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An Aromatic-Dependent Mutant of the Fish Pathogen *Aeromonas salmonicida* Is Attenuated in Fish and Is Effective as a Live Vaccine against the Salmonid Disease Furunculosis

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***Aeromonas salmonicida* is the etiological agent of furunculosis in salmonid fish. The disease is responsible for severe economic losses in intensively cultured salmon and trout. Bacterin vaccines provide inadequate protection against infection. We have constructed an aromatic-dependent mutant of *A. salmonicida* in order to investigate the possibility of an effective live-attenuated vaccine. The *aroA* gene of *A. salmonicida* was cloned in *Escherichia coli*, and the nucleotide sequence was determined. The codon usage pattern of *aroA* was found to be quite distinct from that of the *vapA* gene coding for the surface array protein layer (A layer). The *aroA* gene was inactivated by inserting a fragment expressing kanamycin resistance within the coding sequence. The *aroA*::Ka^r mutation was introduced into the chromosome of virulent *A. salmonicida* 644Rb and 640V2 by allele replacement by using a suicide plasmid delivery system. The *aroA* mutation did not revert at a detectable frequency ($<10^{-11}$). The mutation resulted in attenuation when bacteria were injected intramuscularly into Atlantic salmon (*Salmo salar* L.). Introduction of the wild-type *aroA* gene into the *A. salmonicida* mutants on a broad-host-range plasmid restored virulence. *A. salmonicida* mutant 644Rb *aroA*::Ka^r persisted in the kidney of brown trout (*Salmo trutta* L.) for 12 days at 10°C. Vaccination of brown trout with 10^7 CFU of *A. salmonicida* 644Rb *aroA* by intraperitoneal injection resulted in a 253-fold increase in the 50% lethal dose (LD_{50}) compared with unvaccinated controls challenged with a virulent clinical isolate 9 weeks later. A second vaccination after 6 weeks increased the LD_{50} by a further 16-fold.**

Aeromonas salmonicida is a gram-negative, nonmotile, psychrophilic bacterium which causes furunculosis, an economically important disease of salmonid fish. The disease can affect a wide variety of both farmed and feral teleost fish, but the principal concern is in the intensive culture of salmonids in which the organism can inflict severe losses (7).

Clinical furunculosis is generally manifested as a hemmorhagic septicemia with tissue necrosis and splenomegaly (24, 29). Disease symptoms can be induced in healthy fish by the injection of purified extracellular products (11). A number of potential virulence factors have been identified (10). These include a glycerophospholipid-cholesterol acyltransferase, which is the major lethal toxin of *A. salmonicida* (25); a 70-kDa serine protease (40) which can cause muscle liquefaction; and a surface array protein layer (A layer) which may function as an adhesin and protects the bacterium from killing by preimmune and immune serum (32, 36, 38).

Despite early successful attempts at vaccination (8), the disease continues to pose a major threat to the fish-farming industry. Recent vaccines have been bacterin preparations (reviewed by Austin and Austin [1] and Hastings [15]), but the efficacy of these preparations has been poor. The nature of the protective antigens remains to be established. The apparent lack of correlation between antibody and protection (30) suggests that cell-mediated immunity may be important.

It is well established that live vaccines stimulate cell-mediated immunity better than do killed whole-cell vaccines. Aromatic-dependent mutants of the following pathogens have been shown to be avirulent and to stimulate protective immunity: *Salmonella typhimurium* (17), *Salmonella typhi* (34), *Salmonella choleraesuis* (33), *Shigella flexneri* (53), *Bordetella pertussis* (42), *Yersinia enterocolitica* (4), and *Bacillus anthracis* (21). *aro* mutants are attenuated because they prevent biosynthesis of *p*-aminobenzoic acid, which is required for folate biosynthesis. The bacteria fail to grow in tissue because of the absence of *p*-aminobenzoic acid and because exogenous folate cannot be taken up.

Cipriano and Starliper (6) reported protection of brook trout and Atlantic salmon with an attenuated strain obtained by repeated laboratory subculture. Recently, Thornton et al. (52) isolated a slowly growing, aminoglycoside-resistant mutant and a rapidly growing pseudorevertant of *A. salmonicida*. Both strains were avirulent, and the pseudorevertant strain conferred protection against challenge when administered by intraperitoneal injection or immersion. Thus, it seems likely that protection can be induced by a live-attenuated vaccine.

Here we describe construction of an aromatic-dependent mutant of *A. salmonicida* and experiments to test its potential as a vaccine to protect fish from experimentally induced furunculosis. The *aroA* gene was cloned and sequenced, and an *aroA* mutant of *A. salmonicida* was constructed by allelic replacement. The *aroA* mutant was shown to be attenuated in both Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.) and to stimulate protective immunity in brown trout.

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TABLE 1. Bacterial strains

Organism and strain	Relevant characteristic	Comment	Source or reference
A. salmonicida			
SPUP1	Nal^r	Virulent, A$^+$ strain isolated from hatchery disease outbreak	Fish disease group, University College, Galway, Ireland
644Rb	Nal^r	Virulent, A$^+$ strain isolated from clinical disease outbreak (in vivo passaged)	Fish disease group, University College, Galway, Ireland
640V2	Nal^r	Virulent, A$^+$ strain isolated from clinical disease outbreak (in vivo passaged)	Fish disease group, University College, Galway, Ireland
SPUP1/M8	AroA$^-$	*aroA*::Ka^r mutant	This study
644Rb/M1	AroA$^-$	*aroA*::Ka^r mutant	This study
644Rb/M2	AroA$^-$	*aroA*::Ka^r mutant	This study
640V2/M1	AroA$^-$	*aroA*::Ka^r mutant	This study
640V2/M2	AroA$^-$	*aroA*::Ka^r mutant	This study
7222V	A layer$^-$	A-layer-deficient mutant	Fish disease group, University College, Galway, Ireland
E. coli			
WL95	P2 lysogen	λ Spi$^-$ selection	26
TB1	Δ*lac-proAB* (φ80*lacZ*ΔM15) *hsdR rpsL*	Host for pUC plasmids	54
AB1321	*proA2 aroA2 his4 thi1 lacY1*		51
S17-1	RP4 2-Tc::Mu Km::Tn7 Tra$^+$ *recA* Tp^r Sm^r	Mobilizing donor for conjugation	48
71-18	Δ*lac-proAB* (F′ *proAB lacI^q*ZΔM15)	M13 propagation	54

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The properties of the bacterial strains and plasmids are listed in Tables 1 and 2.

Bacteriological media. *Escherichia coli* strains were grown in L broth (LB) or on L agar (LA) (43). *A. salmonicida* strains were grown in brain heart infusion (BHI; Difco) broth or Trypticase soy agar or broth (TSA or TSB; Difco). The following antibiotics were used at the indicated concentrations: for *E. coli*, ampicillin (100 μg/ml), tetracycline (10 μg/ml), chloramphenicol (20 μg/ml), and kanamycin (40 μg/ml); for *A. salmonicida*, nalidixic acid (30 μg/ml), tetracycline (2 μg/ml), ampicillin (15 μg/ml), chloramphenicol (3 μg/ml), and kanamycin (40 μg/ml).

M9 minimal media (43) was used for *E. coli*. Fe_2SO_4 (10 μg/ml) was added to the medium for the growth of strains AB1321 and AB1360, as was *p*-aminobenzoic acid (10 μg/ml), where appropriate.

