 2,256 *E. ictaluri* mutants an efficient process.

Among the 13 attenuated mutants we identified, redundant mutations were present in two genes. Three mutants (EiAK Mut02, EiAKMut03, and EiAKMut08) harbored transposon insertion in *gcvP* at different locations, and two mutants (EiAK Mut01 and EiAKMut07) harbored transposon insertion in *rseB* at the same location. The independent isolation of mutations in the same gene indicates that the BLMS procedure was effective in detecting true gene targets.

Our results indicate that BLMS is an effective procedure for development of live attenuated vaccines. Thirteen mutants were identified that are significantly attenuated compared to

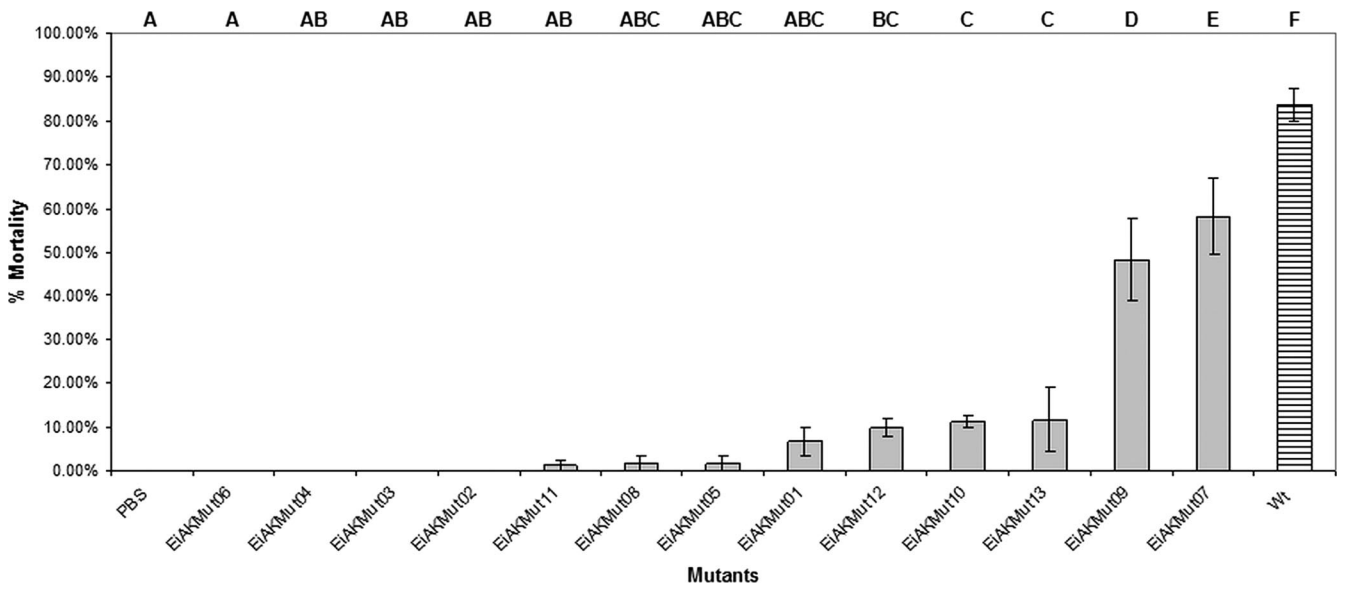

FIG. 4. Results of virulence trial. The percent mortalities are the mean of four replicate tanks per treatment. PBS is the saline control, and Wt is the parent strain 93-146. Capital letters above each bar indicate statistical groupings. Groups marked with the same capital letter do not show statistically significant differences ($P < 0.05$).

the virulent parent strain 93-146. Of these mutants, three (EiAKMut02, EiAKMut08, and EiAKMut05) demonstrate potential to be more effective live attenuated vaccines than the current commercially available vaccine, AQUAVAC-ESC. In addition, EiAKMut12 only caused 4.45% mortality and provided good protection; with dose adjustment, it may also have potential to be an effective vaccine.

