The Aspartate-Semialdehyde Dehydrogenase of *Edwardsiella ictaluri* and Its Use as Balanced-Lethal System in Fish Vaccinology

Javier Santander¹, Wei Xin¹, Zhao Yang¹,², Roy Curtiss¹*  
¹The Biodesign Institute, Center for Infectious Diseases and Vaccinology, Arizona State University, Tempe, Arizona, United States of America, ²State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China

**Abstract**

*asdA* mutants of Gram-negative bacteria have an obligation requirement for diaminopimelic acid (DAP), which is an essential constituent of the peptidoglycan layer of the cell wall of these organisms. In environments deprived of DAP, i.e., animal tissues, they will undergo lysis. Deletion of the *asdA* gene has previously been exploited to develop antibiotic-sensitive strains of live attenuated recombinant bacterial vaccines. Introduction of an *Asd*⁺ plasmid into a Δ*asdA* mutant makes the bacterial strain plasmid-dependent. This dependence on the *Asd*⁺ plasmid vector creates a balanced-lethal complementation between the bacterial strain and the recombinant plasmid. *E. ictaluri* is an enteric Gram-negative fish pathogen that causes enteric septicemia in catfish. Because *E. ictaluri* is a nasal/oral invasive intracellular pathogen, this bacterium is a candidate to develop a bath/oral live recombinant attenuated *Edwardsiella* vaccine (RAEV) for the catfish aquaculture industry. As a first step to develop an antibiotic-sensitive RAEV strain, we characterized and deleted the *E. ictaluri asdA* gene. *E. ictaluri* Δ*asdA*01 mutants exhibit an absolute requirement for DAP to grow. The *asdA* gene of *E. ictaluri* was complemented by the *asdA* gene from *Salmonella*. Several *Asd*⁺ expression vectors with different origins of replication were transformed into *E. ictaluri* Δ*asdA*01. *Asd*⁺ vectors were compatible with the pEI1 and pEI2 *E. ictaluri* native plasmids. The balanced-lethal system was satisfactorily evaluated in vivo. Recombinant GFP, PsPA, and LcrV proteins were synthesized by *E. ictaluri* Δ*asdA*01 harboring *Asd*⁺ plasmids. Here we constructed a balanced-lethal system, which is the first step to develop an antibiotic-sensitive RAEV for the aquaculture industry.

---

**Introduction**

Aspartate β-semialdehyde dehydrogenase (Asd; EC 1.2.1.11), a highly conserved homodimeric enzyme encoded by the *asd* gene, is involved in the conversion of β-aspartyl phosphate to aspartate β-semialdehyde. Asd is an enzyme common to the biosynthesis of the essential amino acids lysine, threonine, methionine, and isoleucine. It also performs a key step in the production of diaminopimelic acid (DAP), a required component for the peptidoglycan synthesis of Gram-negative and some Gram-positive bacterial cell walls [1,2,3,4] and an immediate precursor to lysine. *asd* mutants have an obligation requirement for DAP, and in the absence of DAP they undergo lysis. This has been demonstrated by gene-knockout studies with *Legionella pneumophila* [5], *Salmonella* Typhimurium [6] and *Streptococcus mutans* [7].

The Asd enzyme is also found in plants, where lysine is synthesized via the DAP pathway [8,9]. In contrast, mammalian cells neither synthesize nor use DAP as a substrate in any metabolic pathway, and lysine is not synthesized since it is an essential amino acid that is obtained from dietary sources [5,10,11]. Also lysine, threonine, methionine, and isoleucine are essential amino acids in the diet of teleostei fish [12,13,14,15,16,17], suggesting the absence of both the DAP/lysine synthesis pathway and Asd enzyme in fish cells.

Since DAP is absent from mammalian tissues, deletion of the *asd* gene has been exploited to develop a balanced-lethal system for vaccine delivery vehicles using a cloned *asd* gene as a selective marker in place of antibiotic-resistance markers, which are totally impractical in vivo [6]. Introduction of an *Asd*⁺ plasmid into *asd* mutants makes the bacterial strain plasmid-dependent. This dependence on the *Asd*⁺ plasmid vector creates a balanced-lethal complementation between the bacterial strain and the recombinant plasmid [18]. *Asd*⁺ vectors introduced into live recombinant attenuated *Salmonella* vaccines have been used to deliver heterologous antigens [19]. The construction of live attenuated recombinant bacterial vaccines not only require the absence of antibiotic-resistance markers in their recombinant plasmid, but also in their chromosomal deletions.

*Edwardsiella ictaluri*, a Gram-negative bacterial pathogen, is the cause of enteric septicemia in catfish, which causes losses estimated at $50–80 million annually [20]. The current USDA licensed vaccine, live *E. ictaluri* AQUAVAC-ESC® (Intervet Inc.), has been selected by multiple passages in increased concentrations of the antibiotic rifampicin [21,22,23]. The selected spontaneous mutant
strain presented an attenuated phenotype missing part of the lipopolysaccharide (LPS) [20,24]. Although there are FDA and USDA regulations against the use of antibiotic resistance in live attenuated bacterial vaccines for birds, mammals, and humans, the catfish industry currently allows antibiotic-resistant vaccine strains. Despite the fact that the current vaccine against enteric septicemia in catfish is antibiotic resistant, by using this vaccine we have learned that *E. ictaluri* live attenuated vaccines can be easily delivered to young fish and stimulate both humoral and cellular immunity of long duration [25,26]. These results provide guidance to design live attenuated antibiotic-sensitive vaccines for the catfish aquaculture.

As a first step in developing an antibiotic-sensitive live recombinant *E. ictaluri* vaccine strain (RAEV), we adapted suicide vector technology [27] to *E. ictaluri* to construct defined unmarked chromosomal deletion mutations, for instance the *asd* deletion. Two *E. ictaluri* *asd* genes were identified, a functional *asdA* and a non-functional *asdB* pseudogene. The *asdA* gene was deleted by using the described suicide vector technology. Using Asd expression vectors [19], we developed a balance-lethal system compatible with *E. ictaluri* native plasmids, to express and secrete heterologous proteins through the type II secretion system. The virulence of the *E. ictaluri AsdA* mutant, harboring an AsdA expression vector, was evaluated in vivo in the catfish (*Ictalurus punctatus*) and in the zebrafish (*Danio rerio*) host models. Here we report the first balance-lethal-vehicle-host system in *E. ictaluri*, a key in constructing antibiotic-sensitive live RAEV for the catfish industry.

**Results**

**Sequence analysis**

To develop a balanced-lethal system we first characterized the *asd* genes present in *E. ictaluri*. The genome of *E. ictaluri* has two *asd* gene sequences, *asdA* (gene ID 7960734) and *asdB* (gene ID 7959931). Sequence and structural alignment between functional representative bacterial Asd proteins reveals that 22 amino acid residues (~6%) are strictly conserved out of 367 residues in *E. ictaluri* AsdA (Fig. 1). *E. ictaluri* AsdA has 28%, 81%, 82%, 84%, and 97% amino acid similarity to the Asd of *Streptococcus mutans*, *Salmonella enterica*, *Escherichia coli*, *Yersinia* (*F. pestis* and *Y. ruckeri*), and *E. tarda*, respectively. The overall domain organization of *E. ictaluri* AsdA is similar to other Gram-negative Asd-family members, presenting an N-terminal domain comprising the NAD binding site and a C-terminal catalytic domain (Fig. 1). The same set of key functional groups in the active sites (Cys-135, Gln-162, Gln-241, Arg-267, and His-274) are conserved in *E. ictaluri* AsdA and likely have the same catalytic mechanism as other Asd enzymes (Fig. 1).

