***Yersinia ruckeri* lipopolysaccharide is necessary and sufficient for eliciting a protective
immune response in rainbow trout (*Oncorhynchus mykiss*, Walbaum)**

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ABSTRACT

Enteric redmouth disease (ERM), caused by *Yersinia ruckeri*, has been controlled successfully using immersion-applied bacterin vaccines for several decades. While the host response to vaccination and the mechanism of protection of this vaccine have been elucidated, the bacterial components eliciting protection have remained unclear. Here we show that highly purified serotype O1 *Y. ruckeri* lipopolysaccharide (LPS) is sufficient to induce a protective response to experimental challenge in rainbow trout (*Oncorhynchus mykiss*). Dose response experiments demonstrated that *Y. ruckeri* LPS at doses of 1 ng/fish and above resulted in essentially complete protection and doses as low as 0.01 ng/fish (1.38 ng/kg) resulted in significant protection, thus demonstrating the extremely high potency of this immunogen. Analysis of the *Y. ruckeri* genome identified a cluster of putative O-antigen biosynthetic genes specific to serotype O1 strains. This cluster primarily consisted of genes encoding proteins predicted to function in the biosynthesis of legionamic acid, a nonulosonic acid known to be part of the O-polysaccharide repeat of O1 *Y. ruckeri*. Mutation of the *nab2* gene, a nonulosonic acid biosynthesis gene (*nab* gene), resulted in production of severely truncated forms of LPS. Vaccination with bacterin vaccines derived from the *nab2* mutant and its wild type parent strain demonstrated that LPS is a required component of the whole-cell bacterin vaccine and suggests that LPS is the only cellular component contributing to the protective response elicited by this vaccine. We speculate that the exceptionally high potency of *Y. ruckeri* LPS accounts for the unusual success of this vaccine when delivered by immersion.

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50 **1. Introduction**

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52 *Yersinia ruckeri* is the etiologic agent of enteric red mouth disease (ERM), a hemorrhagic
53 septicemia of salmonid fish species [1-3] . This disease is most prevalent in intensive rainbow
54 trout (*Oncorhynchus mykiss*) farms where it can cause high rates of mortality if left untreated.
55 Most ERM outbreaks are caused by serotype O1 strains of this pathogen, isolates of which form
56 a biochemically and genetically homogenous group defined by their shared lipopolysaccharide
57 (LPS) antigens. *Y. ruckeri* was first identified as a significant rainbow trout pathogen in Idaho in
58 the 1950s and was initially described as having a limited geographic range: however, serotype
59 O1 strains are now found in all areas of the world where rainbow trout are cultured [1-
60 3]. Vaccines for ERM were successfully developed, licensed and commercialized in the late
61 1970s and consist of killed whole-cell preparations of serotype O1 *Y. ruckeri* strains [4, 5]. The
62 route of administration, efficacy and duration of protection have been well defined for this
63 vaccine and have shown that long-lasting protection is afforded by oral, bath or injection
64 vaccination [4, 6-8]. In commercial use these vaccines are typically applied by brief (30 second)
65 immersion of fish *en masse* allowing simultaneous vaccination of many fish with minimal
66 handling stress. The ease with which this vaccine can be delivered with high efficacy is unique
67 and likely accounts for its success and worldwide use. The success of this vaccine has prompted
68 its use as a model-system for better understanding the induction of a protective immune response
69 in rainbow trout. Several laboratories have described the effect of vaccination or infection on
70 host expression of immune-related genes [9-13] and the mechanism of protection has recently
71 been elucidated [11, 14].