A mutant screening strategy such as BLMS is particularly effective at elucidating multifactorial mechanisms of pathogen resistance to host defenses. Pathogen resistance to host phagocytosis is known to be complex, often requiring the appropriate expression of many virulence genes. We have previously shown that *E. ictaluri* resistance to the complement cascade is multifactorial (28). Our results from the current study indicate that *E. ictaluri* resistance to catfish serum and neutrophils is indeed multifactorial; none of the mutants we identified demonstrated complete susceptibility to either serum or neutrophils.

Several of the mutants we identified were novel, but one of the mutants identified by BLMS was not a surprise. EiAK Mut13, which had reduced resistance to serum, has an insertion in *ugd*. This gene encodes UDP-glucose 6-deyhydrogenase and is located in the *E. ictaluri* O polysaccharide (OPS) biosynthesis operon (28); thus, it is likely that EiAKMut13 has altered OPS biosynthesis. We have previously reported that a mutant defective in OPS biosynthesis, 93-146 R6, has significantly reduced resistance to catfish serum (28), and EiAK Mut13 has a similar phenotype. Interestingly, 93-146 R6 is also similar to EiAKMut13 in that it is not very effective as a live attenuated vaccine by immersion.

BLMS proved effective at identifying virulence genes in plasmids as well as in the chromosome. *E. ictaluri* contains two native plasmids, pEI1 and pEI2, that are consistently present in channel catfish isolates (36). The sequences of both plasmids are available (18), but most of these plasmids' gene functions are poorly defined. Our results agree with previously reported findings that pEI1 is important in *E. ictaluri* virulence (46). EiAKMut04 has an insertion in a gene encoding a hypothetical protein (p1) located on pEI1. The protein has >50% identity with *Salmonella* effector proteins with leucine-rich repeats that are secreted through a type III secretion system. The 618-amino-acid protein appears to be in a monocistronic operon. Interestingly, the *orf1* gene on pEI1 was also identified as a virulence gene by signature-tagged mutagenesis (46). EiAK Mut10 has a mutation in putative open reading frame 4 (p4) of pEI1. Open reading frame 4 has similarity to putative RNA one modulator protein, which is involved in plasmid replication.

EiAKMut05 has an insertion in the *sdhC* gene, which encodes one of four subunits of the succinate dehydrogenase complex. Succinate dehydrogenase is part of the aerobic respiratory chain and the tricarboxylic acid (TCA) cycle, oxidizing succinate to fumarate while reducing ubiquinone to ubiquinol. It is closely related to fumarate reductase, which catalyzes the reverse reaction. SdhC is one of the two subunits that anchors the complex in the cytoplasmic membrane (35). Although SdhC has a similar function, hydrophobicity, and protein size compared to the membrane-binding subunit from fumarate reductase (FrdC), SdhC and FrdC do not share significant sequence identity (52). In *E. coli* and *Salmonella*, succinate dehydrogenase is known to contribute to pathogenicity. The organic acids formate and succinate have a protective effect in stationary-phase cells against killing effects of antimicrobial bactericidal permeability-increasing protein, which appears to disrupt the bacterial respiratory chain (3). Maintenance of protective levels of formate and succinate requires the activity of formate dehydrogenase and succinate dehydrogenase, respectively. A *sdhCDA* mutant of *S. enterica* serovar Typhimurium was slightly attenuated, but complete attenuation was achieved by succinate dehydrogenase and fumarate reductase double mutation (33). In *E. ictaluri*, *sdhC* is the first gene in a