A. salmonicida strains were grown on defined media to test for the Aro$^-$ phenotype. It consisted of M9 salts (43), glucose, $MgSO_4$, and $CaCl_2$ at the concentrations used in the *E. coli* minimal medium with the addition of *Aeromonas* salts (14) to 1% (vol/vol). All amino acids except the aromatic amino acids tryptophan, phenylalanine, and tyrosine were added to 100 μg/ml to form the aromatic compound-deficient medium. Complete defined medium contained 10 μg of *p*-aminobenzoic acid per ml and 100 μg of the aromatic amino acids per ml. Alternatively, a semidefined medium was used when amino acids were provided in the form of acid-hydrolyzed casein (1% [wt/vol]; Difco), which lacks tryptophan.

Attempts were made to select Aro$^+$ revertants of *A. salmonicida aroA*::Ka^r by growth in liquid minimal medium without aromatic supplements. The mutant was grown in 100 ml of BHI broth to the stationary phase. Cells were washed in sterile saline and were then inoculated into 1 liter of *Aeromonas* liquid minimal medium (as described above but without agar) and shaken (150 rpm) at 22°C for 21 days.

TABLE 2. Plasmids

Plasmid	Relevant characteristic	Comment	Source or reference
pBR322	Ap^r Tc^r	Cloning vector	3
pUC18	Ap^r	Cloning vector, X-Gal selection[a]	54
pSUP202	Tc^r Ap^r Cm^r ColE1 *ori* Mob$^+$	Broad-host-range mobilizable suicide vector	48
pGSS33	Ap^r Tc^r Cm^r Sm^r IncQ Tra$^+$ Mob$^+$	Broad-host-range mobilizable vector	47
pAA2	AroA$^+$ Ap^r	*Pst*I *aroA* fragment in pUC18	This study
pAA1	AroA$^+$ Ap^r Tc^r	*Eco*RI *aroA* fragment in pBR322	This study
pGSSA2	Tc^r Cm^r Sm^r	*Pst*I *aroA* fragment in pGSS33	This study
pSUP202A2	Tc^r Cm^r	*Pst*I *aroA* fragment in pSUP202	This study
pSUP202ΔA2	Tc^r Cm^r	Deletion of *aroA Eco*RI fragment from pSUP202A2	This study
pAAK2	Tc^r Ap^r Ka^r AroA$^-$	*aroA*::Ka^r in pAA1	This study
pSUP202K2	Tc^r Ap^r Ka^r AroA$^-$	*Eco*RI fragment with *aroA*::Ka^r in pSUP202	This study
pSUP202K3	Tc^r Cm^r Ka^r AroA$^-$	*Pst*I fragment with *aroA*::Ka^r in pSUP202	This study

[a] X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Samples were removed at 3-day intervals and tested for the presence of bacteria with the ability to grow on aromatic-deficient minimal agar.

Congo red agar (20) comprised TSA with 30 μg of Congo red (Sigma) per ml. Protease production was detected by incorporating 1% skimmed milk into TSA. Hemolysin production was detected on TSA containing 5% horse erythrocytes.

DNA manipulations. Restriction endonucleases, the DNA polymerase I Klenow fragment, and T4 DNA ligase were obtained from the Boehringer Corporation or Promega and were used according to the recommendations of the manufacturers. Sequencing-grade *Taq* polymerase was purchased from Promega, and T7 DNA polymerase (Sequenase) was obtained from United States Biochemicals. Standard procedures were used for molecular cloning and DNA hybridization (2, 43). DNA probes were prepared by nick translation with [α-^{32}P]dATP (Amersham).

Preparation of *A. salmonicida* genomic DNA. A 5-ml culture of *A. salmonicida* was grown statically for 48 h in TSB at 22°C, and 0.2 ml was inoculated into 100 ml of fresh TSB in a 250-ml Erlenmeyer flask, which was incubated with shaking (200 rpm) for 28 h at 22°C. The cells were harvested at 10,000 × *g* for 10 min and washed twice in 10 mM Tris-HCl–25 mM EDTA–150 mM NaCl (pH 7.5) (TE_1). The pellet was freeze-thawed twice at −20°C, washed again in TE_1, resuspended in 2 ml of 1.0 M NaCl, and left on ice for 1 h with intermittent vortexing. The cells were pelleted at 10,000 × *g* for 10 min and resuspended in 0.5 ml of 10 mM Tris-HCl–25 mM EDTA (pH 7.5) (TE_2) containing lysozyme (Sigma) at 5 mg/ml. The cells were left on ice for 20 min. The viscous lysate was diluted by the addition of 1 ml of TE_2, and the DNA was precipitated by the addition of 2 volumes of cold (−20°C) ethanol. The DNA was spooled, dissolved in 5 ml of TE_2, reprecipitated, and dissolved in TE_2. It was extracted twice with Tris-saturated phenol and then subjected to repeated phenol-chloroform extractions. The DNA was precipitated with ethanol, spooled, washed twice in 70% alcohol, and dissolved in 2 to 3 ml of 10 mM Tris-HCl–1 mM EDTA (pH 8.0).

Construction and screening of an *A. salmonicida* gene library. *A. salmonicida* chromosomal DNA was partially digested with *Sau*3AI and size fractionated on sucrose gradients (43), and 15- to 20-kb fragments were ligated to *Bam*HI-digested λ2001 vector DNA (22). The ligated DNA was then packaged in vitro (43). Phage particles were propagated on *E. coli* WL95 to select for recombinants (26). λ recombinants carrying the *aroA* gene of *A. salmonicida* were identified by infecting *E. coli* AB1321*aroA* and selecting for the growth of complementing plaques on minimal agar without aromatic supplements. A high-titered stock of one λ*aroA* recombinant phage was prepared, and phage particles were purified by sedimentation in CsCl density gradients. Phage DNA was isolated by formamide extraction (37).

Subcloning the *aroA* gene. λ*aroA* contained an insert of ca. 20 kb. Fragments were subcloned into plasmid vectors and transformed into *E. coli* AB1321*aroA*. A 4.3-kb *Pst*I fragment which complemented the *aroA* mutation of AB1321 was cloned in pUC18, forming pAA2, and a 2.0-kb *Eco*RI fragment was cloned in pBR322, forming pAA1 (Fig. 1). The 4.3-kb *Pst*I fragment was also subcloned into the broad-host-range vector pGSS33 (47). This plasmid, designated pGSSA2, was stably inherited in both *E. coli* and *A. salmonicida*.

Construction of *aroA*::Ka^r. In order to isolate an Aro$^-$ mutant of *A. salmonicida*, a kanamycin resistance determinant was inserted into the coding sequence of the *aroA* gene. A *SalI-Xho*I fragment from Tn*5* was ligated into the unique *Sal*I site in the *aroA* fragment of pAA1 (Fig. 1). Recombinants were selected on agar containing tetracycline and kanamycin, and one, designated pAAK2, no longer complemented the *aroA* mutation of *E. coli* AB1321, indicating that the *aroA* gene had been inactivated.

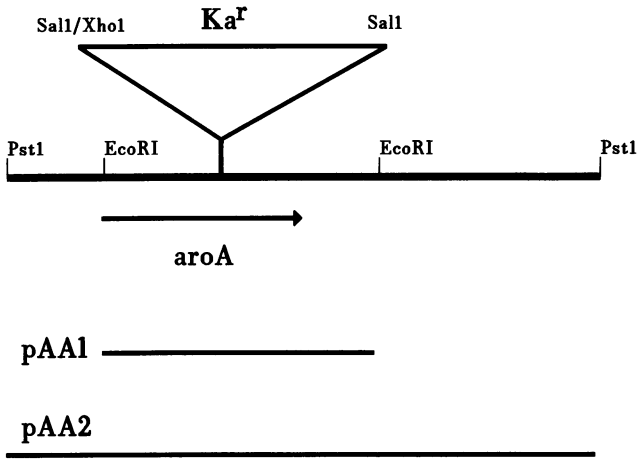

FIG. 1. Restriction map of *aroA* locus. The thick line represents the 4.3-kb *Pst*I chromosomal fragment harboring the *aroA* gene. The arrow indicates the direction of transcription of the *aroA* gene and the extent of the coding region. The kanamycin fragment inserted into the coding sequence in mutant constructions (see the text) is indicated by the open triangle. The lines at the bottom indicate the extent of chromosomal sequences cloned in plasmids pAA1 and pAA2.

