

Entericidin Is Required for a Probiotic Treatment (*Enterobacter* sp. Strain C6-6) To Protect Trout from Cold-Water Disease Challenge

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***Flavobacterium psychrophilum* causes bacterial cold-water disease in multiple fish species, including salmonids. An autochthonous *Enterobacter* strain (C6-6) inhibits the *in vitro* growth of *F. psychrophilum*, and when ingested as a putative probiotic, it provides protection against injection challenge with *F. psychrophilum* in rainbow trout. In this study, low-molecular-mass (≤ 3 kDa) fractions from both *Enterobacter* C6-6 and *Escherichia coli* K-12 culture supernatants inhibited the growth of *F. psychrophilum*. The ≤ 3 -kDa fraction from *Enterobacter* C6-6 was analyzed by SDS-PAGE, and subsequent tandem mass spectroscopy identified EcnB, which is a small membrane lipoprotein that is a putative pore-forming toxin. Agar plate diffusion assays demonstrated that *ecnAB* knockout strains of both *Enterobacter* C6-6 and *E. coli* K-12 no longer inhibited *F. psychrophilum* ($P < 0.001$), while *ecnAB*-complemented knockout strains recovered the inhibitory phenotype ($P < 0.001$). In fish experiments, the engineered strains (C6-6 Δ *ecnAB* and C6-6 Δ *ecnAB*<pET101::*ecnAB*>) and the wild-type strain (C6-6) were added to the fish diet every day for 38 days. On day 11, the fish were challenged by injection with a virulent strain of *F. psychrophilum* (CSF 259-93). Fish that were fed C6-6 had significantly longer survival than fish fed the *ecnAB* knockout strain ($P < 0.0001$), while fish fed the complemented knockout strain recovered the probiotic phenotype ($P = 0.61$). This entericidin is responsible for the probiotic activity of *Enterobacter* C6-6, and it may present new opportunities for therapeutic and prophylactic treatments against similarly susceptible pathogens.**

Probiotics are defined as live or dead microbial feed supplements that provide health benefits to the host animal (1). Probiotic strains of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* (2) have been used successfully in people and terrestrial animals, including poultry (3) and cattle (4), for the past century. Probiotic supplementation has gained attention from the aquaculture industry during the last 2 decades, especially for shrimp production, where antibiotic treatment has been reduced dramatically in favor of supplementing feed with commercially available probiotics composed of *Bacillus subtilis* and *Vibrio alginolyticus* (5, 6). In aquaculture, a probiotic is further defined as a live microbial cell that, when administered via the feed or to the rearing water, benefits the host by improving disease resistance, health and growth performance, feed utilization, stress response, and meat quality (7, 8). The mechanism of action of probiotics has been defined vaguely as improving the host's "microbial balance" (9) or the balance of the ambient environment, but molecular mechanisms are rarely investigated further, even though a number of applied studies have demonstrated probiotic efficacy against a variety of Gram-negative and Gram-positive pathogens in fish and shellfish (7).

In the case of salmonid species, such as Atlantic salmon (*Salmo salar* Linnaeus) and rainbow trout (*Oncorhynchus mykiss* Walbaum), for which global annual production exceeds 2.9 megatons and \$13.7 billion (10), probiotics may have a significant economic benefit while reducing the therapeutic demand for antibiotics. Bacterial cold-water disease (BCWD) (also called rainbow trout fry syndrome [RTFS]), which is caused by *Flavobacterium psychrophilum* (11), is a significant challenge for salmonid aquaculture. The Gram-negative bacterium *F. psychrophilum* causes acute and commonly fatal septicemic infections in young salmonid fry (12, 13) and other fish species (14) and severe economic losses in older fish populations, due to reduction of product value, ana-

tomical deformities, and high treatment costs (15–17). Currently, there is no commercial vaccine available to control BCWD (18). Improved rearing conditions (e.g., stress reduction, reduced stocking densities, cleaner raceways, adjusted feeding strategies, and improved disinfection protocols) and antimicrobials are the only practical control measures. In the United States, oxytetracycline and florfenicol are approved for treatment of BCWD, but drug resistance may be emerging, and concerns about the environmental impact of these treatments make it imperative to find new strategies (19–22).

A probiotic strain, *Enterobacter* sp. strain C6-6, was recently isolated from trout intestines and was shown to inhibit *F. psychrophilum* on agar diffusion plates (23) while reducing mortality during *F. psychrophilum* challenge studies (24). The agar diffusion data indicated that the cell-free supernatant of this probiotic strain could inhibit *F. psychrophilum* growth *in vitro*. A preliminary study showed that treating the supernatant with proteinase K resulted in a loss of inhibition (see Fig. S1 in the supplemental ma-

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terial). Consequently, we hypothesized that the probiotic *Enterobacter* strain C6-6 inhibits growth of *F. psychrophilum* *in vitro* and reduces mortality *in vivo* via the activity of a soluble bactericidal protein. The objective of this study was to identify the soluble inhibitor and to determine if it is required to protect fish from *in vivo* challenge with *F. psychrophilum*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Enterobacter* strain C6-6 was grown in tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% MgSO₄ · 7H₂O, 0.05% CaCl₂ · 2H₂O, pH 7.2) broth (25) at 37°C with shaking for up to 48 h for inhibition experiments, on Luria-Bertani (LB) agar and in LB medium (Difco) at 37°C with shaking for maintenance and bacterial engineering, and in tryptic soy (TS) broth (Difco) at 15°C with shaking for preparation of cells that were used in the *in vivo* experiments. Supernatant of C6-6 was harvested by first pelleting cells from TYES culture (1,600 × g for 10 min at 15°C), and then the decanted supernatant was immediately filter sterilized (0.22-μm Millex-GP syringe filter units; EMD Millipore). Sterilized supernatant was used immediately in subsequent experiments. *Escherichia coli* ATCC 29947 (strain K-12) was made nalidixic acid resistant (Nal^r) by serial passages on LB agar plates containing increasing concentrations of antibiotic until the strain was capable of growing in the presence of 30 μg/ml nalidixic acid (26). The K-12 strain was maintained and harvested using the same methods that were used to process *Enterobacter* C6-6. *E. coli* strain S17 was cultured in LB broth.