While much is known regarding host response to ERM vaccination, the bacterial components necessary for eliciting this protective response remain unclear. Early work optimizing the ERM vaccine demonstrated that vaccination with a phenol or butanol extraction of whole cells did not alter potency of the ERM bacterin, thus suggesting that a crude carbohydrate fraction could provide protection [4]. More recently, *Y. ruckeri* LPS prepared using the aqueous phenol method of Westphal and Jann was shown to elicit protection as well [15]. In both of these studies the purity of these preparations was not determined and the methods used to prepare LPS would not guarantee a high degree of purity. In addition, the concentration of carbohydrate/LPS used was not reported and the approaches used in these studies can assess only if carbohydrate/LPS is capable of eliciting a response, while we were interested in determining the relative role LPS plays as a component of the whole cell vaccine. In the present study, we demonstrate that highly purified *Y. ruckeri* serotype O1 LPS is a potent immunogen and sufficient alone for eliciting a protective response against homologous challenge. We also created a defined *Y. ruckeri* LPS biosynthetic mutant lacking LPS and used this mutant to show that LPS is an essential component of the whole cell vaccine. Together these results suggest that LPS is the only cellular component contributing to the protective response elicited by the ERM bacterin vaccine.

2. Materials and methods

2.1 Bacterial strains, plasmids and media and growth conditions

Unless otherwise noted *Y. ruckeri* CSF007-82 (serotype O1 and Biotype 1) was grown at 24°C in Trypticase soy broth (TSB) or Trypticase soy agar (TSA). *Escherichia coli* strains (Top10 and SM10λpir) were grown at 37°C in Luria media. When necessary ampicillin was added to growth media at a concentration of 100 µg/ml for *E. coli* and 200 µg/ml for *Y. ruckeri*.

2.2 Molecular biological methods

DNA purification, restriction endonuclease digestions, DNA ligation, transformation and agarose gel electrophoresis were all performed using standard protocols [16]. DNA was amplified by PCR using HotStartTaq *Plus* Master Mix kit (Qiagen) according to manufacturer protocols.

2.3 Construction and genetic complementation of *Y. ruckeri nab2* mutant strain JH-101

The *Y. ruckeri nab2* mutant was generated by marker exchange-eviction mutagenesis [17] using a *sacB*-containing suicide vector pTW-MEV [18]. The *nab2* gene was first PCR-amplified using primers nab21F (5'- GGT CAT TCT CAC TTG GCC G-'3) and nab21R (5'- ATA TCC CGG TGC TGC TTT T-'3). These primers were designed to amplify the entire *nab2* ORF in addition to 447 bp upstream of the gene's predicted translational start and 79 bp downstream of the predicted stop codon. This PCR product was cloned into the pCR2.1 vector using the TA-Cloning® Kit (Life Technologies) resulting in plasmid pTW600. To create a deletion within *nab2*, pTW600 was cut with the endonuclease HpaI and re-ligated

under conditions favoring intramolecular ligation resulting in a plasmid containing a 291 bp HpaI deletion within the *nab2* ORF. This deletion causes an *in frame* excision of 97 amino acids spanning from codon 11 to 108 and thus is predicted to result in a Nab2 derivative protein lacking 27% of its amino acid sequence. This *nab2* deletion derivative was subcloned as a NotI-SpeI fragment into the NotI-XbaI sites of the suicide vector pTW-MEV resulting in plasmid pTW601. This plasmid was then mobilized to *Y. ruckeri* strain CSF007-82 by conjugation and ampicillin selection used to identify plasmid cointegrants. Clones containing site-specific plasmid integration were identified by selection onto media containing ampicillin and confirmed by PCR. Cointegration-containing clones were grown overnight (16-18 h) in the absence of selection and subsequently plated onto media containing 10% sucrose in order to select for sucrose-resistant clones that had undergone a homologous recombination event leading to excision of *sacB*. Sucrose-resistant and ampicillin sensitive clones were identified and screened by PCR to identify a successful gene replacement clone containing the mutated *nab2* allele. The wild type *nab2* gene was cloned onto a multicopy plasmid for complementation analysis by amplifying the *nab2* fragment described above using primers nab21F, and nab21R (shown above) followed by PCR cloning this fragment into the pBAD-TOPO vector. The resulting plasmid pJH601 and the empty pBAD-TOPO vector were mobilized to *Y. ruckeri* strains by electroporation [19].