An *aroA* mutation with longer flanking sequences was constructed in plasmid vector pSUP202. The 4.3-kb *aroA*-containing *Pst*I fragment from pAA2 was subcloned into the unique *Pst*I site of pSUP202, forming plasmid pSUP202A2 (Tc^r Cm^r Ap^s AroA$^+$). The 2-kb *Eco*RI fragment of pSUP202A2 carrying *aroA* was deleted, forming pSUP202ΔA2 (Tc^r Cm^r Ap^s).

The deleted 2.0-kb *Eco*RI *aroA* fragment of pSUP202ΔA2 was replaced by the 4.2-kb *aroA*::Ka^r *Eco*RI fragment from pAAK2 by cleavage with *Eco*RI, ligation, and selection for Ka^r Cm^r transformants. One recombinant containing the *aroA*::Ka^r fragment in the correct orientation with respect to flanking chromosomal sequences was named pSUP202K3.

DNA sequencing. Chain termination reactions were carried out by using the Klenow fragment of DNA polymerase I, T7 DNA polymerase, and *Taq* polymerase. Template DNA consisted of single-stranded DNA prepared from phages M13 tg130 or M13 tg131 (23, 31) or was denatured double-stranded plasmid DNA (18). Oligonucleotides were made with an Applied Biosystems PCR Mate oligonucleotide synthesizer. Multiple alignments of protein sequences were generated by using the CLUSTAL alignment program (16).

Bacterial conjugation. The *E. coli* strains were grown for 18 h in LB at 37°C with shaking, and *A. salmonicida* was grown statically in TSB for 3 days at 22°C. The *E. coli* culture (20 μl) and the *A. salmonicida* culture (100 μl) were mixed on the surface of a sterile 0.45-μm-pore-size filter (Millipore) placed on a dried LA plate and incubated at 30°C for 4 h. The filter was placed in 1 ml of saline, and the bacteria were resuspended by vortexing. Dilutions were plated on TSA containing antibiotics and incubated at 22°C for up to 4 days.

Vaccination and LD_{50} determinations. Atlantic salmon (*Salmo salar* L.) or brown trout (*Salmo trutta* L.) weighing between 10 and 100 g were kept in freely flowing dechlorinated tap water at ambient temperature (range, 5 to 14°C), except for the experiment for *A. salmonicida* persistence in the kidney, for which the water was maintained at 10°C with immersible heaters. Fish were anesthetized in a solution of benzocaine (55 ppm) before intraperitoneal injection with 0.1 ml of an unwashed culture of *A. salmonicida aroA* grown in BHI broth (10^7 cells). Groups of four or five fish were challenged by intramuscular injection of dilutions of cultures of a virulent strain. The 50% lethal dose (LD_{50}) determinations were calculated according to the method of Reed and Muench (41).

In vivo passage of *A. salmonicida*. *A. salmonicida* strains were passaged in fish to increase virulence. Two colonies from a TSA plate were emulsified in 0.5 ml of phosphate-buffered saline (PBS), and 0.1 ml was injected intramuscularly into a group of five Atlantic salmon or brown trout. *A. salmonicida* was isolated from the kidneys of dead fish by streaking on a TSA plate. This was repeated four to six times until the time to death was 2 days.

Serum antibody measurements. Blood was removed from the caudal vein, and the serum was diluted serially twofold in PBS. An equal volume of formalin-killed cells of the A-layer-deficient mutant 7222V was added and incubated overnight at 22°C. The titer was recorded as the reciprocal of the last dilution which caused agglutination. Antibody titer measurements were performed for pooled sera from groups of five 10- to 15-g fish.

Nucleotide sequence accession number. The *aroA* coding sequence has been submitted to the GenBank data base under accession number L05002.

RESULTS

Cloning the *aroA* gene of *A. salmonicida*. A library of *A. salmonicida* genomic DNA was constructed in λ2001. Recombinants that could complement the *aroA* mutation in *E. coli* AB1321 were selected. One complementing phage, λ*aroA*, contained an insert of ca. 20 kb. Two fragments which complemented the *aroA* mutation of AB1321 were subcloned from λ*aroA*: a 2.0-kb *Eco*RI fragment in pBR322 (pAA1) and a 4.3-kb *Pst*I fragment in pUC18 (pAA2). Restriction mapping indicated that the *Eco*RI fragment in pAA1 was contained within the *Pst*I fragment of pAA2 (Fig. 1).

Nucleotide sequencing of the *aroA* gene. The DNA sequence of the 2.0-kb *Eco*RI fragment of pAA1 was obtained (Fig. 2). The complete open reading frame is not entirely contained within the *Eco*RI fragment. However, it is very likely that the ATG codon partly overlaps the GAATTC *Eco*RI site (see the legend to Fig. 3).

The predicted amino acid sequence of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase; EC 2.5.1.19) enzyme encoded by the *aroA* gene of *A. salmonicida* was deduced and aligned with the EPSP synthases of other gram-negative bacteria by using the CLUSTAL multiple alignment program (Fig. 3). The EPSP synthases were found to be highly conserved, with 37.6% residue identities.

The codon usage pattern of the *A. salmonicida* EPSP synthase is given in Table 3. Also included is the recently reported sequence of the *A. salmonicida* A-layer protein (encoded by *vapA*) (5). The two genes have rather different codon usage patterns. The G+C content of *aroA* (60 mol%) is much higher than that of *vapA* (49 mol%). The difference is most pronounced at the third positions of codons where *aroA* is similar to that of genes from the closely related species *Aeromonas hydrophila* (5).

Construction of *A. salmonicida aroA*::Ka^r. In order to isolate an Aro^- mutant of *A. salmonicida* by allelic replacement, the *aroA* gene was inactivated by inserting the kanamycin resistance marker of Tn*5* at the *Sal*I site of pAA1, forming pAAK2. This plasmid failed to complement the *aroA* mutation in *E. coli* AB1321. The *aroA*::Ka^r region of pAAK2 was cloned on a 4.2-kb *Eco*RI fragment into the *Eco*RI site of the broad-host-range suicide vector pSUP202, forming pSUP202K2, which was transformed into the *E. coli* S17-1. Filter matings were then performed between *E. coli* S17-1 (pSUP202K2) and *A. salmonicida* SPUP1. Transconjugants selected on agar containing nalidixic acid and kanamycin occurred at a frequency of 10^{-2} per recipient. The majority of Ka^r transconjugants (85%) were Aro^+, while 15% were Aro^-. Southern hybridization analysis of representative Aro^+ and Aro^- transconjugants demonstrated that both classes arose following integration of the entire pSUP202K2 plasmid into the chromosome at the *aroA* locus (Fig. 4a). Chromosomal DNA was digested with *Cla*I and hybridized to plasmid pAA1 labelled with ^{32}P. This detected two differently sized bands in the Aro^- and Aro^+ isolates. The *aroA* gene is located on a 3.5-kb *Cla*I fragment which contains within it the 2.0-kb *Eco*RI *aroA* fragment. Thus, the predicted structure for the wild-type or mutated *aroA* locus is a single hybridizing *Cla*I fragment. The probe is also homologous to the suicide plasmid pSUP202K2 which contains a single *Cla*I recognition site. The detection of two hybridizing *Cla*I fragments is consistent with the integration of the entire pSUP202K2 plasmid into the wild-type *aroA* locus, thereby introducing a novel *Cla*I site in this region of the chromosome. Thus, the two hybridizing bands detected in the Aro^- and Aro^+ isolates correspond to the two junction fragments generated by the integration event. The nature of the integration event also permits an explanation of the observed phenotype of these strains. The 5′ *Eco*RI site is located within the coding sequence of the *aroA* gene (Fig. 2). Integrants resulting from a crossover 5′ to the insertion have an Aro^- phenotype because the *aroA* gene becomes detached from its natural promoter. Integrants resulting from a crossover 3′ to the insertion have an Aro^+ phenotype because the wild-type *aroA* gene remains attached to its natural promoter. Precise plasmid excision, which presumably occurred by a crossover on the same side of the insertion, resulting in restoration of the Aro^+ phenotype, occurred at a frequency of 10^{-7}. It was not possible to isolate derivatives of these Aro^- integrants when the plasmid had been eliminated by a crossover on the other side of the insertion (allelic replacement) to form Aro^- Ka^r Tc^s Ap^s colonies. This could be explained by the low frequency of the second recombination event.