F. psychrophilum strains CSF 259-93 (27) and B17 (27) were grown in TYES at 15°C with shaking for at least 24 h or by culture for several days on TYES agar plates. Supernatant of CSF 259-93 was harvested by pelleting the cells at 1,600 × g for 10 min at 15°C and sterilizing them with 0.22-μm syringe filters or Anotop 25 syringe filter units (Whatman) with a 0.1-μm pore size. Harvesting of CSF 259-93 for the infection challenge followed the methods of Burbank et al. (24). CFU per ml were counted using the drop-plate method originally described by Chen et al. (28).

Size-exclusion experiments. Supernatants of freshly harvested *Enterobacter* C6-6 and *E. coli* K-12 were size fractionated by use of 3-kDa centrifugal filtration units (Amicon) at 1,600 × g. The concentrated supernatant composed of the >3-kDa fraction was diluted back to the starting volume before use. Using a Bioscreen-C MBR instrument (Growth Curves USA) and 100-well honeycomb plates, triplicate wells were filled with 100 μl of the flowthrough (≤3 kDa) or the >3-kDa fraction and 100 μl of diluted CSF 259-93 culture and grown at 15°C for 5 days. The CSF 259-93 used for these experiments was grown in 1 × TYES to turbidity for 3 to 5 days, and then a 1:1,000 dilution was made in 2 × TYES. The Bioscreen-C MBR system measured the optical density at 600 nm every 3 h for 5 days after a brief period of shaking (5 s). Control wells contained 100 μl of CSF 259-93 grown with 100 μl of exhausted supernatant of CSF 259-93.

Protein separation and identification. *Enterobacter* C6-6 and *E. coli* K-12 were cultured in 25-ml volumes for 24 to 48 h in TYES. The respective supernatants were harvested as described above and immediately frozen in liquid nitrogen and stored at -20°C. The frozen samples were then freeze-dried (Thermo Savant MicroModulyo) and eluted with 2 ml of phosphate-buffered saline (PBS). These >10-fold-concentrated supernatants were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29). Resultant gels were stained using Sypro Ruby protein gel stain (Life Technologies), and the protein band of interest was excised (*Enterobacter* C6-6 sample only), digested with trypsin, and submitted for liquid chromatography-tandem mass spectrometry (LC-MS/MS) (University of Idaho Environmental Biotechnology Institute, Moscow, ID). Results were analyzed using a Mascot search (Matrix Science).

Generation of *ecnAB* deletion mutants. Initial efforts to delete the *ecnAB* locus, using data from a previously sequenced strain of *Enterobacter* (NCBI accession no. NC_009436.1), apparently failed due to insufficient

sequence identity with *Enterobacter* C6-6. To circumvent this issue, we sequenced strain C6-6 by using a MiSeq instrument (Illumina) to quickly identify the sequence flanking the *ecnAB* locus. We were not attempting to produce a closed and annotated sequence for the C6-6 genome. A genomic library was made using a TruSeq DNA LT sample prep kit (Illumina) per the manufacturer's instructions. Briefly, 1 μg of genomic DNA was sheared using a Covaris M220 focused ultrasonicator (peak incident power = 50 W; duty factor = 20%; cycle/burst [count] = 200; 65-s duration). The sheared DNA was then end repaired and adenylated, and Illumina adaptors containing bar-coded sequences were ligated to the fragments. After gel purification, a short 10-cycle PCR was performed to amplify the library. Finally, the purified library was analyzed using a model 2100 Bioanalyzer (Agilent Technologies) and quantified using a ddPCR library quantification kit (Bio-Rad) with a QX100 ddPCR system (Bio-Rad). The library was diluted to 2 nM, hydrolyzed in 0.2 M NaOH, and diluted to a flow cell loading concentration of 500 pM. The library was sequenced using an Illumina MiSeq benchtop sequencer with sequencing-by-synthesis technology per the manufacturer's protocol. The run was set for "Generate FASTQ only" workflow in Illumina Experiment Manager. A 600-cycle MiSeq v.3 reagent cartridge (Illumina) was used to sequence the library with a paired-end 2 × 301 sequencing run. Data were demultiplexed automatically on the MiSeq instrument and imported into CLC Genomics Workbench v. 6.5.1 (Qiagen). A *de novo* assembly was executed, and the resulting contigs were queried against BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST>) to locate the *ecnA* and/or *ecnB* gene fragments.