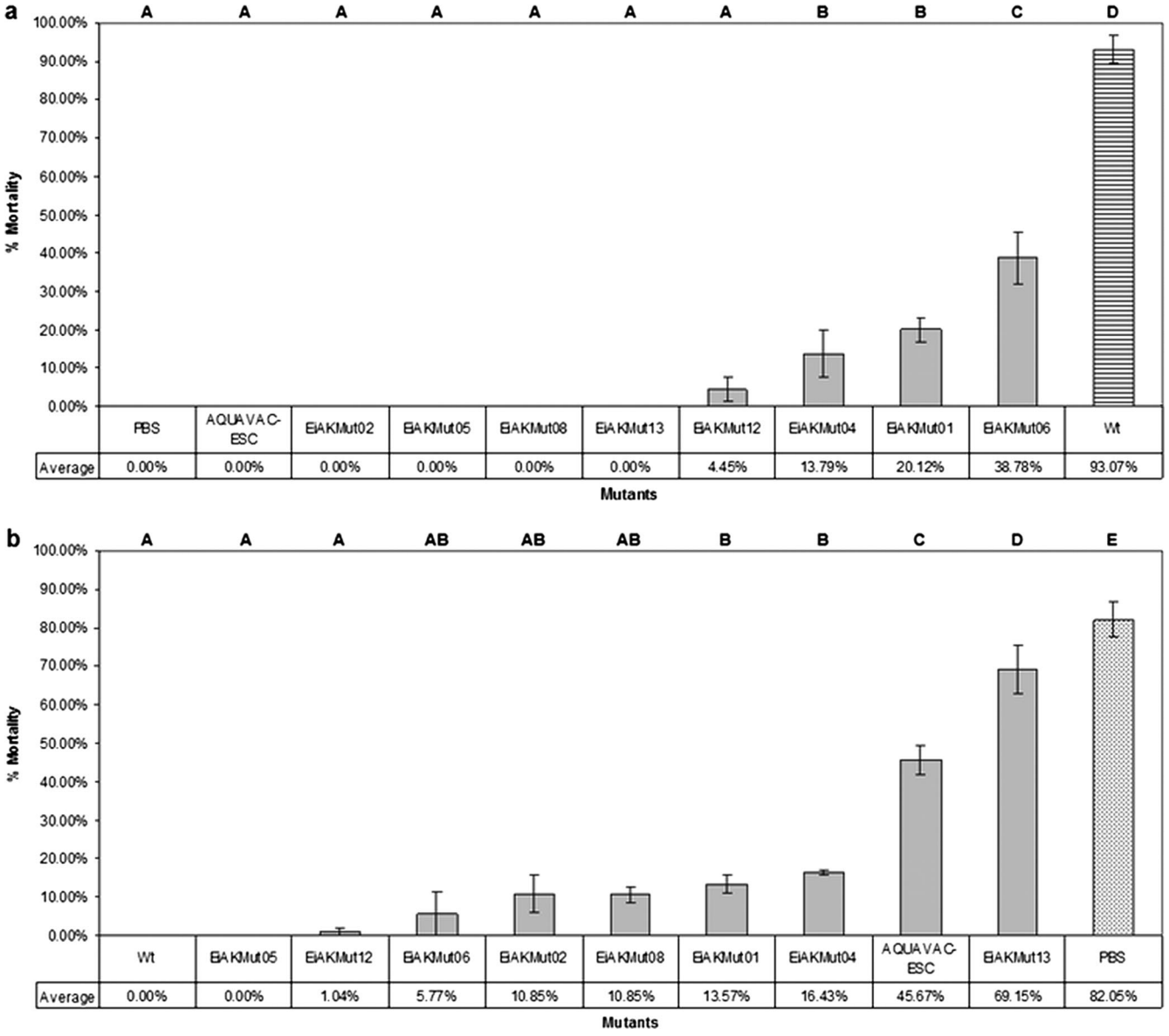

FIG. 5. Results of vaccine efficacy trial. (A) Percent mortalities resulting from vaccination; (B) percent mortalities resulting from challenge with parent strain 93-146 21 days postvaccination. The percent mortalities are the mean of four replicate tanks per treatment. PBS is the saline control, Wt is the parent strain 93-146, and AQUAVAC-ESC is a commercial live attenuated vaccine. Capital letters above each bar indicate statistical groupings. Groups marked with the same capital letter do not show statistically significant differences ($P < 0.05$).

polycistronic operon that encodes the four components of succinate dehydrogenase. *E. ictaluri* also encodes the formate dehydrogenase complex in its genome; however, attenuation of *E. ictaluri* was achieved with *sdh* mutation without a need for generating double mutants in *frd* genes.

In addition to *sdhC*, two other mutants had insertions in enzymes in the TCA cycle. EiAKMut11 was mutated in *sucA*, which encodes 2-oxoglutarate dehydrogenase E1 component, and EiAKMut12 was mutated in *mdh*, which encodes malate dehydrogenase. The isolation of three mutants encoding TCA cycle enzymes in our screening is a strong indication of the importance of the TCA cycle in *E. ictaluri* resistance to catfish neutrophils.