2.3 Lipopolysaccharide extraction and analysis by sodium dodecyl sulfate (SDS)-PAGE

High molecular weight LPS was purified using a modified hot phenol extraction method that included 3 consecutive differential ultracentrifugation steps [20]. Purified LPS used for

vaccination was verified by SDS-PAGE and composition analysis carried out by the Complex Carbohydrate Research Center at the University of Georgia. The Hitchcock and Brown method [21] was used to detect the lower molecular weight LPS forms (truncated forms) present in the *nab2* mutant strain. LPS was resolved by SDS-PAGE and visualized by Silver Staining as described previously [20].

2.4 Experimental infection

All vaccination-challenge experiments were carried out using rainbow trout grown at the Research Division of Clear Springs Foods, Inc. The experiments shown in Figures 1 and 5 were conducted concurrently and shared PBS controls, while the rest of the experiments were conducted independently. For each of the these experiments, two replicate groups of 25 fish (mean weight, 7.2 grams) were exposed to a dilution of a culture of *Y. ruckeri* strain CSF007-82 containing 1×10^9 viable bacteria/mL for one hour in a static bath with supplemental oxygen. Cells for the challenge were grown for 72 hours at 15°C in TSB. After challenge, each group of 25 fish was then placed in a 19 L tank that received 15°C ultra-violet treated spring water. During the challenge fish were fed once daily by hand to avoid overfeeding due to infection-caused loss of appetite. Fish were monitored for 28 days and mortalities removed and recorded daily. In all cases replicates were pooled as there was no significant difference between the curves at a 95% confidence level using the Log-rank (Mantel-Cox) test. Mortality due to *Y. ruckeri* infection was confirmed by plating kidney tissue onto TSA from at least 20% of daily mortalities in each tank and confirming *Y. ruckeri* by serotyping. When necessary, fish were euthanized by MS-222 (200 mg/L) overdose. Stock and test fish were

confirmed free of bacterial infection just prior to experimentation using standard microbiological methods. The experiments and general animal care were carried out at Clear Springs Foods Inc. using protocols approved by the Clear Springs Foods Animal Care and Use Committee.

2.5 Vaccine production and delivery

Vaccines consisted of formalin-inactivated whole cells of the *nab2* LPS mutant, its isogenic wild type parent, complemented mutant strain and vector control strain. To grow the cells, 5 mL TSB cultures were started from a frozen seed stock and allowed to grow with shaking (250 RPM) for 18 h at 24 °C. These cultures were then used to seed larger cultures by adding 0.1 mL of starter culture to 500 mL of fresh media put into a 2800 mL Fernbach flask (Pyrex no. 4220). Cultures were grown with shaking (250 RPM) for 18 h at 24 °C. Cell inactivation was accomplished by the addition of formalin to 0.8% v/v and holding at 4 °C for at least 24 h. Prior to formalin killing, the cell concentration was estimated by direct plate counting. Sterility of the vaccines was tested by inoculating 10 mL of vaccine into 95 mL TSB media and verifying that no growth occurred after 14 days of incubation at 24 °C. Vaccine preparations were diluted to the necessary concentrations in Phosphate buffered saline (PBS) and were delivered under sedation (immersion in 90 mg/L of MS-222) by intraperitoneal (IP) injection of 0.1 mL using a 1.0 mL Tuberculin syringe (27G1/2 inch needle). A total of 160 fish per treatment were vaccinated (mean weight, 2 g) and kept in 380 L tanks that received constant temperature (15°C) single pass spring water. Fish were fed daily at rate of 1 % bodyweight/day.

2.6 Statistical analysis

Difference in survival was determined using the product limit method of Kaplan and Meier and the Log-rank (Mantel-Cox) test was used to compare survival curves. All statistical analysis was carried out using the GraphPad Prism 5 software package (GraphPad Software Inc., San Diego, CA, USA).