An approximately 430-bp region encompassing the *ecnAB* locus was deleted from both the *Enterobacter* C6-6 and *E. coli* K-12 chromosomes, but using two different methods. The method of gene splicing by overlap extension (SOE) (30) was used to generate the *Enterobacter* C6-6 deletion, with minor modifications. Briefly, primers were designed to amplify approximately 200-bp regions flanking both sides of the *ecnAB* locus (Table 1) (primer pairs *ecnAB_A1F/ecnAB_A1R* and *ecnAB_A2F/ecnAB_A2R*). The resulting products were combined and used as the template in a second round of PCR, using only the *ecnAB_A1F* and *ecnAB_A2R* primers, to produce a spliced PCR product of approximately 400 bp. After size confirmation by gel electrophoresis, the PCR product was purified using a QIAquick PCR purification kit (Qiagen). *E. coli* S17 with plasmid pDM4 (31) was cultured overnight in LB with 34 μg/ml chloramphenicol, and plasmid DNA was isolated using a PureYield plasmid miniprep system (Promega) according to the manufacturer's recommendations. The pDM4 plasmid contains an R6K origin of replication that requires λ*pir* to replicate. This allows development of the genetic construct by use of a λ*pir* strain of *E. coli* while preventing replication in *Enterobacter* C6-6. As a consequence, only *Enterobacter* cells that undergo allelic exchange will be detected during the screening process. The purified PCR template and the suicide vector pDM4 were both digested using the restriction enzymes *SacI* and *XhoI* for 3 h at 37°C, purified again, quantified by using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), and ligated using T4 DNA ligase (New England BioLabs). The ligated product was then transformed into electrocompetent *E. coli* S17 λ*pir* (32) and selected on LB plates with chloramphenicol. Successful transformants were grown in LB broth with chloramphenicol overnight at 37°C, the plasmid was subsequently isolated, and the insert was verified using PCR and agarose gel electrophoresis (primer pairs pDM4-up/pDM4-down and *ecnAB_A1F/ecnAB_A2R*) (Table 1). The pDM4 plasmids with inserts of the correct length were sequenced (Eurofins Genomics) and confirmed using Sequencher software (version 4.10.1; Gene Codes Corporation). The plasmids were then transformed into electrocompetent *Enterobacter* C6-6, using 1,600 V, 25 μF, and 400 Ω, according to the method of Khampang et al. (33). Transformants were recovered on LB plates containing chloramphenicol (34 μg/ml) overnight. Individual colonies were then cultured in LB broth in the presence of 10% sucrose (wt/vol). Sucrose-resistant, chloramphenicol-sensitive colonies were evaluated by PCR for the loss of

TABLE 1 Primers used in this study

Primer	Sequence (5'–3')
<i>ecnAB</i> _A1F	AGTCGAGCTCCTGAAGGGTGACACTGCTGG
<i>ecnAB</i> _A1R	CGGATATCACACTGCCCTGCCTGAATATTACAGGC
<i>ecnAB</i> _A2F	AATATTCAGGCAGGGGCAGTGTGATATCCGGACGG
<i>ecnAB</i> _A2R	AGTCCTCGAGCTCTGGATGGAAGGCGCCA
pDM4-up	TAACGGCAAAGCACC GCC
pDM4-down	GATAACAATTTGTGGAATTCCGG
<i>ecnAB</i> -pET101-F	CACCATGATGAAACGTACGATTAA
<i>ecnAB</i> -pET101-R	TTAATGGTGATGGTGTGATGATGGTTCTGCGCTTTCGTCGCT
K12_ <i>ecnAB</i> _KO_P1	TGCGGCCTGCAGGCTGCACCATCACTTATTCAGGTCAGAGTGTGTAGGCTGGAGCTGCCTTCG
K12_ <i>ecnAB</i> _KO_P2	AAAAACAACCGGCACGACAGCTGCTGCTACCCTTATTGCCATATGAATATCCTCCTTA
<i>ecnAF</i> Screen	CGTGAAGTAATGCGGTTGTG
<i>ecnBR</i> Screen	AAAAACAACCGGCACGACAC
C1	TTATACGCAAGGCGACAAGG
C2	GATCTTCCGTCACAGGTAGG
T7F	TAATACGACTCACTATAGGG
M13R	CAGGAAACAGCTATGACC
K12 <i>ecnAB</i> CompR	CAATAACTAAGATAATCCAGGACAT
<i>ecnA</i> F (screen)	CGTGAAGTAATGCGGTTGTG

ecnAB by using primers *ecnAB*_A1F and *ecnAB*_A2R (Table 1), and selected clones were designated *Enterobacter* C6-6 Δ *ecnAB*.

An *E. coli* K-12 Δ *ecnAB* mutant was constructed by using site-directed gene deletion as described by Datsenko and Wanner (34) (this procedure did not work with *Enterobacter* C6-6). Briefly, primers were generated to amplify the chloramphenicol resistance cassette from pKD3 (34). These primers were designed with 5' extensions that included 40 nucleotides complementary to the flanking regions of the K-12 *ecnAB* locus (primers K-12_ *ecnAB*_KO_P1 and K-12_ *ecnAB*_KO_P2) (Table 1). The resulting PCR product was column purified (Qiagen), digested with DpnI (New England BioLabs) for 2 h at 37°C, and purified again. *E. coli* K-12 containing plasmid pKD46 (34) was grown in super optimal broth (SOB; 2% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl₂, 10 mM MgCl₂, 10 mM MgSO₄) supplemented with 1 mM L-arabinose and 100 µg/ml ampicillin to an optical density at 600 nm of 0.6 to 0.8 with shaking at 30°C. The Red helper plasmid pKD46 (34) expresses the λ Red recombinase that facilitates allelic exchange. This plasmid can be cured at 37°C due to its temperature sensitivity. Electrocompetency was achieved by washing *E. coli* K-12 <pKD46> twice in ice-cold distilled water and twice in 10% glycerol, with the final cell concentration increasing 100-fold. Electrocompetent cells (80 µl) were pulsed with approximately 150 ng of PCR product, using a Gene Pulsar 1 instrument (Bio-Rad), and immediately resuspended with SOC medium (35), followed by recovery on a shaker at 30°C for 1 h. To select for transformants, the cells were then incubated on LB plates with chloramphenicol at 30°C overnight. Successful replacement of the gene of interest with the chloramphenicol marker was confirmed by PCR amplification using primers for sequences within the chloramphenicol cassette and genomic primers that immediately flanked the gene of interest (primer pair *ecnAF*Screen/C1 and primer pair C2/*ecnBR*Screen) (Table 1) (34).