Construction of *aroA*::Ka^r cassette with longer flanking sequences. In order to construct a suicide plasmid with longer chromosomal sequences flanking the Ka^r insert, the 4.3-kb *Pst*I *aroA* fragment from pAA2 was cloned in the *Pst*I site of pSUP202, forming pSUP202A2. The 4.2-kb *Eco*RI fragment containing *aroA*::Ka^r from pSUP202K2 was then inserted in place of the 2.0-kb *Eco*RI fragment of pSUP202A2 carrying the wild-type *aroA* gene, forming plasmid pSUP202K3 (*aroA*::Ka^r).

Isolation of *A. salmonicida aroA*::Ka^r by allelic replacement. The suicide plasmid pSUP202K3 was mobilized from *E. coli* S17-1 into *A. salmonicida* SPUP1 (Nal^r) at a frequency of 10^{-2} per recipient. Approximately 5% of Ka^r transcon-

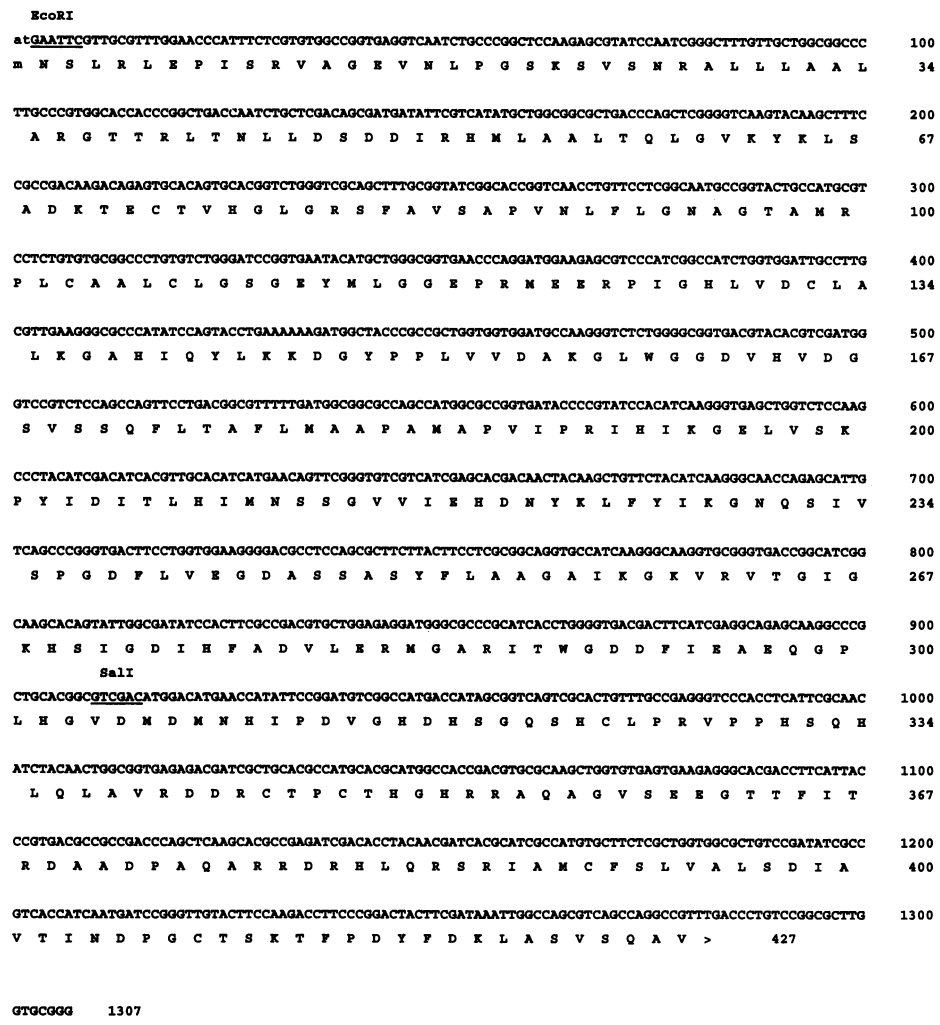

FIG. 2. Nucleotide and deduced amino acid sequence of the *aroA* gene of *A. salmonicida*. The nucleotide sequence of the *aroA* gene is presented in the top line, with the deduced amino acid sequence below it in the one-letter amino acid code. Note that the existence of the methionine start codon has not been determined but is included here directly preceding the codon for Asn for the reasons described in the legend to Fig. 3. >, a translational stop codon. The *Eco*RI and *Sal*I sites are underlined.

jugants were both Aro$^-$ and were susceptible to tetracycline and to chloramphenicol, the expected phenotype of cells in which allele replacement has occurred at the *aroA* locus. This was confirmed by Southern hybridization analysis (Fig. 4b). Genomic DNA from wild-type and mutant cells was cleaved separately with *Eco*RI and *Cla*I and transferred to nitrocellulose. The membrane was hybridized with pAA1 (which carries the wild-type *aroA* gene and also hybridizes to pSUP202) labelled by nick translation. A single hybridizing band occurred in all lanes, with that of the Aro$^-$ mutants being 2.2 kb longer than the corresponding band from the wild type (Fig. 4b). This corresponds to the size of the *Sal*I-*Xho*I fragment inserted in the *aroA* gene in plasmid pSUP202K2. No bands corresponding to pSUP202 sequences were seen, demonstrating that the plasmid had been eliminated.

Reversion tests performed with *A. salmonicida aroA*::Ka^r failed to reveal any Aro$^+$ revertants among 10^{11} bacteria. This was in contrast to the aforementioned Aro$^-$ integrants which reverted at a frequency of 10^{-7}. Mutants grew normally on minimal agar to which aromatic supplements had been added.

