JOURNAL OF BACTERIOLOGY, May 2008, p. 3467–3474 Vol. 190, No. 10
0021-9193/08/$08.00+0 doi:10.1128/JB.00075-08

Cold Shock Exoribonuclease R (VacB) Is Involved in *Aeromonas hydrophila* Pathogenesis▽

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Received 15 January 2008/Accepted 6 March 2008

In this study, we cloned and sequenced a virulence-associated gene (*vacB*) from a clinical isolate SSU of *Aeromonas hydrophila*. We identified this gene based on our recently annotated genome sequence of the environmental isolate ATCC 7966^T of *A. hydrophila* and the *vacB* gene of *Shigella flexneri*. The *A. hydrophila* VacB protein contained 798 amino acid residues, had a molecular mass of 90.5 kDa, and exhibited an exoribonuclease (RNase R) activity. The RNase R of *A. hydrophila* was a cold-shock protein and was required for bacterial growth at low temperature. The *vacB* isogenic mutant, which we developed by homologous recombination using marker exchange mutagenesis, was unable to grow at 4°C. In contrast, the wild-type (WT) *A. hydrophila* exhibited significant growth at this low temperature. Importantly, the *vacB* mutant was not defective in growth at 37°C. The *vacB* mutant also exhibited reduced motility, and these growth and motility phenotype defects were restored after complementation of the *vacB* mutant. The *A. hydrophila* RNase R-lacking strain was found to be less virulent in a mouse lethality model (70% survival) when given by the intraperitoneal route at as two 50% lethal doses (LD_{50}). On the other hand, the WT and complemented strains of *A. hydrophila* caused 80 to 90% of the mice to succumb to infection at the same LD_{50} dose. Overall, this is the first report demonstrating the role of RNase R in modulating the expression of *A. hydrophila* virulence.

A human diarrheal isolate SSU of *Aeromonas hydrophila* possesses various pathogenic mechanisms that contribute to the overall virulence of this bacterium in the host. Most importantly, our laboratory has described type II, type III, and type VI secretion system effector proteins that are involved in the disease state (gastroenteritis or septicemia) in animal models (11, 15, 17, 21, 32, 44–46, 48). However, to better understand the full virulence potential of any pathogen, it is important to identify new pathogenic factors and/or mechanisms that could be involved in their virulence. This is crucial since the expression of different virulence genes could be contributing factors leading to disease depending upon the anatomical niche where the organisms colonize and the environment in those regions which dictates the differential expression of genes (18).

Studies by Tobe et al. (50) identified a novel chromosomal gene, *vacB*, which was required for the production of *S. flexneri* virulence factors (e.g., IpaB, IpaC, IpaD, and VirG) from the large plasmid (230 kb) of this microorganism. More recently, it was shown that the *vacB* gene in *S. flexneri* and *Escherichia coli* strains encoded the 3′-5′ exoribonuclease RNase R (10). The deduced size of VacB (virulence-associated) protein is approximately 92 kDa, and it is located at 95 min on the *E. coli* chromosome, a position consistent with the earlier mapping studies of the gene encoding RNase R (10). These investigators provided evidence that RNase R has an essential cell function, in addition to its role in bacterial virulence, and hence they renamed the *vacB* gene to *rnr* (RNase R) based on its sequence similarity to another exoribonuclease, RNase II (5). Exoribonucleases (RNase R, polynucleotide phosphorylase [PNPase], and others) belong to the RNR family that has homologs widespread in most sequenced prokaryotic and eukaryotic genomes (6, 49), and they mediate many aspects of RNA metabolism and, in particular, the quality control of rRNA (9). More recent studies revealed multifaceted roles of exoribonucleases in the regulation of gene expression in pathogenic bacteria (3, 39, 55). For example, in *Streptococcus pyogenes* it was found that exoribonuclease PNPase activity was rate limiting for the decay of *sagA* and *sda* transcripts that coded for streptolysin S and streptodornase in the late exponential growth phase of the bacterium (3). PNPase enhanced the ability of *Yersinia pseudotuberculosis* and *Y. pestis* to withstand the killing activities of murine macrophages and was required for the optimal functioning of the *Yersinia* T3SS (39). In *Salmonella enterica* PNPase, apart from being a regulator of the cold shock response, it also functioned in tuning the expression of virulence genes and bacterial fitness during infection (55).