Complementation of *ecnAB*. The *Enterobacter* C6-6 *ecnAB* locus and flanking sequence were amplified using *Pfu* Turbo Hotstart DNA polymerase (Agilent Technologies) and primers *ecnAB*-pET101-F and *ecnAB*-pET101-R (Table 1). The amplicon was immediately ligated into pET101/D-TOPO-vector (Invitrogen) and transferred into chemically competent TOP10 cells (Invitrogen). Selection was accomplished on LB agar (Difco) containing 50 µg/ml ampicillin (Sigma). Transformants were then cultured in LB broth supplemented with 100 µg/ml ampicillin overnight, and the plasmid was harvested using a PureYield plasmid miniprep system (Promega). The plasmid (100 to 300 ng) with the *ecnAB* locus was introduced into electrocompetent *Enterobacter* C6-6 Δ *ecnAB* cells according to the method of Khampang et al. (33), using a Gene Pulsar 1 instrument

(1,600 V, 25 mF, and 400 Ω; Bio-Rad). Cells were immediately resuspended with SOC medium (35) and incubated for 1 h at 37°C on a shaker. Finally, transformants were selected on LB plates supplemented with 100 µg/ml ampicillin overnight at 37°C. The complemented strain was designated *Enterobacter* C6-6 Δ *ecnAB*<pET101::*ecnAB*>. This vector incorporates a C-terminal His tag into the recombinant protein. We confirmed that the presumptive EcnB protein was present by Western blotting with and without induction using IPTG (isopropyl-β-D-thiogalactopyranoside) (see Fig. S2 in the supplemental material).

An *E. coli* C6-6 Δ *ecnAB* complement was created using a similar strategy. Briefly, the *E. coli* K-12 *ecnAB* locus was amplified using Platinum *Taq* Polymerase and the primer pair K12*ecnAB*CompR/*ecnAF*Screen (Table 1). After ligating the resulting amplicon into the pCR2.1-TOPO-vector (Invitrogen), TOP10 cells (Invitrogen) were chemically transformed and selected on LB agar with 100 µg/ml ampicillin. Transformants were cultured in LB broth with 100 µg/ml ampicillin overnight, and the plasmid was isolated using a PureYield plasmid miniprep system (Promega). Approximately 100 ng of plasmid was introduced into chemically competent *E. coli* K-12 Δ *ecnAB* cells by heat shock at 42°C for 30 s. Cells were immediately resuspended with SOC medium (35) and allowed to recover for 1 h at 37°C on a shaker. Finally, transformants were selected on LB plates supplemented with 34 µg/ml chloramphenicol and 100 µg/ml ampicillin. Successful complements were confirmed using the primer pairs T7F/M13R, *ecnAF*Screen/C1, and C2/*ecnBR*Screen (Table 1).

Inhibition plate assays. Inhibition plate assays were used to detect the inhibitory phenotype of *Enterobacter* C6-6 against *F. psychrophilum* CSF259-93 strain B17 (36). Strain B17 was used because it does not exhibit a gliding motility phenotype that would otherwise complicate inhibition plate assays (our unpublished data). B17 was inoculated into 20 ml of TYES broth 5 days prior to the experimental setup, grown at 15°C on a shaker, and adjusted to an optical density at 600 nm of approximately 0.2 in a 50-ml conical tube. The culture (75 µl) was evenly spread onto a TYES plate by use of glass beads to grow a "lawn" of *F. psychrophilum* on the agar surface. Plates were then allowed to dry briefly at 15°C. *Enterobacter* and *E. coli* strains were inoculated into 20 ml of TYES broth 2 days prior at 37°C and pelleted at 1,600 × g for 10 min at 15°C. The pellet was resuspended in 5 ml of TYES. The TYES plates were divided in quarters, and 30 µl of each respective test strain as well as exhausted supernatant of *F. psychrophilum* B17 was deposited on the agar surface in triplicate locations. Plates were incubated at 15°C for 5 to 10 days, until an *F. psychrophilum* B17 lawn was visible. The plates were photographed and analyzed with the software ImageJ (<http://imagej.nih.gov/ij/>). Areas of clearance were normalized by

TABLE 2 Treatment groups for the trout challenge trial

Treatment group ^a	Basal diet supplement ^b	Relative % survival (mean ± SEM) ^c
C6-6 wild type	Oil + <i>Enterobacter</i> strain C6-6	58.1 ± 5.59
<i>ecnAB</i> knockout	Oil + <i>Enterobacter</i> C6-6 Δ <i>ecnAB</i>	19.4 ± 22.3
<i>ecnAB</i> complement	Oil + <i>Enterobacter</i> C6-6 Δ <i>ecnAB</i> <pET101:: <i>ecnAB</i> >	80.7 ± 9.68
No-probiotic control	Oil only	Reference
Mock infection	Nothing added	100 ± 0

^a Fish were fed the treatment diet for 10 days before experimental subcutaneous challenge with *Flavobacterium psychrophilum* strain CSF 259-93 (1.03×10^7 CFU/fish). Treatment groups were fed the assigned diets and were then divided into three independent replicate tanks at the time of injection challenge with CSF 259-93. The mock infection group was treated with TYES medium. Tanks were subsequently monitored for an additional 28 days.

^b The basal diet consisted of an amount equivalent to 3% of body weight of Bio-Olympic Fry (1.5-mm pellet size; Bio-Oregon) with 5% (wt/vol) menhaden oil.

^c Relative percent survival (RPS) was calculated relative to the survival of the no-probiotic control group (see Materials and Methods for the formula). Numbers in bold represent groups with statistically significant differences from the no-probiotic control group ($P < 0.001$).

dividing by their respective drop/colony areas, and the means and standard errors were calculated for 7 to 14 independent replicates.