Vaccine potency was estimated by calculating the relative percent survival (RPS) according to Amend [22] as follows:

$$RPS = 1 - \left(\frac{\% \text{vaccinate mortality}}{\% \text{control mortality}} \right) 100$$

3. Results and Discussion

3.1 Purified *Y. ruckeri* LPS is a potent protective antigen

In order to determine the role purified LPS plays in eliciting a protective immune response rainbow trout were injection-vaccinated with doses of purified serotype O1 *Y. ruckeri* LPS ranging from 100- 0.01 ng per fish. These experiments demonstrated that doses of 1 ng/fish and above resulted in essentially complete protection under conditions that resulted in nearly 90% mortality in mock-vaccinated controls (Fig. 1). Lower doses consisting of 0.1 ng/fish and 0.01 ng/fish resulted in an RPS of 58% and 40%, respectively and demonstrated a dose-response

effect. These results demonstrate that *Y. ruckeri* LPS alone is sufficient to confer protection against homologous challenge.

The purpose of these studies was to investigate the role of *Y. ruckeri* LPS in the induction of an acquired protective response. However, LPS is well known to act as a strong stimulator of innate immune mechanisms in diverse eukaryotic species including fish [23, 24]. This response, in many vertebrates, is dependent on the conserved Lipid A portion of the LPS molecule and is thought to be independent of the bacterial source of the LPS [25]. In rainbow trout, commercially available *E. coli* LPS preparations have been used to investigate induction of such innate immune mechanisms [23, 24, 26, 27], although the stimulating factors in these preparations appear not be LPS, but rather, contaminating peptidoglycan [26]. The conditions used here (challenge at 420 degree days after vaccination) were designed to investigate long-term acquired immunity: however, it is still possible that the typically short-term and non-specific mechanisms induced by LPS preparations could be responsible for the protection observed under the conditions used in these experiments. To test this hypothesis, an independent experiment was conducted comparing LPS from *E. coli* 0111:B4 (purchased from Sigma) delivered by injection at doses of 10, 100 and 1000 ng/fish to the 10 ng dose of *Y. ruckeri* LPS shown previously to confer complete protection. Vaccination with the *E. coli* 0111:B4 LPS conferred no protective effect against *Y. ruckeri* challenge at 420 degree days after vaccination at any of the doses tested, whereas *Y. ruckeri* LPS at a dose of 10 ng/fish again conferred complete protection (Fig. 2). These results suggest that, under the conditions tested herein, the protective response observed is likely not caused by the induction of innate immunity by conserved LPS structures but rather a specific and long lasting adaptive immune response.

3.2 Identification of LPS biosynthetic genes and creation of an isogenic LPS mutant strain.

Our results demonstrate that *Y. ruckeri* LPS alone is sufficient to induce a protective response against homologous challenge. However, this result does not discount the possibility that other cellular components of the whole-cell bacterin vaccine contribute to the induction of a protective response. We therefore sought to define the role of LPS as a protective component of the whole-cell ERM vaccine by comparing the protection elicited by vaccination with a defined isogenic mutant lacking LPS to its parent strain. To do this, it was first necessary to identify putative LPS biosynthesis genes and then use these sequences to create a defined LPS mutant by marker-exchange mutagenesis. The O-polysaccharide (O-PS) repeat of *Y. ruckeri* O1 is known to contain a derivative of 8-epi-legionamic acid (Leg), a nonulosonic acid (NulO) related to sialic acid [28]. We therefore searched the recently published [29] genome sequence of serotype O1 strain CSF007-82, searching the annotation of this genome for genes with putative functions consistent with roles in the biosynthesis of legionamic or similar nonulosonic acids [30]. This approach yielded a cluster of contiguous ORFs including putative proteins predicted to function in nonulosonic acid biosynthesis (nab genes). Specifically the *nab1*, *nab2* and *nab3* genes encode proteins representing the core nonulosonic acid biosynthetic enzymes predicted to function in the biosynthesis of a NulO from its precursor UDP-GlcNAc [30]. The characteristics, putative functions and accession numbers of these genes are presented in Table 1. Similar nab gene clusters have been identified in NulO producing members of the *Vibrionaceae* [31, 32].