EiAKMut02, EiAKMut03, and EiAKMut08 all had insertions in *gcvP*, which encodes a protein that is part of the glycine cleavage system. The glycine cleavage system is a loosely associated four-subunit enzyme complex that catalyzes the reversible oxidation of glycine to form 5,10-methylenetetrahydrofolate, which serves as a one-carbon donor. Expression of the glycine cleavage enzyme system is induced by glycine (32, 44), and *gcv* mutants are unable to use glycine as a one-carbon source and excrete glycine (41). GcvP is a 104-kDa protein that catalyzes the decarboxylation of glycine. In *E. ictaluri*, *gcvP* is the third gene in a three-gene operon; it is located downstream of *gcvH* and *gcvT*, which encode subunits of the glycine cleavage system. To our knowledge, the glycine cleavage system has not been linked with virulence previously, but our study showed that this protein is critical for both neutrophil and

TABLE 1. Summary of insertion identification results

Mutants	Type[a]	Gene identification	GenBank accession no.	Location[b]
EiAKMut01	N	Negative regulator of sigma E activity (*rseB*)	ZP_00822625	*MAR2xT7*^TAttgcgggtttcggtatgaatggttc
EiAKMut02	NS	Glycine cleavage system protein P (*gcvP*)	ZP_00834320	*MAR2xT7*^TAcccgctgcctgatcccggcctcggc
EiAKMut03	NS	Glycine cleavage system protein P (*gcvP*)	ZP_00834320	*MAR2xT7*^TAtatcgctcgccgtcttggcgcggtc
EiAKMut04	NS	Hypothetical protein pEI1_p1	NP_061805	*MAR2xT7*^TAtactctccctctgtttgtggtccca
EiAKMut05	NS	Succinate dehydrogenase/fumarate reductase, cytochrome *b* subunit	ZP_00828037	*MAR2xT7*^TAgaataccgatggcgataaagacggc
EiAKMut06	N	Electron transport complex protein RnfB	YP_070683	*MAR2xT7*^TAccggcagggtacgaccattgccgcg
EiAKMut07	N	Negative regulator of sigma E activity (*rseB*)	ZP_03069723	*MAR2xT7*^TAttgcgggtttcggtatgaatggttc
EiAKMut08	NS	Glycine cleavage system protein P (*gcvP*)	ZP_00834320	*MAR2xT7*^TAgttggcgttaaggatcgccaccgtg
EiAKMut09	N	Fimbrial chaperon protein	BAC55513	*MAR2xT7*^TAccacgctggatgaacaggttcgtaa
EiAKMut10	N	Putative RNA one modulator protein pEI1_p4	NP_061808	*MAR2xT7*^TAattcccaccgctcgccgcaagatcg
EiAKMut11	N	2-Oxoglutarate dehydrogenase E1 component (*sucA*)	ABV40371	*MAR2xT7*^TActtgaccgatcccgattcagtggag
EiAKMut12	N	Malate dehydrogenase (*mdh*)	YP_404893	*MAR2xT7*^TAttcagaacgccggcactgaggtagt
EiAKMut13	S	UDP-glucose 6-dehydrogenase (*ugd*)	AAL25636	*MAR2xT7*^TAtaccttatcgctttgcaccgcgccg

[a] N, neutrophil sensitive; S, serum sensitive; NS, neutrophil and serum sensitive.
[b] *MAR2xT7*, mariner transposon; ^, insertion point; TA, two-base TA duplication; lowercase letters, 25-bp flanking unique gene sequences of *E. ictaluri*.

serum resistance in *E. ictaluri*. The phenotypes of all three mutants were very consistent in their response to serum and neutrophils, as well as in the catfish host.