Introduction of the *aroA*::Ka^r mutation into virulent strains of *A. salmonicida*. *A. salmonicida* SPUP1, which was used in preliminary allele replacement experiments, was found to have lost virulence for brown trout (both the wild type and mutant derivatives failed to cause disease when 10^6 CFU were injected intramuscularly). Accordingly, the *aroA*::Ka^r mutation was transferred into freshly passaged, highly virulent strains 644Rb and 640V2. The mutants were isolated as described above by using the minimum number of laboratory subcultures and were then stored at −70°C. The frequency of transfer of pSUP202K3 and the frequency of isolation of bona fide allelic replacement recombinants were the same as those for strain SPUP1. The structure of chromosomal mutants was verified by Southern hybridization (data not shown). Wild-type and mutant strains were indistinguishable when grown on nutrient agar or in broth. Their growth rates in BHI broth were identical, and mutant and wild-type bacteria expressed the same levels of extracellular hemoly-

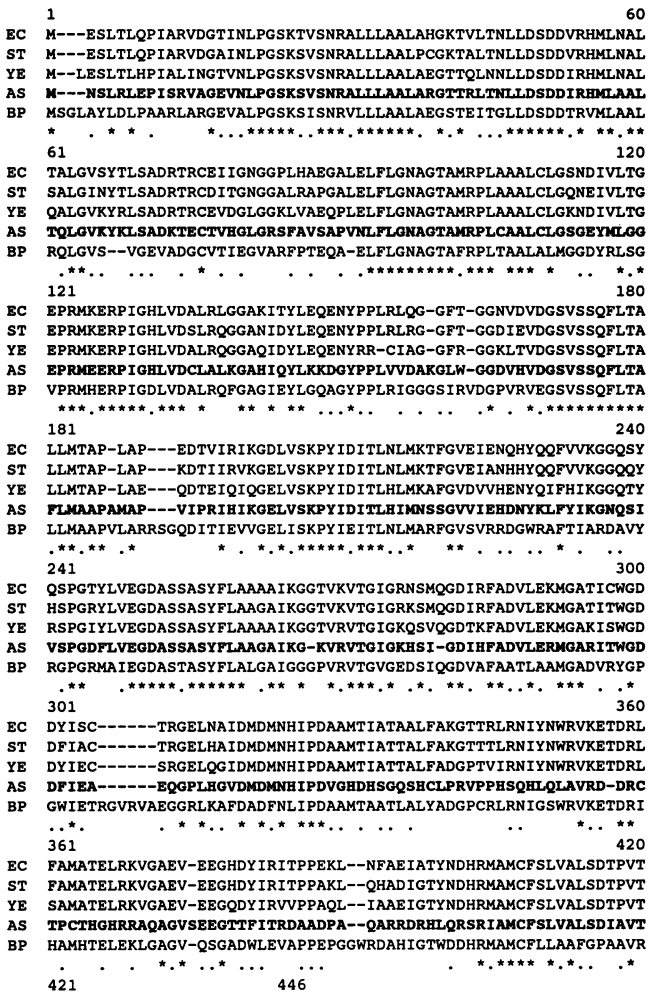

FIG. 3. CLUSTAL computer alignment of the protein sequences encoded by the *aroA* genes of gram-negative bacteria. Amino acid residues conserved across all species are indicated by an asterisk, with conservative substitutions indicated by dots. Gaps (–) are introduced to produce the best alignment. The numbering system does not correspond to any individual amino acid position in original sequences but simply refers to the amino acids' positions in the alignment. The *A. salmonicida* sequence is indicated in boldface type. The methionine start codon has not been identified but is included in the position shown on the basis of a consideration of the length and sequence of the other gram-negative proteins. The other available gram-negative sequence from *Salmonella gallinarum* (13) is not shown in the figure but is identical to the *S. typhimurium* sequence, with the exception of four conservative amino acid substitutions in nonconserved regions. The position of these differences is indicated on the *S. typhimurium* sequence in boldface type. The species from which the sequence is derived are indicated at the left of the figure: EC, *E. coli* (9); ST, *S. typhimurium* (49); YE, *Y. enterocolitica* (35); AS, *A. salmonicida* (this study); and BP, *B. pertussis* (27).

sin and protease. The *aroA* mutants also produced pigment and retained the A-layer surface array protein as determined by the Congo red binding assay and their ability to autoaggregate in broth.

Virulence of *A. salmonicida aroA*. Groups of four Atlantic salmon parr (10 to 15 g) were injected intramuscularly with 10^6 cells of the wild-type strains 640V2 and 644Rb and their isogenic Aro$^-$ mutants. All fish injected with the wild-type strains died within 3 days, while none of the fish receiving the Aro$^-$ mutants died or showed any signs of illness over a 14-day period. Thus, salmon parr were completely resistant to challenge with mutant bacteria, whereas the wild-type cells subjected to the same number of laboratory subcultures were fully virulent. In order to demonstrate that the observed attenuation was due exclusively to the *aroA* mutation, the wild-type *aroA* gene was transferred into *A. salmonicida* 644Rb *aroA*::Ka^r on a broad-host-range plasmid, pGSSA2. An inoculum of 10^6 CFU killed four fish when injected intramuscularly, and bacteria recovered from the kidney of the dead fish had the expected phenotype (Aro$^+$ Ka^r Cm^r Tc^r Sm^r). This showed that the attenuation of Aro$^-$ recombinants was not the result of laboratory subculture.

Persistence of *A. salmonicida* 644Rb *aroA* in vivo. Forty brown trout (100 g) were maintained at 10°C and injected intramuscularly with 3.4×10^7 bacteria. Groups of four fish were sacrificed at 2-day intervals, and viable counts of bacteria recovered from the kidneys were determined (Fig. 5). Bacteria were detected up to approximately 12 days postinjection and were no longer detectable after 14 days.

Protection experiments with *A. salmonicida* 644Rb *aroA*. Brown trout were injected intraperitoneally with 5.4×10^7 CFU of 644Rb *aroA*::Ka^r. The agglutinating antibody titer was shown to rise from <2 to 128 at 6 weeks after injection. A challenge experiment was performed on a group of fish 6 weeks after vaccination by injection with doses of virulent bacteria ranging from 5×10^4 to 5×10^1 CFU. Only two mortalities were recorded at the highest inoculum, suggesting an LD_{50} of about 10^4. The LD_{50} of this challenge strain for unvaccinated fish (measured at week 9) was 7.2×10^1 CFU. Some of the vaccinated fish received a second intraperitoneal injection of the Aro$^-$ mutant (10^7 CFU) 6 weeks after the first inoculation. After a further 3 weeks, challenge experiments were performed to determine the LD_{50} for vaccinated and unvaccinated fish. The LD_{50} for unvaccinated fish was 7.1×10^1. In contrast, the LD_{50} was 1.8×10^4 in fish vaccinated once and 3×10^5 in fish vaccinated twice. This demonstrates that vaccination of brown trout with Aro$^-$ *A. salmonicida* confers substantial protection against challenge with a highly virulent strain.

DISCUSSION

This paper describes the isolation of an insertion mutation in the *aroA* gene of *A. salmonicida*. The first *aroA*::Ka^r construct in the suicide vector pSUP202 failed to yield the desired double recombinants. Instead, all the Ka^r colonies tested had arisen by a single crossover that resulted in plasmid integration. Because the *Eco*RI fragment carrying *aroA* lacked the promoter, ribosome binding site, and ATG codon, integration events resulting from a crossover in *aroA* sequences 5′ to Ka^r insertion were Aro$^-$, whereas those that resulted from recombination 3′ to the insertion were Aro$^+$. Attempts to isolate derivatives of the pSUP202K2 integrants which were Aro$^-$ by excision of the plasmid failed. The fact that *aroA*::Ka^r double recombinants were readily isolated with pSUP202K3 when the Ka^r insert is flanked by longer stretches of homologous chromosomal DNA suggests that the recombination frequency is determined, at least in part, by the length of flanking sequences.