The sequence and structural alignment between representative bacterial AsdB proteins reveals that 52 amino acid residues (~15%) are strictly conserved out of 336 in *E. ictaluri* AsdB (Fig. 2). The *E. ictaluri* AsdB has 30%, 32%, 40%, 75%, and 99% amino acid similarity to the AsdB of *Streptococcus mutans*, *Mycobacterium marinum*, *Vibrio cholerae*, *F. pestis*, and *E. tarda*, respectively. In contrast to AsdA, the overall domain organization of *E. ictaluri* AsdB is similar to other Gram-positive Asd-family members. However, *E. ictaluri* AsdB lacks key functional groups in the active sites (Cys-135, Gln-162, and Arg-267) and likely has no catalytic activity.

The guanine plus cytosine (G+C) content found in the *E. ictaluri* *asdA* gene was 62%, significantly higher than the 54% of G+C found in the *Escherichia coli asdA* gene. Overall DNA comparison of the *asdA* gene showed that the *E. ictaluri* *asdA* gene shared 72% identity with the *Escherichia coli asdA* gene.

In terms of phylogeny, the bacterial Asd family is subdivided into two structural branches consisting of the enzymes from Gram-negative and Gram-positive bacteria [20] (Fig. 3). The *E. ictaluri* AsdA enzyme belongs to the Gram-negative branch, in contrast to AsdB that belongs to the Gram-positive branch (Fig. 3). *Edwardsiella* species comprise a lineage that diverged from the ancestral trunk before the divergence of some other enteric bacteria, such as *Salmonella* and *Escherichia* [29,30]. The phylogenetic position of the *E. ictaluri* AsdA enzyme corresponds with the *E. ictaluri* genome phylogenetic position (Fig. 3). Inside of the AsdB branch, a non-functional AsdB branch composed of *Edwardsiella* and *Yersinia* AsdB sequences was identified (Fig. 3), indicating that these non-functional AsdB proteins may have a common origin.

**Construction and characterization of *asdA* mutants**

The construction of *E. ictaluri* *ΔasdA* mutants was performed first by using pEZ101, a pR112 (Cm) base suicide vector (Tables 1 and 2). pEZ101 was conjugated from *Escherichia coli* χ7213 to *E. ictaluri* J100 and *E. ictaluri* J102 using the methods described for *E. ictaluri* [31] and *Escherichia coli* [32]. The selection of transconjugants was carried out in BHI agar supplemented with Col, DAP, and Cm. We did not recover transconjugants by using pEZ101. Therefore, we constructed and used pEZ102, a pMEG-575 (Cm, Amp) base suicide vector (Table 2). The selection of transconjugants was carried out in BHI agar supplemented with Col, DAP, and Cm (1 μg/ml), transconjugants were not recovered using pEZ101 (Cm) or pEZ102 (Amp, Cm). Certainly, these results indicate that Col selection and Cm-base suicide vectors are not useful to genetically manipulate *E. ictaluri*.

Single colonies of *E. ictaluri* transconjugants harboring pEZ102 (Colr, Amps), were grown in BHI, TSB or LB supplemented with DAP and Col at 28°C for 6 h with aeration (180 r.p.m.). The selection was performed in BHI, TSA and LB agar plates supplemented with DAP, Col, and 5% sucrose at 28°C for 4–5 days. BHI sucrose selection agar did not provide selection, due to *E. ictaluri* overgrowth. TSA and LB sucrose selection agar presented a satisfactory selection. Positive mutants were screened for Colr, Ampr, and DAPr. Several *E. ictaluri* ΔasdA mutants were recovered from TSA and LB sucrose-selection agar plates. The genotype was verified by PCR, and the phenotype by growth in the absence of DAP and no growth in the absence of DAP (Fig. 4). The biochemical profile, evaluated by API20E, did not present any difference between the wild type and ΔasdA01 mutant strains. *E. ictaluri* strains were identified as *Edwardsiella* sp (code 4004000). These results confirmed that the AsdB present in *E. ictaluri* is non-functional, since deletion of *asdA* is enough to preclude cell growth in the absence of DAP. Thus, AsdB can be considered a pseudo gene in *E. ictaluri*.

We evaluated reutilization of DAP by the *E. ictaluri* ΔasdA01 mutants released from lysed ΔasdA cells grown in absence of DAP. Washed cells of *E. ictaluri* J112 ΔasdA01 were diluted from 103 to 1010 CFU/ml in BHI Col. The estimated minimum number of *E. ictaluri* ΔasdA01 cells needed to support growth in absence of DAP was 1.3×108–2.7×109 CFU/ml. This is because of DAP-less death and reuse of DAP to permit growth on media without DAP.

The amount of DAP in the cell wall of *Escherichia coli* has been estimated at ~3.5×1010 molecules [33]. Based on the results obtained for the minimum number of *E. ictaluri* ΔasdA01 cells
Figure 1. Sequence alignment among representative members of the AsdA family. The secondary structure at the top of the alignment corresponds to the *E. ictaluri* AsdA enzyme (spirals represent $\alpha$-helix; arrows represent $\beta$-sheet). Conserved amino acids residues are indicated in grey. The stars indicated the key catalytic active site residues (Cys-135, Gln-162, Glu-241, Arg-267, and His-274). The AsdA sequences were obtained from NCBI's Entrez Protein database for *Edwardsiella ictaluri* YP_002935083.1; *Edwardsiella tarda* YP_003297386.1; *Escherichia coli* AP_004358.1; *Salmonella Typhi* NP_807591.1; *Salmonella Paratyphi* A YP_152515.1; *Salmonella Typhimurium* AAB69392.1; *Shigella flexneri* YP_690789.1; *Shigella sonnei* YP_312455.1; *Citrobacter koseri* YP_001456333.1; *Enterobacter cloacae* AP_004355.1; *Enterobacter aerogenes* YP_001142146.1; *Sodalis glossinidius* YP_456010.1; *Vibrio cholerae* YP_001217562.1; *Pseudomonas aeruginosa* NP_251807.1; *Erwinia carotovora* atrosepticum YP_052242.1.

doi:10.1371/journal.pone.0015944.g001
needed to support growth in the absence of DAP, and the calculated amount of DAP molecules per cell of *Escherichia coli*, we estimated that the minimum number of DAP molecules to support growth is \(4.5 \times 10^{14} - 9.5 \times 10^{14}\) molecules of DAP/ml in the growth media. We evaluated the growth of *E. ictaluri* in 10^10 to 10^20 molecules of DAP/ml in BHI Col.