EiAKMut01 and EiAKMut07 had insertions in *rseB*, which encodes a negative regulator of sigmaE (34). RseB is a periplasmic protein that stimulates binding of RseA to sigmaE, thereby assisting RseA in tethering sigmaE to the cytoplasmic membrane (11). Although mutations in *rseA* cause increased sigmaE activity (15, 34), a *rseB* mutant shows wild-type sigmaE activity under inducing conditions and exhibits a small increase in sigmaE activity under noninducing conditions (15). In *E. ictaluri*, *rseB* is the third gene in a polycistronic operon. It is downstream of *rpoE*, which encodes sigmaE, and *rseA*, and it is upstream of *rseC*, which encodes a positive regulator of sigmaE. SigmaE is required for *Salmonella* virulence (22) and mediates *Salmonella* resistance to oxidative stress (22, 45) and antimicrobial peptides (13). SigmaE is also required for *Salmonella* to survive intracellularly (6). EiAKMut01 and EiAK Mut07 had consistent phenotypes in serum and neutrophil resistance assays, but although both mutants were attenuated in channel catfish, EiAKMut01 caused significantly lower mortalities than EiAKMut07 following intraperitoneal exposure. This may be because RseB is in a regulatory cascade. Small variations in individual fish host environment may cause variability in the RseA-sigmaE response to host stimuli, which in turn would be amplified through downstream effects on the sigmaE regulon, causing variability in the degree of attenuation. Further work is required to confirm this hypothesis.

EiAKMut06 has an insertion in *rsxB*, which encodes one of six proteins that form a SoxR reducing system in *E. coli* (26). SoxR is a regulatory protein that senses superoxide and nitric oxide and induces expression of a SoxS-mediated oxidative stress response (26). The SoxR reducing system inactivates SoxR, thereby turning off the oxidative stress response. In *E. coli*, when any of the six *rsx* genes are mutated, SoxS is constitutively expressed, leading to induction of oxidative stress response. In *Salmonella*, SoxS is not essential for virulence (16), but SoxS was found to contribute to virulence in an *E. coli* mouse pyelonephritis model (7). In *E. ictaluri*, *rsxB* is the second in the six gene *rsx* operon.

The present study resulted in the development of a high-throughput screening method (BLMS) for identification of bacterial virulence genes required for resistance to host defense mechanisms. High-throughput identification of genes required for bacterial virulence complements transcriptomic and proteomic studies that identify bacterial genes and proteins whose expression is altered by host environment. Importantly, BLMS appears to be effective for identification of new candidate live attenuated vaccine strains.

BLMS is limited to assays that can be conducted in tissue culture plates, and it also would not be effective for screening stages of infection that require whole-animal studies. However, BLMS has the advantage that it allows identification of bacterial genes required for resistance to a particular host mechanism of interest. In our particular study, BLMS resulted in elucidation of *E. ictaluri* mechanisms of resistance to channel catfish serum and neutrophils. Because *E. ictaluri* is a member of the *Enterobacteriaceae*, and it shares pathogenic mechanisms with some of the members of this family, it is likely that the genes identified in the present study may be crucial not only for the virulence of *E. ictaluri* but also for other bacterial pathogens with similar infection mechanisms. Thus, TCA enzymes, the glycine cleavage system, the sigmaE regulatory system, and the SoxR reducing system may be important for resistance of other *Enterobacteriaceae* to host phagocytes and complement.