The expression of the *aroA* gene in pAA1 as determined

TABLE 3. Codon usage in *A. salmonicida*

Amino acid	Codon	Codon usage in[a]: *aroA* n	*aroA* RSCU	*vapA* n	*vapA* RSCU	Amino acid	Codon	Codon usage in[a]: *aroA* n	*aroA* RSCU	*vapA* n	*vapA* RSCU
Phe	UUU	2	0.31	10	0.74	Ser	UCU[b]	2	0.38	11	2.44
Phe	UUC	11	1.69	17	1.26	Ser	UCC	10	1.88	8	1.78
Leu	UUA	0	0.00	1	0.13	Ser	UCA	1	0.19	0	0.00
Leu	UUG	9	1.26	9	1.17	Ser	UCG	6	1.13	0	0.00
Leu	CUU	3	0.42	5	0.65	Pro	CCU	2	0.36	4	1.23
Leu	CUC	5	0.70	4	0.52	Pro	CCC	6	1.09	0	0.00
Leu	CUA	2	0.28	0	0.00	Pro	CCA	4	0.73	1	0.31
Leu	CUG	24	3.35	27	3.52	Pro	CCG	10	1.82	8	2.46
Ile	AUU	6	0.75	11	1.27	Thr	ACU[b]	2	0.42	23	1.92
Ile	AUC	17	2.13	15	1.73	Thr	ACC	10	2.11	19	1.58
Ile	AUA	1	0.13	0	0.00	Thr	ACA	2	0.42	6	0.50
Met	AUG	12		3		Thr	ACG	5	1.05	0	0.00
Val	GUU[b]	1	0.13	24	2.23	Ala	GCU[b]	4	0.38	42	2.63
Val	GUC	14	1.81	3	0.28	Ala	GCC	20	1.90	8	0.50
Val	GUA	3	0.39	10	0.93	Ala	GCA	4	0.38	10	0.63
Val	GUG	13	1.68	6	0.56	Ala	GCG	14	1.33	4	0.25
Tyr	UAU	0	0.00	2	0.80	Cys	UGU	4	0.89	0	1.00
Tyr	UAC	9	2.00	3	1.20	Cys	UGC	5	1.11	0	1.00
Ter	UAA	0		1		Ter	UGA	0		0	
Ter	UAG	0		0		Trp	UGG	2		4	
His	CAU	9	0.90	2	1.00	Arg	CGU	9	2.16	10	4.29
His	CAC	11	1.10	2	1.00	Arg	CGC	5	1.20	2	0.86
Gln	CAA	6	1.00	11	1.16	Arg	CGA	4	0.96	0	0.00
Gln	CAG	6	1.00	8	0.84	Arg	CGG	3	0.72	1	0.43
Asn	AAU	6	1.09	14	0.72	Ser	AGU	3	0.56	2	0.44
Asn	AAC	5	0.91	25	1.28	Ser	AGC	10	1.88	6	1.33
Lys	AAA	3	0.35	8	0.59	Arg	AGA	1	0.24	0	0.00
Lys	AAG	14	1.65	19	1.41	Arg	AGG	3	0.72	1	0.43
Asp	GAU	13	0.87	19	1.27	Gly	GGU[b]	16	1.64	35	3.18
Asp	GAC	17	1.13	11	0.73	Gly	GGC	19	1.95	7	0.64
Glu	GAA[b]	6	0.80	15	1.58	Gly	GGA	1	0.10	1	0.09
Glu	GAG	9	1.20	4	0.42	Gly	GGG	3	0.31	1	0.09

[a] The usage of each codon is presented as the number of occurrences (*n*) and as the relative synonymous codon usage (RSCU); the latter was calculated as the number of codons observed divided by the number expected if all codons for an amino acid were used equally.
[b] Codon appearing at a significantly higher frequency in *vapA* than in *aroA*; significance assessed by the chi-square test ($P < 0.025$).

by complementation of the *aroA* mutation in *E. coli* AB1321 is somewhat surprising because the *Eco*RI fragment is in the wrong orientation to be expressed from the *tetR* promoter, which transcribes across the *Eco*RI site in pBR322 (50). There are no documented promoters that transcribe across the *Eco*RI site in a clockwise direction (50). However, some transcription must occur to generate sufficient levels of EPSP synthase to complement the *aroA* defect in *E. coli* AB1321. The ribosome binding site and ATG initiation codon are also absent. Low levels of translation initiation must be occurring in adjacent plasmid sequences or internally in the gene, but it is not obvious from examination of the sequence how this happens.

Chu et al. (5) have recently reported the sequence of the *vapA* structural gene encoding the surface array protein which forms the A layer. Interestingly, the codon usage patterns (Table 3) and moles percent G+C content of the two genes are quite different; the G+C content of *aroA* at the third position of codons (72 mol%) is much higher than that of *vapA* (49 mol%). The base composition and codon usage of *aroA* are similar to those of genes from the closely related species *A. hydrophila* (5) and are as might be expected for a lowly expressed gene from a somewhat-G+C-rich genome (the *A. salmonicida* genomic G+C content is 55 mol%). Thus, the codon usage of *vapA* requires explanation. In other instances in which a gene has been found to have an anomalous base composition, it has been suggested that the gene may been acquired by recent horizontal transfer from a species with a different base composition (for example, see reference 12). Thus, *vapA* could have originated in a more A+T-rich genome.

Alternatively, codon usage in other gram-negative bacteria (notably enterobacteria) is known to vary with the level of gene expression (19). While *aroA* is expressed at a low level, *vapA* is probably very highly expressed, and its codon usage may reflect that of a highly expressed gene in *A. salmonicida*. The contrast between *aroA* and *vapA* may parallel the situation in the G+C-rich enteric species *Serra-*

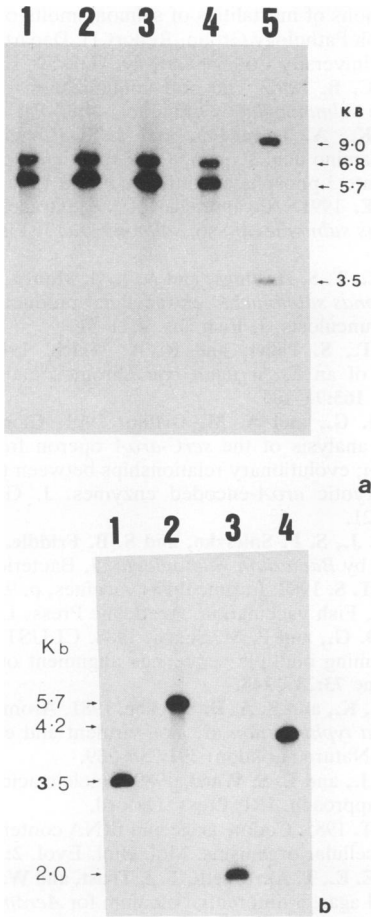

FIG. 4. Southern hybridization analysis of pAAK2 plasmid integrants (a) and *A. salmonicida aroA*::Ka^r (b). For panel a, chromosomal DNA (3 μg) isolated from 1 Aro$^+$ (lane 5) and 4 Aro$^-$ (lanes 1 to 4) transconjugants was cleaved with *Cla*I, and the products were separated on a 0.8% agarose gel. The contents of the gel were then transferred to a nitrocellulose membrane and probed with pAA1, which was labelled with ^{32}P by nick translation. The resultant autoradiogram (exposed for 3 days at −70°C) is shown. The estimated sizes (in kilobases) of the hybridizing fragments are indicated on the right. For panel b, chromosomal DNA was isolated from an *aroA* mutant of strain SPUP1 and cleaved separately with *Cla*I and *Eco*RI. Wild-type chromosomal DNA was included as a control. The products were separated on a 0.8% agarose gel, the gel contents were transferred to a nitrocellulose membrane, and the membrane was then probed with plasmid pAA1 labelled by nick translation. Lanes: 1, wild-type DNA cleaved with *Cla*I; 2, mutant DNA cleaved with *Cla*I; 3, wild-type DNA cleaved with *Eco*RI; 4, mutant DNA cleaved with *Eco*RI.

tia marcescens (genomic G+C content, 59 mol%), in which the G+C content at silent sites in genes varies from >80 mol% in lowly expressed genes to <50 mol% in highly expressed genes (45). In *vapA*, five particular U-ending codons (GUU, encoding Val; UCU, Ser; ACU, Thr; GCU, Ala; and GGU, Gly) and an A-ending codon (GAA or Glu) occur at frequencies higher than those in *aroA* (Table 3). All of these codons also occur at higher frequencies in highly expressed *E. coli* genes (46). This may indicate that tRNA abundances and anticodons (the factors which determine which codons are optimal) are similar in the two species. Knowledge of other highly expressed genes from *A. salmonicida* may resolve this question.