Fish and feed preparation. Rainbow trout fry were hatched at the Aquaculture Research Institute at the University of Idaho (Moscow, ID) and then transferred to the College of Natural Resources at the University of Idaho ($n = 500$; ~4 g each). Upon arrival, five freshly euthanized fish (see below) were tested for bacterial contamination by probing liver, spleen, and kidney tissues with sterile inoculation loops and streaking samples onto TS agar (Difco) and TYES agar. Additionally, a section of hind gut was aseptically dissected and the intestinal mucus squeezed onto a TS agar plate and streaked across the plate surface. All cultures were incubated for 96 h at 15°C. Remaining fish were divided into five groups and acclimated in flowthrough tanks with 500 liters of dechlorinated municipal water (10 to 15°C) and aeration through air stones. Fish were fed a commercial salmonid diet (Bio-Olympic Fry, 1.5 mm; Bio-Oregon) at a rate of 3% of body weight per day for 1 week for acclimation. Fish were assigned to treatment groups (Table 2) and were fed the same commercial salmonid diet with or without the addition of 5% menhaden oil (Table 2). After injection challenge on day 11 (see below), fish were divided into triplicate groups of 25 and transferred to 20-liter flowthrough tank systems (3 separate tanks per treatment group) with dechlorinated municipal water (10 to 15°C) and supplemental aeration by air stones.

Feed with the probiotic strains was prepared according to the method of Burbank et al. (24), with minor modifications. Briefly, the quantity of feed (g) was calculated daily, and rations were prepared fresh every day in 50-ml conical tubes. One colony of each strain (*Enterobacter* C6-6, *Enterobacter* C6-6 Δ *ecnAB*, or *Enterobacter* C6-6 Δ *ecnAB*<pET101::*ecnAB*>) was inoculated into 15 ml of TS broth without antibiotics and cultured on a shaker for 24 h at 15°C. The cells were pelleted at $1,600 \times g$ for 10 min and resuspended in 7.5 ml menhaden oil. A 5% menhaden oil-cell suspension (vol/wt) was mixed into the feed (except for the mock infection control), resulting in 10^6 to 10^8 bacterial cells per g as determined by the drop-plate method (28). Feeding started 10 days before infection and ended at 28 days postinfection.

Infection challenge. CSF 259-93 was grown in TYES broth at 15°C for 72 h before infection and harvested by centrifugation at $1,600 \times g$ for 15 min at 15°C. The supernatant was discarded, and the pellet was resuspended in $1 \times$ TYES to an optical density at 525 nm of 0.2. The number of CFU per ml was determined using the drop-plate method (28). Fish were withheld from feed for 24 h and then injected subcutaneously with 25 μ l of the virulent strain CSF 259-93 (1.03×10^7 CFU/fish) or with 25 μ l $1 \times$ TYES (mock-infected groups). Feeding was resumed 24 h after infection.

Daily mortalities were recorded, and at least 25% of dead fish per tank were evaluated for signs of BCWD and isolation of yellow-pigmented bacteria by culturing of the liver, spleen, and kidneys on TYES agar at 15°C for at least 96 h. The relative percent survival (RPS) was calculated according to the method of LaFrentz et al. (37), using the following formula: $RPS = [1 - (\text{cumulative percent mortality of treatment group/cumulative percent mortality of positive-control group})] \times 100$. Animal challenge experiments conformed to a protocol approved by the University of Idaho Institutional Animal Care and Use Committee.

Statistical analysis. SigmaPlot (v.11.0; Systat Software) was used for all statistical analysis. One-way analysis of variance (ANOVA) and Tukey's multiple-comparison test were used to evaluate results from the size-exclusion and inhibition plate assays. Kaplan-Meier curves were plotted for the survival analysis, based on pooled data across replicate treatment tanks ($n = 3$ per treatment), and the statistical differences were assessed by employing a log rank test and Tukey's multiple-comparison test. Because each treatment tank served as an independent replicate, we also compared relative percentages of survival between treatments by using ANOVA and Tukey's multiple-comparison test. The threshold for significance was a P value of < 0.05 .

Nucleotide sequence accession number. The contig identified to contain *ecnA* and *ecnB* was annotated and submitted to GenBank under accession no. [KM407562](https://www.ncbi.nlm.nih.gov/nuclot/KM407562).

RESULTS

The inhibitory protein has a mass of ≤ 3 kDa. A preliminary proteinase digest indicated that inhibition was likely caused by a soluble protein (see Fig. S1 in the supplemental material). Under the assumption that this putative protein would have a low mass, we fractionated supernatants from *Enterobacter* C6-6 and *E. coli* K-12 (≤ 3 kDa and > 3 kDa). These fractions, unfractionated supernatants, and a control that was composed of exhausted supernatant from CSF 259-93 culture were incubated with diluted CSF 259-93. The unfractionated supernatants of *Enterobacter* C6-6 and the ≤ 3 -kDa fractions inhibited the growth of CSF 259-93 ($P < 0.001$) (Fig. 1), while the larger fraction from *Enterobacter* C6-6 (> 3 kDa) did not have a significant effect on CSF 259-93 culture ($P > 0.05$). Unexpectedly, the *E. coli* K-12 strain produced an identical phenotype, where unfractionated medium and the ≤ 3 -kDa fraction inhibited growth of CSF 259-93 ($P < 0.001$) (Fig. 1), while the larger fraction (> 3 kDa) did not affect growth of *F. psychrophilum* ($P > 0.05$).

LC-MS/MS identified entericidin as a candidate protein. Supernatants of *Enterobacter* C6-6 and *E. coli* K-12 were concentrated by freeze-drying and separated using SDS-PAGE (Fig. 2). For both supernatants, a dense band was present in the mass range of approximately 3 kDa. The *Enterobacter* C6-6 band was excised and submitted to liquid chromatography and tandem mass spectrometry. This analysis identified three hypothetical proteins (Ent638_0594, Ent638_3274, and Ent638_3824), an lpp repeat-containing protein, and an entericidin B membrane lipoprotein. The latter protein has been implicated in cell lysis (38), and consequently, we focused our subsequent analysis on this protein.