Figure 2. Sequence alignment among representative members of the AsdB family. The secondary structure at the top of the alignment corresponds to the S. mutans AsdB enzyme (spirals represent \(\alpha\)-helix, arrows represent \(\beta\)-sheet). Conserved amino acid residues are indicated in grey. The stars indicate the key catalytic active site residues not present in AsdB from *Edwardsiella*. The AsdB sequences were obtained from NCBI’s Entrez Protein database for *Streptococcus mutans* NP_721384.1; *Edwardsiella ictaluri* YP_002934124; *Edwardsiella tarda* YP_003296462; *Bacillus cereus* YP_001217630.1; *Legionella longbeachae* CBJ10915; *Legionella pneumophila* YP_096311.1; *Kantharosomas axonopodis* NP_643032.1; *Kantharosomas campestris* NP_637897.1; *Mycobacterium tuberculosis* YP_001853481.1.

doi:10.1371/journal.pone.0015944.g002

---

**Table 1.** Comparison of key catalytic active site residues among the AsdB family. The residues are indicated in grey. The stars indicate the key catalytic active sites not present in AsdB from *Edwardsiella*.

| Sequence | 
|----------|
| *Streptococcus mutans* NP_721384.1 |
| *Edwardsiella ictaluri* YP_002934124 |
| *Edwardsiella tarda* YP_003296462 |
| *Bacillus cereus* YP_001217630.1 |
| *Legionella longbeachae* CBJ10915 |
| *Legionella pneumophila* YP_096311.1 |
| *Kantharosomas axonopodis* NP_643032.1 |
| *Kantharosomas campestris* NP_637897.1 |
| *Mycobacterium tuberculosis* YP_001853481.1 |
| *Mycobacterium marinum* YP_001853481.1 |

---

**Figure 2.** Sequence alignment among representative members of the AsdB family. The secondary structure at the top of the alignment corresponds to the *S. mutans* AsdB enzyme (spirals represent \(\alpha\)-helix, arrows represent \(\beta\)-sheet). Conserved amino acid residues are indicated in grey. The stars indicate the key catalytic active site residues not present in AsdB from *Edwardsiella*. The AsdB sequences were obtained from NCBI’s Entrez Protein database for *Streptococcus mutans* NP_721384.1; *Edwardsiella ictaluri* YP_002934124; *Edwardsiella tarda* YP_003296462; *Bacillus cereus* YP_001217630.1; *Legionella longbeachae* CBJ10915; *Legionella pneumophila* YP_096311.1; *Kantharosomas axonopodis* NP_643032.1; *Kantharosomas campestris* NP_637897.1; *Mycobacterium tuberculosis* YP_001853481.1; *Mycobacterium marinum* YP_001853481.1.
did not grow in concentrations below $10^{14}$ molecules of DAP/ml. Our previous estimation about the minimum number of DAP molecules required to support growth was confirmed, indicating that the amount of DAP in the cell wall of *E. ictaluri* is similar to *Escherichia coli*.

It has been reported that lysine, threonine, methionine, and isoleucine are essential amino acids in the diet of teleostei fish [12,13,14,15,16,17], suggesting the absence of the DAP/lysine synthesis pathway in fish cells. We tested the growth of *E. ictaluri* J112 *ΔasdA01* in different catfish broths (1% of catfish liver, spleen, kidney and meat in BHI) in presence and absence of DAP. *E. ictaluri* J112 *ΔasdA01* was not able to grow in fish broth not supplemented with DAP. *E. ictaluri* J100 wild-type, used as control, grew in all fish broth conditions (data not shown). This result supports the idea that as mammalian cells, fish cells neither synthesize nor use DAP as substrate in any metabolic pathway.

**Complementation of *E. ictaluri* asdA gene and *E. ictaluri* ΔasdA01 mutant**

The structural analysis of *E. ictaluri* AsdA indicated that the overall domain organization is similar to other AsdA-family members and has the same set of key active-site functional groups and therefore the same catalytic mechanism as other Asd enzymes (Fig. 1). To evaluate the likely broad functionality of *E. ictaluri* AsdA enzyme, *asdA* mutants of *Escherichia coli*, *Salmonella enterica* (serovars Typhimurium, and Typh), *Y. pestis*, and *E. ictaluri*, were complemented with the *E. ictaluri* *asdA* gene. Because overproduction of AsdA enzyme increases generation times [19,34] and the synthesis of Asd enzyme is proportional to the copy number of the complementing plasmid, *asdA* mutants were complemented with *E. ictaluri* *asdA* gene with (*P_{asdA}-asdA*) and without its promoter (SD-asd), this last to decrease Asd synthesis, cloned into p15A ori plasmid (*pACYC184*; Table 2).

*Escherichia coli*, *S. enterica* and *P. putida* *ΔasdA* mutants complemented with *E. ictaluri* SD-asd1 presented similar growth rates compared to wild type (Fig. 5), indicating full complementation. *E. ictaluri* *ΔasdA01* mutants complemented with SD-asd1 presented a significantly lower growth rate than the wild type (Fig. 5). This could be due to overproduction or underproduction of Asd. It has been reported that SD-asd constructions do not enable SDAs strains to survive in absence of DAP if the origin of plasmid replication (*ori*) is from pSC101 or p15A. In other words, with these lower-copy-number replicons, the amount of Asd enzyme synthesized is insufficient to enable growth in absence of lysis [19]. To evaluate if the decrease in the generation time of the SD-asd1 complemented *E. ictaluri* *ΔasdA01* strain was due to overproduction or underproduction of AsdA, complementation with *P_{asdA}-asdA* (*pEZ142*) was performed. Complementation of *E. ictaluri* *ΔasdA01* with *asdA* from *E. ictaluri* *ΔasdA01* mutants complemented with *E. ictaluri* *asdA* gene and *E. ictaluri* *ΔasdA01* was detected out of 100. The Asd sequences were obtained from NCBI’s Entrez Protein database for *Edwardsiella tarda*; *E. ictaluri*; *P. aeruginosa*; *S. enterica*, and *Y. pestis*.

**Figure 3. Phylogenetic tree constructed by the unweighted pair group method with arithmetic mean.** Bootstrap values indicate the number of times that a given node was detected out of 100. The Asd sequences were obtained from NCBI’s Entrez Protein database for *Edwardsiella ictaluri* YP_002935083.1; *Edwardsiella tarda* YP_003297386.1; *Escherichia coli* AP_004358.1; *Salmonella Typhimurium* NP_807591.1; *Salmonella Paratyphi A* YP_152515.1; *Salmonella Typhimurium* AAB89322.1; *Shigella flexneri* YP_690789.1; *Shigella sonnei* YP_001456333.1; *Enterobacter cancerogenus* YP_05969786.1; *Enterobacter sp.* YP_001178547.1; *Yersinia pestis* ZP_40615435.1; *Proteus mirabilis* YP_002152826.1; *Aeromonas hydrophila* ABK34977.1; *Aeromonas salmonicida* YP_001214216.1; *Sodalis glossinidius* YP_456010.1; *Vibrio cholerae* YP_002810714.1; *Pseudomonas aeruginosa* NP_251807.1; *Erwinia carovora atroseptica* YP_052242.1; *Streptococcus mutans* NP_721384.1; *Edwardsiella ictaluri* YP_002934124; *Edwardsiella tarda* YP_003296462; *Vibrio cholerae* YP_001217630.1; *Bacillus cereus* YP_085142.1; *Legionella longbeachae* CBJ10915; *Legionella pneumophila* YP_096311.1; *Xanthomonas axonopodis* NP_218225.1; *Mycobacterium marinum* YP_001853481.1; *Mycobacterium tuberculosis* NP_218225.1; *Mycobacterium tuberculosis* YP_002887982.1.

doi:10.1371/journal.pone.0015944.g003

---

**Balanced-Lethal System in *E. ictaluri***

![Phylogenetic tree](image-url)
mutants with pEZ142 decreased the growth rate even more than complementation with SD-asdA. These results suggest that the decreased growth rate in the E. ictaluri ΔΔasdA01 complemented with its own asdA gene is due to overproduction of AsdA. There are differences in the SD regions that could justify part of the difference in the growth rate of E. ictaluri ΔΔasdA01 complemented with its own SD-asdA. The SD region of E. ictaluri asdA gene has an optimal spacing (6 nt) between the SD region and the ATG initiation codon of the mRNA [35] in contrast to the other bacterial species complemented with E. ictaluri SD-asdA (Fig. 5).