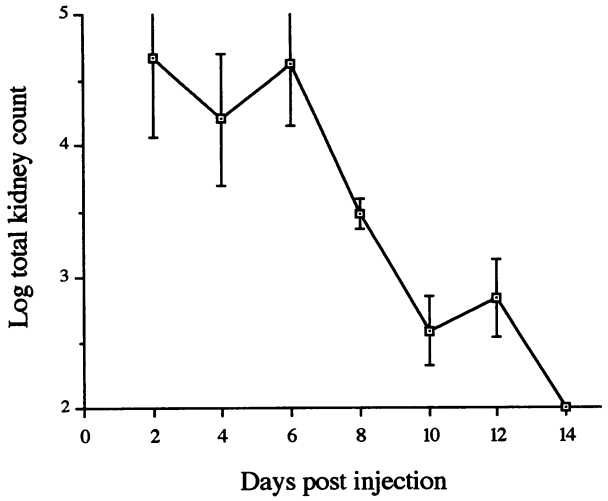

FIG. 5. Persistence of the 644Rb *aroA*::Ka^r in the kidney of brown trout at 10°C. Each point is the mean for four samples and is expressed as the log of the total bacterial count in the kidney. Error bars are ±2 standard error of the mean. All four kidney samples on day 14 yielded negative counts (<log 2).

This is the first report that an aromatic-dependent mutant can act as a live vaccine to prevent a bacterial disease of fish. The level of protection achieved from just a single dose is a considerable improvement from the variable protection achieved by vaccinating with killed whole-cell or subunit vaccines (1, 15). In addition, the 16-fold increase in protection observed in fish which had received a second dose suggests that immunological memory had been stimulated by exposure to the vaccine.

There have been two previous reports of the use of live-attenuated *A. salmonicida* strains as vaccines. Cipriano and Starliper (6) used a laboratory-passaged attenuated derivative of a previously virulent isolate to vaccinate brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*). This strain provided significant protection when administered by intraperitoneal injection or by immersion. The nature of the attenuation was not addressed, but since it had lost its ability to autoagglutinate in broth, it probably lacked the A layer. The strain was obtained by repeated passage in broth and agar over a 12-month period, conditions which are known to attenuate virulent strains of *A. salmonicida* (29). The principal objections to this type of live vaccine are the possibility of reversion to virulence when the organism is administered to a large number of fish and the possibility of establishing carriers in immunized populations, which would violate fish health policies.

A more recent report by Thornton et al. (52) described the isolation of a slowly growing, aminoglycoside-resistant mutant and a rapidly growing pseudorevertant of *A. salmonicida*. Both mutants continued to express a variety of factors associated with pathogenesis but differed morphologically from wild-type cells with respect to A-layer organization, membrane antagonist sensitivity, and aerobic metabolism. The slowly growing resistant mutant had lost virtually all aerobic metabolism, while the pseudorevertant had partially recovered the ability to metabolize certain carbon sources. Both mutants were avirulent and did not persist in tissue. The rapidly growing antibiotic-sensitive pseudorevertant protected chinook salmon (*Oncorhynchus tshawytscha*) from challenge with a heterologous virulent strain when

administered by intraperitoneal injection or immersion. The levels of protection achieved were a 10-fold and 35-fold increase in LD_{50} for injection- and immersion-vaccinated fish, respectively. The inability of these strains to persist in fish tissue allays any fears of creating carrier status in immunized populations. However, the rapid clearance of the vaccine strain from fish tissue (it was cleared from the liver, kidney, and spleen of immersion-vaccinated fish after approximately 48 h) is consistent with the abnormal A layer of the strain (52) and suggests that this defect may limit the ability of the vaccine to elicit an effective immune response. Furthermore, passive immunity studies suggest that the native A layer, or a closely associated antigen, is protective (28, 36). Reversion to virulence is also possible with these spontaneous mutants.

The aromatic-dependent mutant described here offers a number of advantages. Perhaps the most important is the rational approach to attenuation which results in a severe metabolic deficiency affecting seven key metabolic pathways (39) caused by a defined, nonreverting mutation. The *aroA* mutation renders the organism avirulent without affecting its ability to produce virulence determinants, and, most importantly, it does not affect the A layer. This has important implications for the induction of a high level of immunity and is almost certainly a contributing factor to the persistence of these mutants in tissue that is longer than that described by Thornton et al. (52). However, this persistence is finite because of the metabolic deficiency imposed by the *aroA* mutation, thus preventing the occurrence of a carrier state in immunized fish. Finally, the level of immunity achieved here in brown trout by using the *aroA* vaccine strain is 25-fold higher than that achieved by Thornton et al. (52) in terms of LD_{50} compared with unvaccinated controls after a single intraperitoneal dose. Thus, this vaccine approach offers considerable promise as a means of preventing furunculosis in farmed fish.