***ecnAB* is required for *Enterobacter* C6-6 to inhibit *F. psychrophilum* in vitro.** *ecnAB* knockout and complement strains were engineered to demonstrate the role of the *ecnAB* locus in inhibiting *F. psychrophilum*. For these experiments, results from CSF 259-93 were difficult to analyze due to the swarming nature of the strain. Consequently, we report data only for strain B17, because it is deficient in gliding motility. Agar inhibition assays clearly demonstrated that the wild-type *Enterobacter* C6-6, wild-type *E. coli* K-12, and complemented *ecnAB* knockout strains (*Enterobacter* C6-6 Δ *ecnAB*<pET101::*ecnAB*> and *E. coli* K-12 Δ *ecnAB*

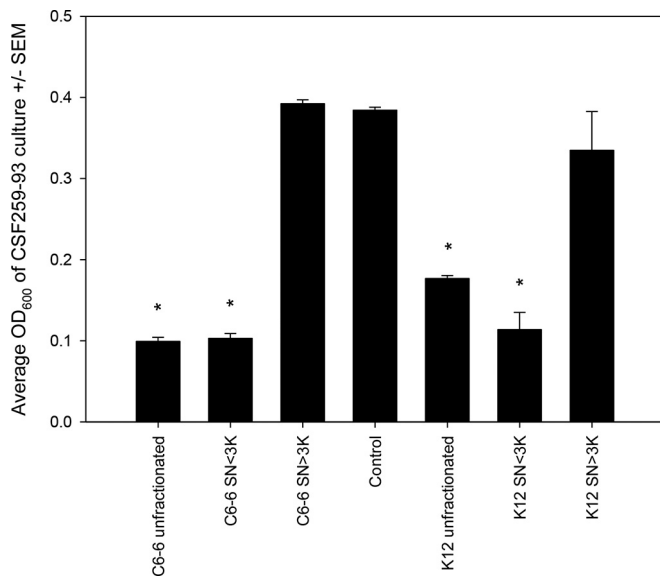


FIG 1 *Flavobacterium psychrophilum* (CSF 259-93) was cultured in the presence of supernatant from *Enterobacter* (C6-6) or *Escherichia coli* (K-12). Unfiltered supernatant and the ≤ 3 -kDa fraction from both species inhibited growth of *F. psychrophilum*, whereas treatment with the higher-mass fraction (> 3 kDa) was indistinguishable from treatment with spent medium from *F. psychrophilum* (Control). Bars represent means plus standard errors of the means (SEM). Asterisks represent differences from the control ($P < 0.05$; one-way ANOVA and Tukey's multiple-comparison test).

$\langle pCR2.1::ecnAB \rangle$) produced readily detectable areas of clearance against *F. psychrophilum* strain B17 ($P < 0.001$) (Fig. 3). No significant zone of clearance was detectable for the *ecnAB* knockout strains (*Enterobacter* C6-6 $\Delta ecnAB$ and *E. coli* K-12 $\Delta ecnAB$).

***ecnAB* is required for *Enterobacter* C6-6 to protect trout fry from BCWD challenge.** A health check at the onset of the fish trial

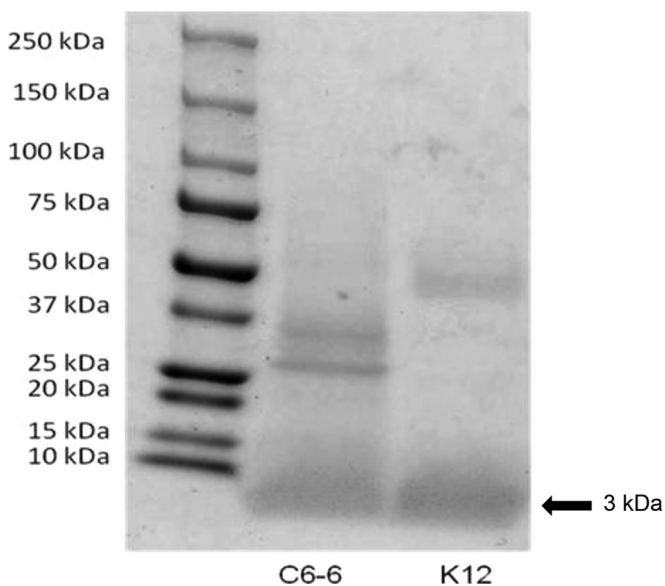


FIG 2 SDS-PAGE (10%) confirmed the presence of proteins of approximately 3 kDa (arrow) from concentrated supernatants that were collected from *Enterobacter* C6-6 and *E. coli* K-12. The C6-6 gel band was excised and submitted for analysis by LC-MS/MS.