Complementation of E. ictaluri ΔΔasdA01 mutants by Gram-positive AsdB enzyme was also evaluated. Streptococcus mutans asdB region (including the full promoter), cloned into pYA575 [36] and S. mutans SD-asdB, cloned into pYA248 [18] complemented E. ictaluri ΔΔasdA01 mutants. However these strains presented lower growth rates than the wild type (Fig. 6). E. ictaluri ΔΔasdA01 mutants

| Table 1. Bacterial strains and plasmids. |
|------------------------------------------|
| **Strain** | **Relevant characteristics** | **Source or reference** |
| Escherichia coli | | |
| y6212 | F− Δ(argF-lacZYA)-U169 glnV44 lacI169 deoR f80lacZΔM15 gyrA96 recA1 relA1 endA1 ΔasdA4 Δ2phf-2::Tn10 thi-1 hsdR17, Tet* | [60] |
| y7213 | thr-1 leuB6 thiA17 glnV44 recA1 ΔΔasdA4 D(2phf-2::Tn10) thi-1 RP4-2-Tc-Mu [Dpir]; Km* | [53] |
| y7232 | endA1 hsdR17 hospital type-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 λpir deoR (f80lacZΔM15) | Lab collection |
| Edwardsiella ictaluri | | |
| J100 | Wild-type; pEI1*; pEI2* API20E 40040057; smooth LPS; Col+ DAP* | [59] |
| J102 | Wild-type; pEI1*; pEI2* API20E 40040057; smooth LPS; Col+ DAP* | ATCC 33202 |
| J111 | J102 derivative; ΔΔasdA01; pEI1*; pEI2* API20E 40040057; smooth LPS; Col+ DAP* | This study |
| J112 | J100 derivative; ΔΔasdA01; pEI1*; pEI2* API20E 40040057; smooth LPS; Col+ DAP* | This study |
| Salmonella enterica | | |
| y3761 | S. Typhimurium UK-1; wild-type | [19] |
| y8958 | S. Typhimurium UK-1 ΔΔasdA33 | Lab collection |
| y9112 | S. Typhi ISP1820 ΔΔasdA33 | Lab collection |
| y9124 | S. Typhi Ty2 ΔΔasdA33 | Lab collection |
| Yersinia pestis | | |
| y10006 | ΔΔasdA12 | Lab collection |

| Table 2. Plasmid used in this study. |
|------------------------------------------|
| **Plasmids** | **Relevant characteristics** | **Source or reference** |
| pYA248 | 3,000 bp, contains 1,071 bp of S. mutans asdA gene; p15A ori | [18] |
| pYA575 | 5,730 bp, contains ~1,330 bp of S. mutans DNA inserted between the EcoRI and HindIII sites of pBR322 plasmid, Amp, Tet, pBR ori | [36] |
| pYA3341 | 2595 bp, plasmid Asd−; pUC ori | [19] |
| pYA3493 | 3113 bp, plasmid Asd−; pBR orI β-lactamase signal sequence-based periplasmic N- terminal secretion plasmid | [19] |
| pYA3620 | 3169 bp, plasmid Asd−; pBR orI β-lactamase signal sequence-based periplasmic N- and C- terminal secretion plasmid | [19] |
| pYA3994 | pBR ori, Asd−, GFP− 3113 bp, Lab collection |
| pYA3840 | 323 bp DNA encoding the LcrV in pYA3493 | [38] |
| pYA4088 | 852 bp DNA encoding the z-helical region of PspA aa 3-285 in pYA3493 | [39] |
| pRE112 | 5,173 bp, Cm, sacB, oriT | [27] |
| pMEG-375 | 8,142 bp, Cm, Amp, lacZ, R6K ori, mob incP, sacR sacB | [52] |
| pACYC184 | 4,245 bp, Tet, Cm, p15A ori | [54] |
| pEZ101 | ΔΔasdA01, pRR112 | This study |
| pEZ102 | ΔΔasdA01, pMEG-375 | This study |
| pEZ140 | SD-asdA, Cm, pACYC184 | This study |
| pEZ142 | PasdA-asdA, Cm, pACYC184 | This study |
complemented with SD-asdB (pYA248), presented the lowest growth rate, suggesting that S. mutans AsdB is probably required in higher levels to fully complement E. ictaluri or S. mutans AsdB do not interact efficiently with E. ictaluri aspartokinase enzymes to transfer the β-aspartyl phosphate to Asd.

Complementation by Asd⁺ vectors to develop a balanced-lethal system in E. ictaluri

The asdA gene from E. ictaluri complemented S. enterica ΔasdA mutants, in addition the Asd enzymes from E. ictaluri and S. enterica share 81% similarity. Therefore, we used the Asd⁺ vectors utilized in live recombinant attenuated Salmonella vaccines [19] to develop a balanced-lethal system in E. ictaluri. The Asd⁺ vectors utilized in this study possess only the SD-asdA gene from S. Typhimurium with a modified start codon from ATG to GTG. E. ictaluri ΔasdA01 mutants were complemented with the asdA gene from S. Typhimurium (Fig. 7). The growth rate of E. ictaluri ΔasdA01 complemented with different copy number of Asd⁺ vectors was similar to the wild type in all cases (Fig. 7). The Asd⁺ vectors were compatible with the native plasmids of E. ictaluri (Fig. 7) and stable for at least 80 generations. These results show the first balanced-lethal system in E. ictaluri.

Expression of genes encoding GFP protein in the AsdA⁺ vector

The synthesis of heterologous proteins, for instance GFP, cloned into Asd⁺ vectors was evaluated in E. ictaluri ΔasdA01 to potentially develop live E. ictaluri recombinant vaccines. First, the synthesis of heterologous proteins was evaluated by using the GFP⁺ Asd⁺ vector pYA4088 (Table 2). E. ictaluri ΔasdA01 mutant strains harboring the GFP⁺ Asd⁺ vector grew in absence of DAP and synthesized GFP⁺ as expected (Fig. 8). The GFP⁺ Asd⁺ vector was compatible with the native plasmids of E. ictaluri in the relaxed conformation (Fig. 8). The GFP⁺ Asd⁺ vector was stable in E. ictaluri ΔasdA01 strains for at least 80 generations. The expression of LcrV and PspA heterologous proteins using AsdA⁺ vectors was also evaluated (see below).

Secretion of heterologous proteins

Secretion of the heterologous antigens by live attenuated recombinant bacterial vaccines has been shown to enhance immunogenicity against the heterologous antigen [37]. The synthesis and secretion of heterologous proteins was evaluated by using the proteins derived from Gram-positive and Gram-negative bacterial strains. PspA-Rx1 from Streptococcus pneumoniae was utilized as a Gram-positive representative and LcrV from Yersinia pestis was utilized as a Gram-negative representative. The heterologous antigens, PspA-Rx1 and LcrV fused to β-lactamase signal sequence, were expressed from the Asd⁺ vectors pYA4088 and pYA3841, respectively (Table 2). Both heterologous proteins were secreted through the type II secretion system. No difference in the growth rate was observed between the recombinant E. ictaluri and the wild-type strain J100.