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REFERENCES

1. **Austin, B., and D. A. Austin.** 1987. Bacterial fish pathogens, p. 111–195. Ellis Horwood Ltd., Chichester, United Kingdom.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley and Sons, Inc., New York.
3. **Bolivar, F., R. L. Rodriquez, P. J. Greene, M. V. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Bowe, F., P. O'Gaora, D. Maskell, M. Cafferkey, and G. Dougan.** 1989. Virulence, persistence, and immunogenicity of *Yersinia enterocolitica* O:8 *aroA* mutants. Infect. Immun. **57**: 3234–3236.
5. **Chu, S., S. Cavaignac, J. Feutrier, B. M. Phipps, M. Kostrzynska, W. W. Kay, and T. J. Trust.** 1991. Structure of the tetragonal surface array protein and gene of *Aeromonas salmonicida*. J. Biol. Chem. **266**:15258–15265.
6. **Cipriano, R. C., and C. E. Starliper.** 1982. Immersion and injection vaccination of salmonids against furunculosis with an avirulent strain of *Aeromonas salmonicida*. Prog. Fish-Cult. **44**:167–169.
7. **Drinan, E., M., R. Palmer, J. O'Kelly, and P. R. Smith.** 1978. Investigations of mortalities of salmon smolts on their transfer to sea (Fish Pathology Group, Report 1). Department of Microbiology, University College Galway, Galway, Ireland.
8. **Duff, D. C. B.** 1942. The oral immunization of trout against *Bacterium salmonicida*. J. Immunol. **44**:87–94.
9. **Duncan, K., A. Lewendon, and J. R. Coggins.** 1984. The complete amino acid sequence of *Escherichia coli* 5-enolpyruvylshikimate 3-phosphate synthase. FEBS Lett. **170**:59–63.
10. **Ellis, A. E.** 1991. An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. J. Fish Dis. **14**:265–277.
11. **Ellis, A. E., T. S. Hastings, and A. L. S. Munro.** 1981. The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. J. Fish Dis. **4**:41–51.
12. **Felmlee, T., S. Pellet, and R. A. Welch.** 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J. Bacteriol. **163**:94–105.
13. **Griffin, H. G., and A. M. Griffin.** 1991. Cloning and DNA sequence analysis of the *serC-aroA* operon from *Salmonella gallinarum*; evolutionary relationships between the prokaryotic and eukaryotic *aroA*-encoded enzymes. J. Gen. Microbiol. **137**:113–121.
14. **Griffin, P. J., S. F. Snieszko, and S. B. Friddle.** 1952. Pigment formation by *Bacterium salmonicida*. J. Bacteriol. **65**:652–659.
15. **Hastings, T. S.** 1988. Furunculosis vaccines, p. 93–111. *In* A. E. Ellis (ed.), Fish vaccination. Academic Press, London.
16. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequences alignment on a microcomputer. Gene **73**:237–244.
17. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291**:238–239.
18. **Howe, C. J., and E. S. Ward.** 1989. Nucleic acid sequencing, a practical approach. IRL Press, Oxford.
19. **Ikemura, T.** 1985. Codon usage and tRNA content in unicellular and multicellular organisms. Mol. Biol. Evol. **2**:13–34.
20. **Ishiguro, E. E., T. Ainsworth, T. J. Trust, and W. W. Kay.** 1985. Congo red agar, a differential medium for *Aeromonas salmonicida*, detects the presence of the cell surface protein array involved in virulence. J. Bacteriol. **163**:1233–1237.
21. **Ivins, B. E., S. L. Welkos, G. B. Knudson, and S. F. Little.** 1990. Immunization against anthrax with aromatic compound-dependent (Aro^-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect. Immun. **58**:303–308.
22. **Karn, J., H. W. D. Matthes, M. J. Gait, and S. Brenner.** 1984. A new selective phage cloning vector, λ2001, with sites for *Xba*I, *Bam*HI, *Hin*dIII, *Eco*RI, *Sst*I and *Xho*I. Gene **32**:217–224.
23. **Kieny, M. P., R. Lathe, and J. P. Lecocq.** 1983. New versatile cloning and sequencing vectors based on bacteriophage M13. Gene **26**:91–99.
24. **Klontz, G. W., W. T. Yasutake, and A. J. Ross.** 1966. Bacterial diseases of the Salmonidae in the western United States: pathogenesis of furunculosis in rainbow trout. Am. J. Vet. Res. **27**:1455–1460.
25. **Lee, K.-K., and A. E. Ellis.** 1990. Glycerophospholipid:cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytolysin of *Aeromonas salmonicida*: LPS stabilizes and enhances toxicity of the enzyme. J. Bacteriol. **172**:5382–5393.
26. **Loenen, W. A. M., and W. J. Brammar.** 1980. A bacteriophage lambda vector for cloning large DNA fragments made with several restrictions enzymes. Gene **10**:249–259.
27. **Maskell, D. J., M. Morrissey, and G. Dougan.** 1988. Cloning and nucleotide sequence of the *aroA* gene of *Bordetella pertussis*. J. Bacteriol. **170**:2467–2471.
28. **McCarthy, D. H., D. F. Amend, K. A. Johnson, and J. V. Bloom.** 1983. *Aeromonas salmonicida*: determination of an antigen associated with protective immunity and evaluation of an experimental bacterin. J. Fish Dis. **6**:155–174.
29. **McCarthy, D. H., and R. J. Roberts.** 1980. Furunculosis of fish—the present state of our knowledge. Adv. Aquat. Microbiol. **2**:293–341.

30. **Michel, C., and B. Faivre.** 1982. Occurrence and significance of agglutinating antibodies in experimental furunculosis of rainbow trout, *Salmo gairdneri* Richardson. J. Fish Dis. **5**:429–432.
31. **Misra, T. K.** 1987. DNA sequencing: a new strategy to create ordered deletions, modified M13 vector and improved reaction conditions for sequencing by dideoxy chain termination methods. Methods Enzymol. **155**:119–139.
32. **Munn, C. B., E. E. Ishiguro, and W. W. Kay.** 1985. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. Infect. Immun. **36**:1069–1075.
33. **Nnalue, N. A., and B. A. D. Stocker.** 1987. Tests of the virulence and live-vaccine efficacy of auxotrophic and *galE* derivatives of *Salmonella choleraesuis*. Infect. Immun. **55**:955–962.
34. **O'Callaghan, D., D. Maskell, F. Y. Liew, C. S. F. Easmon, and G. Dougan.** 1988. Characterization of aromatic- and purine-dependent *Salmonella typhi*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56**:419–423.
35. **O'Gaora, P., D. Maskell, D. Coleman, M. Cafferkey, and G. Dougan.** 1989. Cloning and characterization fo the *serC* and *aroA* genes of *Yersinia enterocolitica*, and construction of an *aroA* mutant. Gene **84**:23–30.
36. **Olivier, G., T. P. T. Evelyn, and R. Lallier.** 1985. Immunogenicity of vaccines from a virulent and an avirulent strain of *Aeromonas salmonicida*. J. Fish Dis. **8**:43–45.
37. **O'Toole, P. O., and T. J. Foster.** 1988. Construction of gene libraries in bacteriophage lambda vectors, p. 171–186. *In* P. Owen and T. J. Foster (ed.), Immunochemical and molecular genetic analysis of bacterial pathogens. Elsevier, Amsterdam.
38. **Parker, N. D., and C. B. Munn.** 1985. Cell surface properties of virulent and attenuated strains of *Aeromonas salmonicida*, p. 97–105. *In* A. E. Ellis (ed.), Fish and shellfish pathology. Academic Press, London.
39. **Pittard, A. J.** 1987. Biosynthesis of the aromatic amino acids, p. 368–394. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
40. **Price, N. C., L. Stevens, D. Duncan, and M. Snodgrass.** 1989. Proteases secreted by strains of *Aeromonas salmonicida*. J. Fish Dis. **12**:223–232.
41. **Reed, L. J., and H. Muench.** 1938. A simple method for estimating 50% end points. Am. J. Hyg. **27**:493–497.
42. **Roberts, M., D. Maskell, P. Novotny, and G. Dougan.** 1990. Construction and characterization in vivo of *Bordetella pertussis aroA* mutants. Infect. Immun. **58**:732–739.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
45. **Sharp, P. M.** 1990. Processes of genome evolution reflected by base frequency differences among *Serratia marcescens* genes. Mol. Microbiol. **4**:119–122.
46. **Sharp, P. M., E. Cowe, D. G. Higgins, D. C. Shields, K. H. Wolfe, and F. Wright.** 1988. Codon usage patterns in *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and *Homo sapiens*; a review of the considerable within-species diversity. Nucleic Acids Res. **16**:8207–8211.
47. **Sharpe, G. S.** 1984. Broad host range cloning vectors for Gram-negative bacteria. Gene **29**:93–102.
48. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
49. **Stalker, D. M., W. R. Hiatt, and L. Comai.** 1985. A single amino acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. J. Biol. Chem. **260**:4724–4728.
50. **Stüber, D., and H. Bujard.** 1981. Organization and transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. USA **78**:167–171.
51. **Taylor, A. L., and M. S. Thoman.** 1964. The genetic map of *Escherichia coli* K-12. Genetics **50**:659–677.
52. **Thornton, J. C., R. A. Garduño, S. G. Newman, and W. W. Kay.** 1991. Surface disorganized, attenuated mutants of *Aeromonas salmonicida* as furunculosis vaccines. Microb. Pathog. **11**:85–89.
53. **Verma, N. K., and A. A. Lindberg.** 1991. Construction of aromatic dependent *Shigella flexneri* 2a live vaccine candidate strains: deletion mutations in the *aroA* and the *aroD* genes. Vaccine **9**:6–9.
54. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.