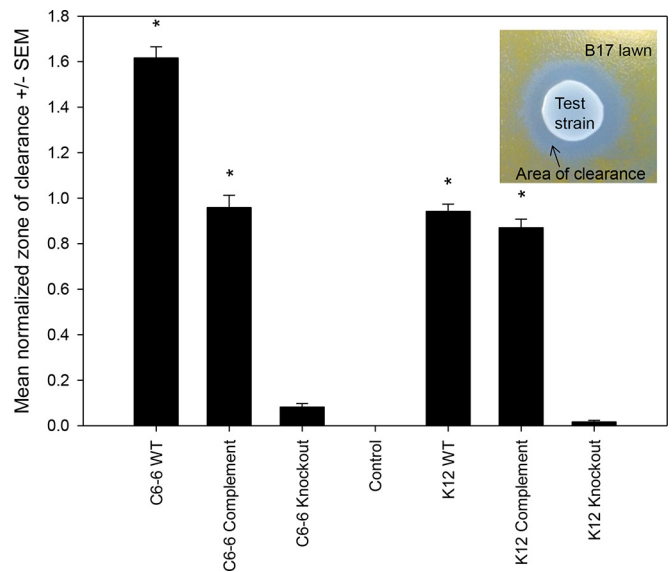


FIG 3 Deletion of the *ecnAB* locus results in loss of the inhibitory phenotype against *F. psychrophilum* strain B17, based on an agar inhibition assay. Areas of clearance were measured as total pixels of clearance divided by the total area of the test strain (center of the inset). Mean values were calculated for 7 to 14 independent replicates, and error bars represent the standard errors of the means. C6-6 WT, *Enterobacter* C6-6; C6-6 Complement, *Enterobacter* C6-6 $\Delta ecnAB \langle pET101::ecnAB \rangle$; C6-6 Knockout, *Enterobacter* C6-6 $\Delta ecnAB$; Control, exhausted supernatant from *F. psychrophilum* B17 culture; K-12 WT, *E. coli* K-12; K-12 Complement, *E. coli* K-12 $\Delta ecnAB \langle pCR2.1::ecnAB \rangle$; K-12 Knockout, *E. coli* K-12 $\Delta ecnAB$. Statistical differences were assessed using one-way ANOVA and Tukey's multiple-comparison test. Asterisks represent differences from the control ($P < 0.001$).

found no evidence of either *Enterobacter* or *F. psychrophilum* from the intestinal contents or liver, spleen, and kidney tissues of selected fish. Three independent groups of trout fry were fed one of several treatment diets (Table 2). As expected, fish that were fed the wild-type *Enterobacter* C6-6 strain and then challenged with CSF 259-93 experienced fewer mortalities than those of the no-probiotic control group ($P < 0.001$) (Fig. 4). Consistent with our hypothesis that *ecnAB* is requisite for the probiotic activity of *Enterobacter* C6-6, the mortality of fish treated with the *ecnAB* knockout strain (*Enterobacter* C6-6 $\Delta ecnAB$) was not different from that of the no-probiotic control group ($P = 0.84$), while the group treated with the complemented strain (*Enterobacter* C6-6 $\Delta ecnAB \langle pET101::ecnAB \rangle$) was not different from the group treated with wild-type *Enterobacter* C6-6 ($P = 0.61$). The RPS values for the groups fed *Enterobacter* C6-6 and *Enterobacter* C6-6 $\Delta ecnAB \langle pET101::ecnAB \rangle$ were 58.1% and 80.7%, respectively, while the group fed *Enterobacter* C6-6 $\Delta ecnAB$ had a 19.4% relative percent survival (Table 2). The mock infection control group experienced no mortalities during the experimental period.

DISCUSSION

Burbank et al. (23) first showed that *Enterobacter* C6-6 culture or spent medium inhibited growth of *F. psychrophilum* in a manner consistent with the presence of a soluble inhibitor. Herein we successfully identified a small protein (entericidin B) in the supernatant of *Enterobacter* C6-6 cultures that is linked to the inhibitory phenotype *in vitro*. Burbank et al. (24) showed that when *Enterobacter* C6-6 was used as an oral probiotic treatment, there was a

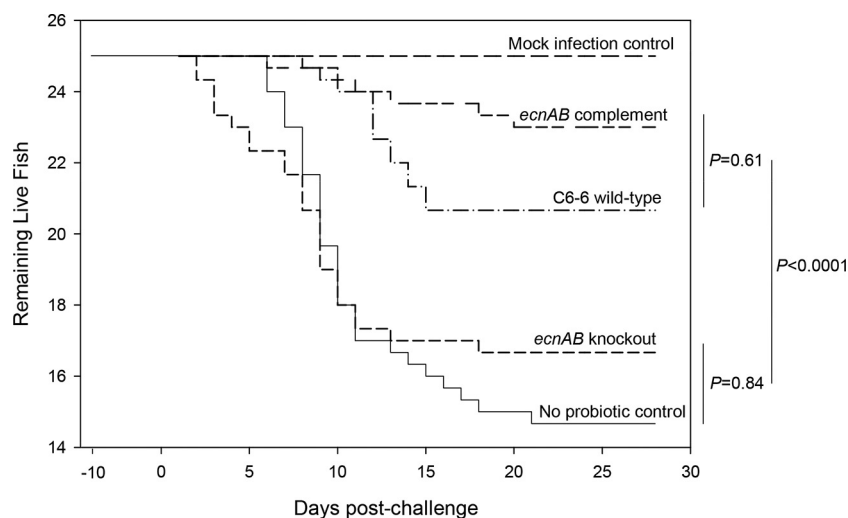


FIG 4 Deletion of *ecnAB* eliminates the probiotic benefit of *Enterobacter* C6-6. Four groups of fish (25 fish per group in triplicate) were fed a standard trout diet with feed supplemented with menhaden oil mixed with “*ecnAB* complement” (*Enterobacter* C6-6 Δ *ecnAB*<pET101::*ecnAB*>), “C6-6 wild-type” (*Enterobacter* C6-6), “*ecnAB* knockout” (*Enterobacter* C6-6 Δ *ecnAB*), or “No probiotic ctrl” (oil only; no added *Enterobacter*). After 10 days of supplemented feeding, these groups were challenged by subcutaneous injection with a virulent strain of *F. psychrophilum* (CSF 259-93). One group of fish (25 fish in triplicate) was fed the standard diet without any supplementation and served as a mock infection control. Daily mortalities were recorded for 28 days after infection. For this analysis, data were pooled across replicate treatment tanks ($n = 3$ per treatment), and statistical analysis was performed using the log rank test.

significant reduction in mortality after injection challenge with the virulent *F. psychrophilum* strain CSF 259-93. At the time of this work, it was unclear if the putative soluble inhibitor protein found by Burbank et al. (23) was responsible for this protection or if protection was conferred by an alternative mechanism, such as cross-reactive immunity, that is consistent with recent findings (39) (see discussion below). We demonstrated that the soluble protein produced by the *ecnAB* locus is required for inhibition of *F. psychrophilum* both *in vitro* and *in vivo*. We did not determine if the protein encoded by the *ecnAB* locus is actively secreted or if this is a passive release mechanism after normal bacterial cell death.