Based on the sequence analysis presented above we hypothesized that this nab gene cluster is necessary for production of *Y. ruckeri* O-PS. To test this, and create a defined LPS mutant strain, an unmarked *nab2* mutation was constructed in wild-type *Y. ruckeri* by marker

exchange mutagenesis resulting in strain JH-101 (see Materials and Methods for details of this construction). Strain JH-101 contains an internal *in frame* deletion of 291 bp spanning from codon 11 to 108 and encoding 97 of the proteins 358 amino acids (27% of protein deleted). *Y. ruckeri* typically forms smooth colonies on solid growth media, a property that is usually indicative of profuse surface polysaccharide production. Strain JH-101 formed waxy, hard colonies that slid intact around the agar surface when pushed with an inoculation loop (not shown). Such “rough” colony morphology is typically indicative of a defect in O antigen or core LPS biosynthesis. To confirm this apparent defect, LPS was prepared using a method for the purification of high-molecular-weight (HMW) LPS. This purified LPS was resolved by SDS PAGE and visualized by silver staining. Using this method both high and low-molecular weight bands observed in the wild-type strain were absent from the strain JH-101 sample (Fig. 3A). This result suggests that the *nab2* mutation in strain JH-101 causes the loss of synthesis of HMW LPS forms. To determine the effect of this mutation on low-molecular-weight (LMW) LPS forms the analysis was repeated using methods that are more suitable for the detection of rough or LMW forms of LPS (Hitchcock and Brown method [21]). The faster migrating band in the *nab2* mutant strain (Fig. 3B) likely corresponds to LPS forms lacking the O-PS repeat, or lacking the O-PS repeat and part of the core region. Composition of the core region of *Y. ruckeri* O1 LPS is unknown, however *Vibrio fischeri* has recently been shown to contain legionamic acid sugars in both the O-antigen repeat and in its core structure [32]. It is therefore possible that *Y. ruckeri* LPS is similarly structured with legionamic acid sugars in both its O-antigen and core regions. Regardless, our results clearly indicate that the *nab2* mutation causes an ablation of HMW LPS synthesis. This defect was fully complemented when just the *nab2* gene was supplied to the mutant strain *in trans* thus demonstrating that this phenotype was not due to a polar effect on

surrounding genes (Fig. 3 panels A and B). In addition, presence of the empty pBAD vector in strain JH-101 had no effect on the LPS production phenotype (Fig. 3 lane 3). The *nab2* mutation in strain JH-101 also caused a loss in reactivity with anti-O1 serotyping antibody (data not shown). These results demonstrate that the *nab2* mutation causes severe truncation of LPS and thus demonstrates that the *nab2* gene plays an essential role in HMW LPS biosynthesis. It is important to note that the *nab2* gene was detected by PCR in all serotype O1 strains of *Y. ruckeri* tested and never in any of the other serotypes (data not shown), thus suggesting that this gene is serotype specific. We are currently examining the potential use of this gene as a target for a PCR-based surrogate serotyping assay.

3.3 LPS is an essential component of the ERM bacterin vaccine

Vaccination-challenge experiments comparing the *nab2* LPS mutant, its isogenic parent, complemented mutant strain and vector control strains, were conducted to investigate the role that the LPS component of the ERM bacterin vaccine plays in eliciting a protective immune response. To accomplish this, whole-cell killed vaccines were prepared from these strains and the protective response compared. Fish vaccinated with the wild-type parent strain displayed 100% survival under conditions where only 11% of the unvaccinated controls survived (Fig. 4). In contrast, the protection induced by vaccination with the LPS-deficient mutant strain (JH-101) was not significantly different from that of the mock vaccinated control group and the vaccine phenotype was completely restored in the complemented mutant strain (Fig. 4). The absence of significant protection elicited by the LPS-deficient mutant strain demonstrates that HMW LPS is an essential component of the whole cell vaccine and suggests that LPS is the only cellular

component contributing to the protective response. Although it is unlikely, we cannot discount the possibility that other cell components contribute to protection but only when LPS is present. This requirement for HMW LPS for protection is consistent with that observed for *V. ordalii*, where only HMW LPS was protective when purified LPS was size fractionated suggesting that presence of the O-antigen repeat was critical for protection [33].