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REFERENCES

1. **Ainsworth, A. J., and C. Dexiang.** 1990. Differences in the phagocytosis of four bacteria by channel catfish neutrophils. Dev. Comp. Immunol. **14:**201–209.
2. **Baldwin, T. J., and J. C. Newton.** 1993. Pathogenesis of enteric septicemia of channel catfish, caused by *Edwardsiella ictaluri*: bacteriologic and light and electron microscopic findings. J. Aquat. Anim. Health **5:**189–198.
3. **Barker, H. C., N. Kinsella, A. Jaspe, T. Friedrich, and C. D. O'Connor.** 2000. Formate protects stationary-phase *Escherichia coli* and *Salmonella* cells from killing by a cationic antimicrobial peptide. Mol. Microbiol. **35:**1518–1529.
4. **Bergman, N. H., E. C. Anderson, E. E. Swenson, B. K. Janes, N. Fisher, M. M. Niemeyer, A. D. Miyoshi, and P. C. Hanna.** 2007. Transcriptional profiling of *Bacillus anthracis* during infection of host macrophages. Infect. Immun. **75:**3434–3444.
5. **Booth, N. J., A. Elkamel, and R. L. Thune.** 2006. Intracellular replication of *Edwardsiella ictaluri* in channel catfish macrophages. J. Aquat. Anim. Health **18:**101–108.
6. **Cano, D. A., M. Martinez-Moya, M. G. Pucciarelli, E. A. Groisman, J. Casadesus, and F. Garcia-Del Portillo.** 2001. *Salmonella enterica* serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. Infect. Immun. **69:**6463–6474.
7. **Casaz, P., L. K. Garrity-Ryan, D. McKenney, C. Jackson, S. B. Levy, S. K. Tanaka, and M. N. Alekshun.** 2006. MarA, SoxS, and Rob function as virulence factors in an *Escherichia coli* murine model of ascending pyelonephritis. Microbiology **152:**3643–3650.
8. **Chan, K., C. C. Kim, and S. Falkow.** 2005. Microarray-based detection of *Salmonella enterica* serovar Typhimurium transposon mutants that cannot survive in macrophages and mice. Infect. Immun. **73:**5438–5449.
9. **Chatterjee, S. S., H. Hossain, S. Otten, C. Kuenne, K. Kuchmina, S. Machata, E. Domann, T. Chakraborty, and T. Hain.** 2006. Intracellular gene expression profile of *Listeria monocytogenes*. Infect. Immun. **74:**1323–1338.
10. **Chen, C. Y., L. Eckmann, S. J. Libby, F. C. Fang, S. Okamoto, M. F. Kagnoff, J. Fierer, and D. G. Guiney.** 1996. Expression of *Salmonella typhimurium rpoS* and *rpoS*-dependent genes in the intracellular environment of eukaryotic cells. Infect. Immun. **64:**4739–4743.
11. **Collinet, B., H. Yuzawa, T. Chen, C. Herrera, and D. Missiakas.** 2000. RseB binding to the periplasmic domain of RseA modulates the RseA-sigmaE interaction in the cytoplasm and the availability of sigmaE RNA polymerase. J. Biol. Chem. **275:**33898–33904.
12. **Collins, L. A., and R. L. Thune.** 1996. Development of a defined minimal medium for the growth of *Edwardsiella ictaluri*. Appl. Environ. Microbiol. **62:**848–852.
13. **Crouch, M. L., L. A. Becker, I. S. Bang, H. Tanabe, A. J. Ouellette, and F. C. Fang.** 2005. The alternative sigma factor sigma is required for resistance of *Salmonella enterica* serovar Typhimurium to antimicrobial peptides. Mol. Microbiol. **56:**789–799.
14. **Daigle, F., J. E. Graham, and R. Curtiss III.** 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Mol. Microbiol. **41:**1211–1222.
15. **De Las Penas, A., L. Connolly, and C. A. Gross.** 1997. The sigmaE-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigmaE. Mol. Microbiol. **24:**373–385.
16. **Fang, F. C., A. Vazquez-Torres, and Y. Xu.** 1997. The transcriptional regulator SoxS is required for resistance of *Salmonella typhimurium* to paraquat but not for virulence in mice. Infect. Immun. **65:**5371–5375.
17. **Faucher, S. P., S. Porwollik, C. M. Dozois, M. McClelland, and F. Daigle.** 2006. Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. Proc. Natl. Acad. Sci. USA **103:**1906–1911.
18. **Fernandez, D. H., L. Pittman-Cooley, and R. L. Thune.** 2001. Sequencing and analysis of the *Edwardsiella ictaluri* plasmids. Plasmid **45:**52–56.
19. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
20. **Fierer, J., L. Eckmann, F. Fang, C. Pfeifer, B. B. Finlay, and D. Guiney.** 1993. Expression of the *Salmonella* virulence plasmid gene *spvB* in cultured macrophages and nonphagocytic cells. Infect. Immun. **61:**5231–5236.
21. **Hebert, P., A. J. Ainsworth, and B. Boyd.** 2002. Histological enzyme and flow cytometric analysis of channel catfish intestinal tract immune cells. Dev. Comp. Immunol. **26:**53–62.
22. **Humphreys, S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts.** 1999. The alternative sigma factor, sigmaE, is critically important for the virulence of *Salmonella typhimurium*. Infect. Immun. **67:**1560–1568.
23. **Karlyshev, A. V., M. J. Pallen, and B. W. Wren.** 2000. Single-primer PCR procedure for rapid identification of transposon insertion sites. BioTechniques **28:**1078–1082.
24. **Karsi, A., and M. L. Lawrence.** 2007. Broad host range fluorescence and bioluminescence expression vectors for gram-negative bacteria. Plasmid **57:**286–295.
25. **Klesius, P. H., and C. A. Shoemaker.** 1999. Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. Adv. Vet. Med. **41:**523–537.