Virulence of E. ictaluri ΔasdA01 strain complemented with the AsdA⁺ plasmid vector in catfish host and zebrafish host models

The idea to develop a balanced-lethal system in a pathogenic bacterial strain is to synthesize heterologous antigens, without the use of antibiotic-resistant genes, in either the plasmid or in the bacterial chromosome. This is the first step towards developing live recombinant bacterial vaccines. The ideal
balanced-lethal system should present nearly the same level of virulence as the wild-type strain with regard to invasion and colonization of lymphoid tissues. We evaluated the virulence of *E. ictaluri* ΔasdA01 mutants with and without the balanced-lethal system in the catfish and zebrafish hosts (Tables 3 and 4). We used pYA3493 AsdA+ since this vector has been used successfully in live recombinant *Salmonella* vaccines [19,38,39,40]. *E. ictaluri* ΔasdA01 was attenuated at the high dose of 10⁸ CFU, but still produced some mortality in catfish (Table 4). *E. ictaluri* ΔasdA01 at a high dose (10⁸ CFU) was not attenuated in zebrafish and all the fish died (Table 3). However, at lower doses (10⁵-10⁶) *E. ictaluri* ΔasdA01 was totally attenuated in zebrafish (Table 3). *E. ictaluri* ΔasdA01 harboring the Asd⁺ vector pYA3493 increased the LD₅₀ one log-fold, from 10⁴ CFU to 10⁵ CFU in orally infected catfish, and two log-fold, from 10³ CFU to 10⁵ CFU, in zebrafish (Tables 3 and 4). Catfish i.p. infected with *E. ictaluri* ΔasdA01 harboring the Asd⁺ vector pYA3493 presented the same level of virulence as *E. ictaluri* wild type (Table 4). From moribund orally infected catfish, *E. ictaluri* ΔasdA (pYA3493) Asd⁺ was recovered from the head-kidney, spleen and liver, indicating that *E. ictaluri* ΔasdA (pYA3493) Asd⁺ colonized these lymphoid tissues.

**Discussion**

To develop a balanced-lethal system in *E. ictaluri*, we first characterized the *asdA* and *asdB* genes present in the genome of *E. ictaluri* (Fig. 1). Deletion of the *asdA* gene precluded the growth of *E. ictaluri* in absence of DAP (Fig. 4), indicating that *asdB* does not encode for a functional protein related to DAP synthesis. This is consistent with the bioinformatic analysis (Fig. 2), which showed that the AsdB enzyme lacked several key amino acid residues at the catalytic active site.

The phylogeny of Asd has two branches, AsdA related with Gram-negatives and AsdB related with Gram-positives [28]. We found a particular group of non-functional AsdB genes in *Edwardsiella* and *Yersinia*. The common origin of AsdB in these bacteria suggests that the genes might have lost their activity through evolution, and that *asdB* could be considered a pseudogene in *Edwardsiella* and *Y. pestis*.

Suicide vector technology has been successfully used in several enteric bacteria to develop antibiotic-sensitive mutants [27]. Using this technology it was possible to construct defined deletion mutations in the absence of antibiotic-resistance markers for the first time in *E. ictaluri* (Fig. 3). During this process, we determined
that *E. ictaluri* is extremely sensitive to Cm, even in the presence of the *cat* gene. The *cat* gene confers high-level resistance to Cm in most bacterial species. It codes for an enzyme called chloramphenicol acetyltransferase which inactivates Cm by covalently linking one or two acetyl groups, derived from acetyl-S-coenzyme A, to the hydroxyl groups on the chloramphenicol molecule [41]. This might indicate that chloramphenicol acetyltransferase is not functional or inefficient in *E. ictaluri*. Further studies are required to answer this.

The current live attenuated *E. ictaluri* vaccine is a rifampicin-resistant strain [22]. Antibiotic resistance in live attenuated bacterial vaccines present a threat to both the animal and to human health, due to the horizontal transmission of genes, in this case by transduction. Recently lytic bacteriophages have been isolated from catfish ponds against *E. ictaluri* [42]. This suggests that temperate phages for *E. ictaluri* that can establish lysogeny might be present in these environments and could spread rifampicin resistance to native environmental bacterial flora. Here we have described a methodology to genetically engineer *E. ictaluri* without the use of antibiotic-resistance genes in the final strain. This advancement opens up the field of *E. ictaluri* live attenuated vaccine development and will provide opportunities for further research into the pathogenesis of this important organism.

Although, *E. ictaluri* Δ*asdA01* is complemented with its own *asdA* gene, the complemented strain did not grow at the same rate as the parental wild-type strain, presenting a higher growth rate. To achieve the right amount of native AsdA in *E. ictaluri* using Asd*+* vectors requires further studies. However, *E. ictaluri* Δ*asdA01* was fully complemented by the *Salmonella* SD-AsdA gene, allowing the development of a balanced-lethal system.

One of the major difficulties in the construction of a balanced-lethal system in *E. ictaluri* is the incompatibility of the Asd*+* vectors with cryptic plasmids present in the bacterial strain. *E. ictaluri* possesses two native autonomous small plasmids, pEI1 and pEI2 [43], that have been implicated in virulence [44]. The Asd*+* expression vectors were compatible with pEI1 and pEI2 native plasmids of *E. ictaluri*, indicating that the origin of replication of these plasmids, ColE1 ori and ColE2 ori-like, respectively [43], are compatible with p15A ori, pBR ori and pUC ori.

*E. ictaluri* was described by Hawke in 1979 [45], and recently sequenced (NCBI's Entrez Genome database NC_012779). Most of its genes encode for putative functions. *E. ictaluri* possesses the machinery for the type II secretion system in its genome. Therefore we evaluated the secretion of proteins by using a β-lactamase signal sequence at the N-terminal end of a recombinant protein [19], a signal required for a protein to be secreted through the system mentioned above. Recombinant proteins, cloned in the AsdA*+* vector and using the β-lactamase signal sequence, were secreted in a similar fashion (Fig. 8) as for a *Salmonella* recombinant vaccine [37], suggesting that the type II secretion system in *E. ictaluri* is fully functional.
Salmonella ΔasdA mutants are totally attenuated in mice orally infected with 10^8 CFU per dose [19]. E. ictaluri ΔasdA01 mutants were not fully attenuated in catfish i.p. or orally infected (Table 4). Zebrafish i.m. infected with doses of 10^7 CFU succumbed to E. ictaluri ΔasdA01 mutant infection (Table 3). Lower doses of E. ictaluri ΔasdA01 mutants (10^6-10^4 CFU) were totally attenuated (Table 3). It has been reported that E. ictaluri contain toxins, like hemolysin [46,47]. We believe that the mortality caused by E. ictaluri ΔasdA01 mutants, either in catfish or zebrafish, is due to a toxic shock-like effect caused by the toxins realized after this DAP dependent mutant lyse in vivo. These toxins probably are not LPS related, since fish and amphibians are resistant to the toxic effects of LPS [48,49]. E. ictaluri ΔasdA01 (pAsdA^+) was attenuated by one log-fold in catfish animal host model (orally infected), and two log-fold in zebrafish. The next step in the construction and design of a live recombinant E. ictaluri vaccine is the attenuation of the bacterial strain without altering colonization of lymphoid tissues and immunogenicity. From moribund orally infected catfish, E. ictaluri ΔasdA (pAsdA^+) were recovered from the

**Table 3.** Survival of zebrafish (*D. rerio*) infected with wild-type and *E. ictaluri ΔasdA01* with and without Asd^+^ vectors.

| E. ictaluri strains | Experiment #1 | | Experiment #2 | |
|---------------------|----------------|------------------|------------------|
|                     | i.m. | Dose (CFU/ml) | Survivors/Total | Dose (CFU/ml) | Survivors/Total |
| J100 wild-type      |       | 1.5×10^8 | 0/25          | 1.2×10^7 | 0/25          |
|                     |       | 1.5×10^5 | 0/25          | 1.2×10^7 | 13/25         |
|                     |       | 1.5×10^4 | 2/25          | 1.2×10^7 | 22/25         |
| J112 ΔasdA01        |       | 3.0×10^8 | 0/10          | 1.7×10^7 | 1/5           |
|                     |       | 3.0×10^7 | 0/10          | 1.7×10^7 | 10/10         |
|                     |       | 3.0×10^6 | 10/10         | 1.7×10^7 | 10/10         |
| J112 ΔasdA01 (pYA3493) |      | 2.1×10^8 | 0/10          | 1.8×10^6 | 0/5           |
|                     |       | 2.1×10^7 | 0/10          | 1.8×10^6 | 0/5           |
|                     |       | 2.1×10^6 | 10/10         | 1.8×10^6 | 5/5           |
| BSG (Control)       |       | None       | 10/10         | None       | 5/5           |

The zebrafish were infected i.m. with 10 μl of the respective *E. ictaluri* strain. 