3.4 Potency of the ERM bacterin vaccine

Our results demonstrate that *Y. ruckeri* LPS is a highly potent protective antigen and is likely the only component of the whole cell vaccine necessary for conferring this protection. We were also interested in determining the potency of LPS when delivered in its native form associated with whole cells as this is how the vaccine is typically delivered in the commercial setting. For this, vaccines consisting of wild-type *Y. ruckeri* were prepared with varying cell numbers ranging from 10^8 to 10 CFU/ 0.1 mL (CFU/dose). Note that 10^8 CFU/fish is the dose used in all of the other vaccination-challenge studies reported here and corresponds to the highest concentration used in this experiment. The resulting post-challenge survival analysis (Fig. 5) demonstrates that doses as low as 10 cells/fish confer significant protection under conditions that result in approximately 90% mortality in unvaccinated controls, while doses of 100 cells/fish and higher confer complete protection. It is important to note that while these low doses confer protection at 28 dpv the higher doses are likely needed to confer the duration of protection necessary to protect fish in the commercial setting. We therefore cannot recommend using ERM bacterin vaccines at these low doses when applied on farm. The potency observed here is extremely high and likely explains why the vaccine is unusually efficacious when delivered by brief immersion.

The immersion vaccine is typically delivered by dipping fish into a bath containing approximately 2×10^9 cells/ mL for 30 seconds. It is not surprising that this application method is protective as our results demonstrate that only 100 cells are needed to be taken up to confer complete protection. This suggests that high potency is required for successful immersion application of the ERM bacterin as opposed to efficient uptake of the vaccine by the fish.

The high level of immunogenicity demonstrated here is similar to that previously reported for LPS purified from *Vibrio ordalii* and used to vaccinate coho salmon against homologous challenge [34]. In these studies, the lowest doses of LPS tested (20 ng/kg fish) resulted in complete protection. Interestingly, *Vibrio* and ERM vaccines are the only bacterin vaccines known to be extremely efficacious when delivered by immersion (Authors Unpublished observation, Tom Goodrich, AquaTactics, personal communication), further suggesting that highly potent LPS antigens are critical for the success of immersion delivered bacterin vaccines.

3.5 Conclusions

Vaccination of rainbow trout with bacterins prepared from a wild-type strain of *Y. ruckeri* serotype O1 and an isogenic LPS mutant indicated that HMW LPS is a necessary component for the function of this vaccine. Dose-response experiments, utilizing purified LPS, showed that purified LPS is a potent immunogen when applied alone. These studies clearly demonstrate the critical role the HMW LPS plays as a component of the ERM bacterin vaccine. Furthermore, we speculate that it is the extremely high potency of this bacterin vaccine that explains why it is uniquely effective when delivered by immersion. Given that LPS appears to be the only bacterin component contributing to the protective response, and that anti-*Y. ruckeri* IgM antibody is the

protective component of serum after vaccination [14], it is likely that anti-HMW LPS IgM is protective, although further work is necessary to confirm this.

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FIGURE LEGENDS

Fig. 1. Effect of vaccination with purified *Y. ruckeri* LPS on mortality induced by exposure to *Y. ruckeri*. Rainbow trout were challenged by immersion exposure to 1.0×10^9 *Y. ruckeri* CSF007-82 cells for one hour in a static bath at 28 dpv. LPS was delivered by IP injection at the following Concentration/fish: open circle, 100 ng; closed square, 10 ng; open square, 1; closed diamond, 0.1 ng; closed circle, 0.01 ng; opened diamond, PBS (mock-vaccination control). Panel A shows survival curves for each treatment and in Panel B the percent mortality at day 28 is shown for each treatment.