26. **Koo, M. S., J. H. Lee, S. Y. Rah, W. S. Yeo, J. W. Lee, K. L. Lee, Y. S. Koh, S. O. Kang, and J. H. Roe.** 2003. A reducing system of the superoxide sensor SoxR in *Escherichia coli*. EMBO J. **22:**2614–2622.
27. **Lagier, B., V. Pelicic, D. Lecossier, G. Prod'hom, J. Rauzier, C. Guilhot, B. Gicquel, and A. J. Hance.** 1998. Identification of genetic loci implicated in the survival of *Mycobacterium smegmatis* in human mononuclear phagocytes. Mol. Microbiol. **29:**465–475.
28. **Lawrence, M. L., M. M. Banes, P. Azadi, and B. Y. Reeks.** 2003. The *Edwardsiella ictaluri* O polysaccharide biosynthesis gene cluster and the role of O polysaccharide in resistance to normal catfish serum and catfish neutrophils. Microbiology **149:**1409–1421.
29. **Lawrence, M. L., M. M. Banes, and M. L. Williams.** 2001. Phenotype and virulence of a transposon-derived lipopolysaccharide O side chain mutant strain of *Edwardsiella ictaluri*. J. Aquat. Anim. Health **13:**291–299.
30. **Lawrence, M. L., R. K. Cooper, and R. L. Thune.** 1997. Attenuation, persistence, and vaccine potential of an *Edwardsiella ictaluri purA* mutant. Infect. Immun. **65:**4642–4651.
31. **Liberati, N. T., J. M. Urbach, S. Miyata, D. G. Lee, E. Drenkard, G. Wu, J. Villanueva, T. Wei, and F. M. Ausubel.** 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. USA **103:**2833–2838.
32. **Meedel, T. H., and L. I. Pizer.** 1974. Regulation of one-carbon biosynthesis and utilization in *Escherichia coli*. J. Bacteriol. **118:**905–910.
33. **Mercado-Lubo, R., E. J. Gauger, M. P. Leatham, T. Conway, and P. S. Cohen.** 2008. A *Salmonella enterica* serovar Typhimurium succinate dehydrogenase/fumarate reductase double mutant is avirulent and immunogenic in BALB/c mice. Infect. Immun. **76:**1128–1134.
34. **Missiakas, D., M. P. Mayer, M. Lemaire, C. Georgopoulos, and S. Raina.** 1997. Modulation of the *Escherichia coli* sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB, and RseC proteins. Mol. Microbiol. **24:**355–371.
35. **Nakamura, K., M. Yamaki, M. Sarada, S. Nakayama, C. R. Vibat, R. B. Gennis, T. Nakayashiki, H. Inokuchi, S. Kojima, and K. Kita.** 1996. Two hydrophobic subunits are essential for the heme b ligation and functional assembly of complex II (succinate-ubiquinone oxidoreductase) from *Escherichia coli*. J. Biol. Chem. **271:**521–527.
36. **Newton, J. C., R. C. Bird, W. T. Blevins, G. R. Wilt, and L. G. Wolfe.** 1988. Isolation, characterization, and molecular cloning of cryptic plasmids from *Edwardsiella ictaluri*. Am. J. Vet. Res. **49:**1856–1860.
37. **Newton, J. C., L. G. Wolfe, J. M. Grizzle, and J. A. Plumb.** 1989. Pathology of experimental enteric septicemia in channel catfish, *Ictalurus punctatus* (Rafinesque), following immersion-exposure to *Edwardsiella ictaluri*. J. Fish Dis. **12:**335–347.
38. **Noya, M., Y. Qian, and A. J. Ainsworth.** 1999. Molecular and functional characterization of channel catfish (*Ictalurus punctatus*) neutrophil collagenase. Vet. Immunol. Immunopathol. **67:**303–316.
39. **Ourth, D. D., and L. M. Bachinski.** 1987. Bactericidal response of channel catfish (*Ictalurus punctatus*) by the classical and alternative complement pathways against bacterial pathogens. J. Appl. Ichthyol. **3:**42–45.
40. **Pfeifer, C. G., S. L. Marcus, O. Steele-Mortimer, L. A. Knodler, and B. B. Finlay.** 1999. *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. Infect. Immun. **67:**5690–5698.
41. **Plamann, M. D., W. D. Rapp, and G. V. Stauffer.** 1983. *Escherichia coli* K12 mutants defective in the glycine cleavage enzyme system. Mol. Gen. Genet. **192:**15–20.
42. **Rubin, E. J., B. J. Akerley, V. N. Novik, D. J. Lampe, R. N. Husson, and J. J. Mekalanos.** 1999. In vivo transposition of mariner-based elements in enteric bacteria and mycobacteria. Proc. Natl. Acad. Sci. USA **96:**1645–1650.
43. **Simon, R., U. Priefer, and A. Piihler.** 1982. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
44. **Stauffer, L. T., S. J. Fogarty, and G. V. Stauffer.** 1994. Characterization of the *Escherichia coli gcv* operon. Gene **142:**17–22.
45. **Testerman, T. L., A. Vazquez-Torres, Y. Xu, J. Jones-Carson, S. J. Libby, and F. C. Fang.** 2002. The alternative sigma factor sigmaE controls antioxidant defenses required for *Salmonella* virulence and stationary-phase survival. Mol. Microbiol. **43:**771–782.
46. **Thune, R. L., D. H. Fernandez, J. L. Benoit, M. Kelly-Smith, M. L. Rogge, N. J. Booth, C. A. Landry, and R. A. Bologna.** 2007. Signature-tagged mutagenesis of *Edwardsiella ictaluri* identifies virulence-related genes, including a *Salmonella* pathogenicity island 2 class of type III secretion systems. Appl. Environ. Microbiol. **73:**7934–7946.
47. **Valdivia, R. H., and S. Falkow.** 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science **277:**2007–2011.
48. **Vidakovics, M. L., J. Paba, Y. Lamberti, C. A. Ricart, M. V. de Sousa, and M. E. Rodriguez.** 2007. Profiling the *Bordetella pertussis* proteome during iron starvation. J. Proteome Res. **6:**2518–2528.