[10.1371/journal.pone.0015944.t003]

Figure 8. Synthesis of heterologous antigens in *E. ictaluri* J112 ΔasdA01 by using AsdA^+^ expression vectors. A. Plasmid profile of J112 (pYA3994); B. Expression of GFP J112 (pYA3994); C. Expression and secretion of *Y. pestis* LcrV antigen by J112 (pYA3840); D. Expression and secretion of *S. pneumoniae* PspA-Rx1 antigen by J112 (pYA4088).

doi:10.1371/journal.pone.0015944.g008
head kidney, spleen and liver, indicating that *E. ictaluri asdA* (pAsd^A^) colonize lymphoid tissues. The increase in attenuation in catfish orally infected with *E. ictaluri ΔasdA* (pAsd^A^) could be used together with other genetic modifications to attenuate *E. ictaluri* in regard to constructing a live RAEV.

In summary, we have described methods to genetically engineer *E. ictaluri* without the use of antibiotic-resistant genes in the final strain. This opens up the field of RAEV development and will provide opportunities for further research into *E. ictaluri* in regard to constructing a live RAEV.

Materials and Methods

Ethics statement
All research involving fish was conducted as per Protocol #09-1042R, approved by the Arizona State University Institutional Animal Care and Use Committee.

Bacterial strains, plasmids, media, and reagents
The bacterial strains and plasmids are listed in Table 1 and 2. Bacteriological media and components are from Difco (Franklin Lakes, NJ). Antibiotics and reagents are from Sigma (St. Louis, MO). LB broth (tryptone, 10 g; yeast extract 5 g; NaCl 10 g; dextrose 1 g, ddH2O 1 L) [50], Bacto-Brain Heart Infusion broth (BHI), and Trypticase Soy Broth (TSB), were used routinely. Antibiotics were added to media, as necessary, at concentrations equal to or lower than their minimal inhibitory concentrations. The bacterial media were supplemented with 1.5% agar, 5% sucrose, colistin sulphate (Col; 12.5 μg/ml), ampicillin (Amp; 100 μg/ml), chloramphenicol (Cm; 25 μg/ml), or kanamycin (Km; 50 μg/ml). Media of 5% sucrose or 2% dextrose were used for determining auxotrophy or trimethoprim resistance, respectively.

Sequence analysis
Nucleotide Basic Local Alignment Search Tool (BLAST) was performed based on the sequences of the putative *asd* genes present in the genome sequence of *E. ictaluri* 93-146 accessed from NCBI’s Entrez Genome database (NC_012779).

Asd sequences used were obtained from NCBI’s Entrez Protein database. Amino acid sequence alignments were performed using the CLC Free Workbench software tool (v. 6.1 CLC bio A/S, Aarhus, Denmark). Protein structural-based alignments were performed by using the web-based interface for ESPript v.2.2 located at http://espritp.ibcp.fr/ESPript/cgi-bin/ESPr ipt.cgi [51]. Phylogenetic position of *E. ictaluri* AsdA protein was performed with CLC Free Workbench version using the unweighted pair group method with arithmetic mean (UPGMA). Bootstrap analysis was performed with 100 resamplings.

Construction and characterization of *asdA* mutants
The recombinant suicide vector pEZ102 (Table 2) carrying the linked flanking regions (5’ 361 bp and 3’ 422 bp) to generate an in-frame deletion of the *asd* gene was constructed as described in [52]. The ΔasdA01 defined deletion mutation encompasses a 1,104 base pair deletion including the ATG start codon not including the TAG stop codon. Primers (primer 1) 5’-ACATGGCATGCAATGCCGTCAACGCCGCAGAAT-3’ and (primer 2) 5’-CCGCTCGAGATGAGGCTACTGCTCTAGCCCGTGC-3’ designed to amplify the upstream *asd* flanking region (361 bp). A SphI site was included in the primer 1 (underlined) and a XhoI site was included in primer 2 (underlined). The downstream *asd* flanking region (422 bp) was amplified by primers (primer 3) 5’-CCGCTCGAGATGAGGCTACTGCTCTAGCCCGTGC-3’ and (primer 4) 5’-CTCGTCGAGATGAGGCTACTGCTCTAGCCCGTGC-3’ were designed to amplify the upstream *asd* flanking region (361 bp). A SphI site was included in the primer 1 (underlined) and a XhoI site was included in primer 2 (underlined). The downstream *asd* flanking region (422 bp) was amplified by primers (primer 3) 5’-CCGCTCGAGATGAGGCTACTGCTCTAGCCCGTGC-3’ and (primer 4) 5’-CTCGTCGAGATGAGGCTACTGCTCTAGCCCGTGC-3’. A XhoI site was included in primer 3 (underlined) and a XhoI site was included in primer 4. The flanking regions were amplified from *E. ictaluri* J100, ligated, cloned into pRE112 and pMEG-375, and then digested with SphI and XhoI. The resulting plasmids were designated pEZ101 and pEZ102, respectively. To construct the *E. ictaluri* ΔasdA01 mutant, the suicide plasmid was conjugationally transferred from *Escherichia coli* C7213 [53] to *E. ictaluri* wild-type strains J100 and J102. Strains containing single-crossover plasmid insertions (*E. ictaluri* asd^A^::pAsd^A^) were isolated on BHI agar plates containing Col, Amp, and DAP. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was

Table 4. Survival of catfish (*I. punctatus*) infected with *E. ictaluri* wild type and *E. ictaluri* ΔasdA01 with and without Asd^A^ vectors.

| Experiment #1 | Experiment #2 |
|---------------|---------------|
| i.p.          | Oral          |
| **E. ictaluri strains** | **Dose (CFU/ml)** | **Survivors/Total** | **Dose (CFU/ml)** | **Survivors/Total** |
| J100 wild-type | 1.5 x 10^8   | 0/6             | 1.2 x 10^8   | 1/7             |
|               | 1.5 x 10^5   | 0/6             | 1.2 x 10^5   | 2/7             |
|               | 1.5 x 10^4   | 1/6             | 1.2 x 10^4   | 4/7             |
| J112 ΔasdA01   | 3.0 x 10^6   | 3/7*            | 1.7 x 10^6   | 7/8*            |
| J112 ΔasdA01 (pYA3493) | 2.1 x 10^6 | 2/6             | 1.8 x 10^6   | 4/7             |
|               | 2.1 x 10^5   | 2/6             | 1.8 x 10^5   | 5/7             |
| BSG (Control) | None          | 6/6             | None          | 6/6             |

The catfish were infected i.p. with 100 μl and orally with 20 μl of the respective *E. ictaluri* strain. *death within 48 h.*

doi:10.1371/journal.pone.0015944.t004

December 2010 | Volume 5 | Issue 12 | e15944
selected by using the satB-based sucrose sensitivity counterselection system [27]. The colonies were screened for Amp\(^r\), Col\(^r\) and for growth only in presence of DAP. DAP colonies were screened by PCR using primer 1 and 4. Biochemical profiles of \(E.\) ictaluri strains were determined using the API 20E system (bioMérieux, Marcy l’Etoile, France).