The *ecnAB* locus encodes two small peptides, designated EcnA and EcnB (44 and 48 amino acids, respectively) (38). *ecnA* is located upstream from *ecnB*, with 106 intervening bases that include a putative translation terminator and promoter region. The locus was first described by Bishop et al. (38) as a putative toxin-antitoxin system in *E. coli* because of hypothesized similarities to a plasmid addiction module, *mazEF*, that has been studied in *E. coli* (40). EcnA was designated the antitoxin that neutralizes the cell toxin EcnB based on the observation that overproduction of EcnB resulted in a greater degree of bacteriolysis, while expression of *ecnA* and *ecnB* in *cis* produced only minor bacteriolytic activity. Bishop et al. (38) further suggested that *ecnB* expression is promoted by a stationary-phase sigma promoter that is active only under hyperosmotic and starvation culture conditions, and consequently this putative toxin-antitoxin system could serve a role akin to programmed cell death. This is a novel but controversial idea because, for instance, deletion of the putative *ecnA* antitoxin gene alone should be lethal, but this is not the case for the *E. coli* Keio collection (41).

If the protein produced by the *ecnAB* locus is secreted and bacteriolytic as we hypothesize, then it clearly has a much broader spectrum of activity than that of a classic bacteriocin (42). For example, sequence homologues of *ecnB* have been annotated in

the chromosomes of diverse species of proteobacteria, indicating that this trait is highly conserved and may play a largely unappreciated role in structuring microbial communities (see Fig. S4 in the supplemental material). As an example of this potential, we originally intended *E. coli* K-12 to serve as a negative-control strain for our phenotypic assays, but we instead discovered that K-12 also inhibits growth of *F. psychrophilum*, and we confirmed the role of the *E. coli* *ecnAB* locus by using a gene knockout and complementation strategy. Additionally, we also used our inhibition assay to confirm that *Enterobacter* C6-6 inhibits growth of *Flavobacterium columnare* (see Fig. S3), the etiologic agent of columnaris disease, which is a devastating disease of cold- and warm-water fish species (43).

While a secreted or passively released toxin could easily explain the *in vitro* activity against *F. psychrophilum*, the mechanism by which the toxin functions *in vivo* is not clear. Our *in vitro* data suggest that a direct interaction is required for the entericidin to inhibit *F. psychrophilum*, but this presents a conundrum for our experimental model, whereby the majority of the *Enterobacter* C6-6 bacteria reside in the gut, while the pathogen challenge occurs through subcutaneous injection of *F. psychrophilum*. *Enterobacter* species will colonize the skin of fish (44), possibly via fecal contamination of the water, and subsequently may interact directly with the pathogen at the site of the injection. Our lab experience suggests that inhibition is quickly diluted, and fish skin generally supports low population densities of bacteria (44), so it is unclear if bacteria on the skin surface can play a role in inhibition following injection challenge. It is possible that large quantities of the toxin are released systemically by the gastric *Enterobacter* C6-6 flora, but our *in vitro* experiments showed no evidence that large quantities of toxin are produced.

Another scenario might involve coresidency in leukocytes. Professional phagocytic cells in fish, especially those from the spleen, which have less production of reactive oxygen species (ROS), are probably responsible for disseminating *F. psychrophilum*.

lum *in vivo* (45). If these cells also harbor *Enterobacter* C6-6, then the proximal condition could be satisfied. Alternatively, de Vries et al. (46) described the *ecnAB* locus as being essential for *Moraxella catarrhalis* to adhere to the upper and lower respiratory tracts of humans. Consequently, it is possible that these membrane lipoproteins serve a dual role as lytic peptides and adhesion molecules. If these proteins are freely available *in vivo*, they may directly affect *F. psychrophilum* while competing for adhesion sites within the fish host. Testing for the distribution of the toxin (e.g., by Western blotting) in different host tissues might resolve this question.

Indirect mechanisms may also be involved with *in vivo* protection. For example, as has been speculated for other probiotics (47), it is possible that the entericidin enhances barrier functions and the innate immune response. In this scenario, the entericidin could elicit release of antimicrobial compounds into the mucus or induce production of a cross-reactive IgM that helps to protect against *F. psychrophilum* in mucus and blood (48). LaPatra et al. (39) recently demonstrated that intraperitoneal injection of live and formalin-killed *Enterobacter* C6-6 produced a protective immune response against challenge with *F. psychrophilum* CSF 259-93, while treatment with culture supernatant failed to induce a significant increase in the titer of antibodies against *F. psychrophilum*.

Regardless of the mechanism, it is clear from the current study that the entericidin locus is requisite for the probiotic activity of *Enterobacter* C6-6, but further work is needed to understand the mechanism of action *in vivo*. Given the apparent conservation of entericidin, it is conceivable that it plays a large and unappreciated role in structuring microbial communities, and this is potentially the case for mammalian microbiota as well. The small size of these peptides (~48 amino acids for EcnB) also means that it may be possible to efficiently produce large quantities for use in different microbiological and industrial applications.

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Kenneth D. Cain has applied for intellectual property protection for the probiotic strain C6-6. Ponnerassery S. Sudheesh works as a postdoctoral fellow in the Cain lab. None of the remaining authors have a commercial affiliation.

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