Fig. 2. Effect of vaccination with purified E.coli 0111:B4 (Sigma product number, L4391) LPS on mortality induced by exposure to wild-type *Y. ruckeri*. Rainbow trout were challenged by immersion exposure to 1.0×10^9 *Y. ruckeri* CSF007-82 cells for one hour in a static bath at 28 dpv. LPS was delivered by IP injection at the following concentration/fish: open

circle, 1000 ng *E. coli* LPS; closed square, 100 ng *E. coli* LPS; open square, 10 ng *E. coli* LPS; closed circle, 10 ng *Y. ruckeri* LPS; opened diamond, PBS (mock-vaccination control). Panel A shows survival curves for each treatment and in Panel B the percent mortality at day 28 is shown for each treatment.

Fig. 3: SDS-PAGE analysis of *Y. ruckeri* LPS. (A) Purified HMW LPS fractionated on a 12.5 % SDS-PAGE gel. (B) Low-molecular weight LPS fractionated on a 16 % SDS-PAGE gel. Strains tested were as follows: lane 1, CSF007-82 (wild-type parent); 2, JH-101 (*nab2* mutant); 3, JH-101/pBAD-TOPO (*nab2* mutant/vector); 4, JH-101/ pJH601 (*nab2* mutant/complementing clone). Gels were stained using an Ag-LPS silver staining method. Arrows indicate LMW bands common to both gels.

Fig. 4. Effect of bacterin vaccines on mortality induced by exposure to wild-type *Y. ruckeri*. Rainbow trout were challenged by immersion exposure to 1.0×10^9 *Y. ruckeri* CSF007-82 cells for one hour in a static bath at 28 dpv. Vaccines were delivered by IP injection at a concentration of 10^8 cells/fish. Bacterin vaccines tested were prepared from the following strains: closed circle, CSF007-82 (wild-type parent); open circle, JH-101 (*nab2* mutant); closed square, JH-101/pBAD-TOPO (*nab2* mutant/vector); open square, JH-101/ pJH601 (*nab2* mutant/complementing clone); diamond, PBS (mock-vaccination control). Panel A shows survival curves for each treatment and in Panel B the percent mortality at day 28 is shown for each treatment.

Fig. 5. Potency of whole-cell bacterin vaccine. Rainbow trout were challenged by immersion exposure to 1.0×10^9 *Y. ruckeri* CSF007-82 cells for one hour in a static bath at 28 dpv. Vaccine was delivered by IP injection at indicated concentrations: closed diamond, 10^8 cells/fish; open circle, 10^6 cells/fish; closed square, 10^4 cells/fish; open square, 10^3 cells/fish; open triangle, 10^2 cells/fish; closed circle, 10 cells/fish; open diamond, PBS mock. Panel A shows survival curves for each treatment and in Panel B the percent mortality at day 28 is shown for each treatment.

Table 1.

OFR	Size (aa)	Gene designation	Accession number	Putative function
1	389	<i>nab3</i>	WP_038241923.1	UDP-N-acetylglucosamine 2-epimerase (EC 5.1.3.14)
2	358	<i>nab2</i>	WP_004723041.1	N-acetylneuraminate synthase (EC 2.5.1.56)
3	212	<i>neuD</i>	WP_038241920.1	4-amino-6-deoxy-N-Acetyl-D-hexosaminyl-(Lipid carrier) acetyltransferase
4	352	<i>pseG</i>	WP_004723045.1	D-glycero-D-manno-heptose 1-phosphate guanosyltransferase
5	321	<i>ORF5</i>	WP_004723048.1	Legionaminic acid biosynthesis protein PtmF
6	229	<i>nab1</i>	WP_004723049.1	Legionaminic acid cytidyltransferase (EC 2.7.7.43)

Fig 1

B

LPS (ng/fish)	100	10	1	0.1	0.01	PBS
Survival (%)	94	90	94	65	48	13

Fig 2

B

	<i>E. coli</i>			<i>Y. ruckeri</i>	
LPS (ng/fish)	1000	100	10	10	PBS
Survival (%)	26	16	19	95	24

Fig 3

Fig 4

Fig 5