**Complementation of asdA gene**

The \(asdA\) gene of \(E.\) ictaluri, with and without its promoter, was cloned into a pACYC184 vector [54] by inactivating the Tet cassette at the \(BamH\)I and XbaI restriction sites. The primers used to amplify \(asdA\) with its promoter (\(F_{asdA}\)-\(XbaI\)) were \(5'\) – TCGTCTAGAATCTTTGAATTAATGAATTCTA – \(3'\) (upstream) and \(5'\) – CCGGGATCGATGACGCGCGCGCGAAGGAATGAGTGCATATGAAAA – \(3'\) (downstream). An \(XbaI\) and \(BamHI\) site were included in these primers, respectively (underlined). To amplify the \(E.\) ictaluri Shine-Dalgarno (SD)-\(asdA\) promoter-less the upstream primer \(5'\) – TCGTCTA-GAAGCCAGGATGGATGAAAGAAA – \(3'\) was used with the downstream primer previously described. An \(XbaI\) site was included in this primer (underlined). The \(E.\) ictaluri promoter-less \(asdA\) includes the SD-AGGA region, 6 bp upstream from the ATG start codon (SD-\(asdA\)). The resulting plasmids, pEZ146 (SD-\(asdA\)) and pEZ146 (\(F_{asdA}\)-\(XbaI\)) were used to complement different \(asdA\) mutant strains. Also \(asd\) from *Streptococcus mutans*, cloned into pYA575 [56] and pYA249 [10], was used to evaluate complementation of \(E.\) ictaluri \(asdA01\) mutants.

To create a balanced-lethal system in \(E.\) ictaluri, several \(Asd^+\) expression vectors harboring the SD-\(asdA\) gene sequence from *Salmonella Typhimurium* UK-1 with different origins of replication, (Table 2) [19] were transformed into \(E.\) ictaluri \(asdA01\) to evaluate their complementation and stability. The growth rate of the complementing strains was evaluated in the absence of DAP. Plasmid stability was evaluated for fifty generations as described by Konjufca et al. [55].

**Expression of heterologous antigens by \(E.\) ictaluri**

\(Asd^+\) expression vectors encoding different heterologous proteins (Table 2) were transformed into \(E.\) ictaluri \(asdA01\) to evaluate the expression and secretion of foreign proteins. First, the green fluorescent protein (GFP) was used to evaluate protein synthesis in the \(E.\) ictaluri \(asdA01\) strain. The vector pYA9994 AsdA\(^+\) GFP\(^-\) without a peptide secretion signal sequence was transformed into \(E.\) ictaluri \(asdA01\) (Table 2). The synthesis of GFP was evaluated by fluorescent microscopy. The synthesis of LcrV and PspA was evaluated by western blot and the secretion was evaluated by subcellular fractionation [37].

**Western blot analysis**

To evaluate the synthesis of heterologous proteins by \(E.\) ictaluri, the strains were grown in 3 ml of BHI at 28°C with aeration (150 r.p.m.). The samples were collected when the culture reached the absorbance of 1.0 (\(O_{D_{600}}\) 1.0–1.0 x \(10^5\) cfu/ml). One ml of culture was collected and prepared for Western blot analysis [56]. The total proteins were normalized by using a nanodrop spectrophotometer (ND-1000, NanoDrop) at 25 mg/ml and separated by 10% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes [56]. Fat-free milk powder solution (5%, wt/vol) in PBS supplemented with 0.05% of Tween 20 (PBS-T) was used for blocking. The membrane was incubated individually with a primary mouse anti-RpoD monoclonal antibody (1:1,000) (Neoclon), rabbit anti-LcrV polyclonal antibody (1:1,000) [57], or rabbit anti-PspA polyclonal antibody (1:10,000) [40], for 1 h at room temperature, washed three times with PBS-T, and then incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG) (Sigma) or anti-rabbit immunoglobulin G (IgG) (Sigma). Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphosphate (BCIP) (Amresco).

**Edwardsiella subcellular fractionation**

 Cultures were grown in BHI at 28°C static to an \(O_{D_{600}}\) of 0.6 and centrifuged at 5,865 g for 10 min. Periplasmic fractions were prepared by a modification of the lysozyme-osmotic shock method [58] as previously described [37]. The supernatant fluid was saved for analysis of secreted proteins. Equal volumes of periplasmic, cytoplasmic, and supernatant fractions and total lysate samples were separated by SDS-PAGE for western blot analysis.

**Determination of LD\(_{50}\) in zebrafish animal host**

Zebrafish infections were performed by the methodology described by Petri-Hanson et al. [59] with modifications. The temperature of the water was 26±1°C and the fish were acclimated during 2 weeks prior to the start of the experimentation. Adult zebrafish (average weight, 0.5 g) were sedated in 100 mg/L tricaine methanesulfonate (MS-222, Sigma) and then injected intramuscularly (i.m.). Groups of zebrafish (typically 15 fish per group) were injected i.m. with 10 µl of the bacterial suspension (\(10^5–10^6\) CFU) into each fish. A 3/10-cc U-100 ultrafine insulin syringe with a 0.5-in.-long (ca. 1-cm-long) 29-gauge needle (catalog no. BD-309301; VWR) was used to inject the fish. Two sets of controls were used: fish that were injected with 10 µl of sterile phosphate-buffered saline containing 0.01% gelatin (BSG) [60] and fish that were not injected. Moribund fish demonstrating clinical signs were euthanized, necropsied, and bacteria isolated as previously described [59]. The fish were fed twice daily with TetraMin Tropical Fish Flake Feed. During the experiments, the fish were observed daily, and every other day water quality was monitored for pH and NO\(_2\) with standard kits. The LD\(_{50}\) was calculated by the method of Reed-Muench [61]. Fish care and use was performed in accordance with the requirements of the Arizona State University, Institutional Animal Care and Use Committee.

**Determination of LD\(_{50}\) in catfish animal host**

Specific-pathogen-free channel catfish (Ictalurus punctatus) fingerlings were used with a mean weight of 18.5±1.3 g. The animals were randomly assigned to treatment groups of 6 to 8 fish each in 100 liter tanks. Each tank was equipped with a self-contained, recirculating, biofiltered, mechanical filtered, and U.V. water treated system with 12 h of illumination daily. The water temperature was 28±1°C during the 2 weeks of acclimatization and during the experiments. The fish were fed daily with commercial Aquamax grower 400 (Purina Mills Inc., St. Louis, MO). During the experiments, the fish were observed daily, and every other day water quality was monitored for pH and NO\(_2\) with standard kits. The LD\(_{50}\) was calculated by the method of Reed-Muench [61]. Moribund animals were necropsied to evaluate presence of \(E.\) ictaluri in kidney, spleen and liver. Fish care and use was performed in accordance with the requirements of the Arizona State University, Institutional Animal Care and Use Committee.
Bacteria 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 to 0.9 (~$10^8$ CFU/ml). Bacteria were sedimented 10 min by centrifugation (5,865 g) at room temperature and resuspended in BSG [60] to densities appropriate for the inoculation.

Acknowledgments

We thank Greg Golden for his assistance with the manuscript editing. We also thank Dr. Wei Sun for his assistance with the manipulation of F. pestis strains, and Rebin Kader, Dr. Maria Dolores Juarez-Rodriguez and Dr. Ascension Torres-Escobar for their suggestions.

Author Contributions

Conceived and designed the experiments: JS. Performed the experiments: JS. Analyzed the data: JS RC. Contributed reagents/materials/analysis tools: JS WX ZY. Wrote the paper: JS RC.