49. **Waterstrat, P. R., A. J. Ainsworth, and G. Capley.** 1991. In vitro responses of channel catfish, *Ictalurus punctatus*, neutrophils to *Edwardsiella ictaluri*. Dev. Comp. Immunol. **15:**53–63.
50. **Waterstrat, P. R., A. J. Ainsworth, and G. Capley.** 1988. Use of a discontinuous Percoll gradient technique for the separation of channel catfish, *Ictalurus punctatus* (Rafinesque), peripheral blood leucocytes. J. Fish Dis. **11:** 289–294.
51. **Weiss, D. S., A. Brotcke, T. Henry, J. J. Margolis, K. Chan, and D. M. Monack.** 2007. In vivo negative selection screen identifies genes required for *Francisella* virulence. Proc. Natl. Acad. Sci. USA **104:**6037–6042.
52. **Wood, D., M. G. Darlison, R. J. Wilde, and J. R. Guest.** 1984. Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of *Escherichia coli*. Biochem. J. **222:**519–534.
53. **Zhao, Y., R. Jansen, W. Gaastra, G. Arkesteijn, B. A. van der Zeijst, and J. P. van Putten.** 2002. Identification of genes affecting *Salmonella enterica* serovar Enteritidis infection of chicken macrophages. Infect. Immun. **70:**5319–5321.