References

1. Paidhungat M, Setlow B, Driks A, Setlow P (2000) Characterization of spores of Bacillus subtilis which lack dipicolinic acid. J Bacteriol 182: 5505–5512.
2. Pavelka MS, Jr., Jacobs WR, Jr. (1996) Biosynthesis of diaminopimelate, the precursor of l-lysine and a component of peptidoglycan, is an essential function of Bacillus subtilis. J Bacteriol 178: 6966–6977.
3. Viola RE (2001) The central enzymes of the aspartate family of amino acid biosynthesis. Acc Chem Res 34: 339–349.
4. Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36: 407–427.
5. Harb OS, Abu Kwaik Y (1998) Identification of the aspartate-beta-semialdehyde dehydrogenase gene of Legionella pneumophila and characterization of a null mutant. Infect Immun 66: 1890–1903.
6. Galan JE, Nakayama K, Curtis R, 3rd (1999) Cloning and characterization of the alg gene of Salmonella typhimurium: use in stable maintenance of recombinant plasmids in Salmoilnella vaccine strains. Gene 94: 29–35.
7. Cardinaux GA, Curtis R, 3rd (1987) Nucleotide sequence of the alg gene of Shigetobaccae mutants: Identification of the promoter region and evidence for attenuator-like sequences preceding the structural gene. J Biol Chem 262: 3344–3353.
8. Vogel HG (1959) On biochemical evolution: lysine formation in higher plants. Proc Natl Acad Sci U S A 45: 1717–1721.
9. Hudson AO, Bless C, Macedo P, Chatterjee SP, Singh BK, et al. (2005) Identification and characterization of bacteriophages specific to the catfish pathogen, Edwardsiella ictaluri. Vaccine 23: 1163–1167.
10. Broad-Kiellsor LL, Portius A, Curtis R, 3rd (1997) Specific detection of Salmonella typhimurium proteins synthesized intracellularly. J Bacteriol 179: 3604–3612.
11. Cersini A, Salvia AM, Bernardini ML (1998) Intracellular multiplication and virulence of Shigella flexneri auxotrophic mutants. Infect Immun 66: 549–557.
12. Halper JE, Delong DC, Mertz ET (1957) Nutrition of salmonid fishes. V. Classification of essential amino acids for chinook salmon. J Nutr 63: 95–105.
13. Halper JE, Shanko WE (1960) Nutrition of salmonid fishes. VIII. Indispensable amino acids for sockeye salmon. J Nutr 72: 340–346.
14. Dupree HK, Halber JE (1970) Amino acids essential for growth of channel catfish, Ictalurus punctatus. Trans Am Fish Soc 99: 90–100.
15. Nose T, Arai S, Lee DL, Hashimot Y (1974) Note on amino-acids essential for growth of young carp. Bull Jap Soc Sci Fish 44: 739–742.
16. Li MH, Robinson EH (1998) Effects of supplemental lysine and methionine in low protein diets on weight gain and body composition of young channel catfish, Ictalurus punctatus. J Aquat Anim Health 10: 297–307.
17. Nakayama K, Kelly SM, Curtis R, 3rd (1988) Construction of an Acp expression-cloning vector: stable maintenance and high level expression of cloned catfish genes, including a Salmonella enterica serovar Typhimurium vaccine. J Bacteriol 170: 6496–6507.
18. Curris RL, Zhang X, Wanda SY, Kang HY, Konjufca V, et al. (2007) Induction of host immune responses using Salmonella-rectored vaccines; Brogden KA, Cowan N, Stantton TB, Zhang Q, Nolan LK, Wannemuehler MJ, eds. Washington, D.C: ASM Press.
19. Russe R, Shoomaker CA, Panagala V, Klesius PH (2009) In vitro and in vivo interaction of macrophages from vaccinated and non-vaccinated channel catfish (Ictalurus punctatus) to Edwardsiella ictaluri. Fish Shellfish Immunol 26: 545–552.
20. Arias CR, Shoomaker CA, Evans JJ, Klesius PH (2003) A comparative study of Edwardsiella ictaluri parent (EIL0) and E. ictaluri rhamycin-resistance (RE-33) isolates using lipopolysaccharides, outer membrane proteins, fatty acids, Biolog, API 20E and genomic analyses. J Fish Dis 26: 415–421.
21. Klesius PH, Shoomaker CA (1999) Development and use of modified live Edwardsiella ictaluri vaccine against enteric septicaemia of catfish. Adv Vet Med 41: 523–537.
22. Shoomaker CA, Klesius PH, Evans JJ (2007) Immunization of eyed channel catfish, Ictalurus punctatus, eggs with monovalent Flavobacterium columnare vaccine and bivalent E. ictaluri and Edwardsiella ictaluri vaccine. Vaccine 25: 1126–1131.
23. Lawrence M, Banes M, Williams M (2001) Phenotype and virulence of a transposon-derived lipopolysaccharide O side-chain mutant of Edwardsiella ictaluri. J Aquat Anim Health 13: 291–299.
47. Williams ML, Lawrence ML (2005) Identification and characterization of a two-component hemolysin from Edwardsiella ictaluri. Vet Microbiol 108: 281–289.
48. Berczi I, Bertok L, Bereznai T (1966) Comparative studies on the toxicity of Escherichia coli lipopolysaccharide endotoxin in various animal species. Can J Microbiol 12: 1070–1071.
49. Iliev DB, Roach JC, Mackenzie S, Planas JV, Goetz FW (2005) Endotoxin recognition: in fish or not in fish? FEBS Lett 579: 6519–6528.
50. Bertani G (1951) Studies on lysisogeny. I. The mode of phage liberation by lysogenic Escherichia coli. J Bacteriol 62: 293–300.
51. Gouet P, Courcelle E, Stuart DI, Metoz F (1999) ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics 15: 305–308.
52. Santander J, Wanda SY, Nickerson CA, Curtiss R, 3rd (2007) Role of RpoS in fine-tuning the synthesis of Vi capsular polysaccharide in Salmonella enterica serovar Typhi. Infect Immun 75: 1382–1392.
53. Roland K, Curtiss R, 3rd, Sizemore D (1999) Construction and evaluation of a delta cya delta crp Salmonella typhimurium strain expressing avian pathogenic Escherichia coli O78 LPS as a vaccine to prevent airsacculitis in chickens. Avian Dis 43: 429–441.
54. Chang AC, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J Bacteriol 134: 1141–1156.
55. Konjufca V, Wanda SY, Jenkins MC, Curtiss R, 3rd (2006) A recombinant attenuated Salmonella enterica serovar Typhimurium vaccine encoding Eimeria acervulina antigen offers protection against E. acervulina challenge. Infect Immun 74: 6785–6796.
56. Sambrook J, Russell W (2001) Molecular Cloning: A Laboratory Manual. Editor: Cold Spring Harbor Press.
57. Branger CG, Sun W, Torres-Escobar A, Perry R, Roland KL, et al. (2010) Evaluation of Pan, HmaR and a modified LcrV protein delivered to mice by live attenuated Salmonella as a vaccine against bubonic and pneumonic Yersinia pestis challenge. Vaccine In Press. Available online 24 October 2010.
58. Witholt B, Bockhout M, Brock M, Kingma J, V Heerikhuizen H, et al. (1976) An efficient and reproducible procedure for the formation of spheroplasts from variously grown Escherichia coli. Anal Biochem 74: 160–170.
59. Curtiss R, III (1963) Chromosomal aberrations associated with mutations to bacteriophage resistance in Escherichia coli. J Bacteriol 89: 28–40.
60. Anderson DP (1984) Fish Immunology. In: Hua SZA, ed. Fish Immunology. Beijing: China Agriculture Press. pp 110–113.