The first report regarding a broad-specificity RNase from *Aeromonas* was published for a gene from *A. hydrophila* AH1133 strain that coded for an RNase I-like RNase (20). In the present study, we describe the identification, cloning, and sequencing of the *vacB* gene from *A. hydrophila* SSU. Further, we demonstrate that VacB indeed is RNase R, since it exhibited exoribonuclease activity, and showed both a cold-sensitive

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▽ Published ahead of print on 14 March 2008.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
A. hydrophila		
SSU		CDC[a]
SSU-R	Rif^r strain of *A. hydrophila* SSU	Laboratory stock
vacB-Ω mutant	Isogenic *vacB* gene mutant of *A. hydrophila* SSU-R strain, Rif^r Sm^r Sp^r	This study
vacB-Ω/pBR322	*vacB*-Ω mutant strain containing pBR322 vector, Rif^r Sm^r Sp^r Ap^r Tc^r	This study
vacB-Ω/pBR322-*vacB*	*vacB*-Ω mutant complemented with the *vacB* gene via pBR322, Rif^r Sm^r Sp^r Ap^r	This study
E. coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcr*BC) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Sm^r) *endA1 nupG*	Invitrogen
JM109	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* λ$^-$ Δ(*lac-proAB*) [F′ *traD36 proA*$^+$*B*$^+$ *lacIZ*Δ*M15*]	Promega
SM10 (λ*pir*)	Km^r *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *pir*	14
K12 CAN20-12E	RNase I$^-$ II$^-$ D$^-$ BN$^-$, Tc^r	7
K12 CAN20-12ER$^-$	RNase I$^-$ II$^-$ D$^-$ BN$^-$ R$^-$, Tc^r Km^r	7
K12 CAN20-12ER$^-$(pBAD)	RNase I$^-$ II$^-$ D$^-$ BN$^-$ R$^-$, Tc^r Km^r Ap^r	This study
K12 CAN20-12ER$^-$(pBAD/*vacB*)	RNase I$^-$ II$^-$ D$^-$ BN$^-$ R$^-$, Tc^r Km^r Ap^r	This study
Plasmids		
pCR2.1	TA cloning vector, Ap^r Km^r	Invitrogen
pCR2.1/*vacB*	TA cloning vector carrying the *vacB* gene, Ap^r Km^r	This study
pHP45Ω	pHP45 plasmid containing a 2-kb Ω element, Ap^r Sm^r Sp^r	33
pCR2.1/*vacB*-Ω	TA cloning vector harboring the *vacB* gene disrupted by Ω element, Ap^r Sm^r Sp^r	This study
pJQ200SK	Suicide vector, P15A origin, *sacB* Gm^r	36
pJQ200SK/*vacB*-Ω	Suicide vector containing the *vacB* gene with Ω element, Gm^r Sm^r Sp^r	This study
pBR322	Ap^r Tc^r	Amersham
pBR322/*vacB*	Contains the *vacB* gene, Ap^r	This study
pBAD/Thio-E	Arabinose-inducible *araBAD* promoter-based vector, Ap^r	Invitrogen
pBAD	Control derivative plasmid, Ap^r	15
pBAD/*vacB*	pBAD expression vector with the *vacB* gene, Ap^r	This study

[a] CDC, Centers for Disease Control and Prevention, Atlanta, GA.

phenotype and that VacB modulated virulence of *A. hydrophila* in in vitro and in vivo models of *Aeromonas* infection.

MATERIALS AND METHODS

Strains, plasmids, and media. Bacterial strains and plasmids used in the present study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria-Bertani (LB) broth and LB agar plates (41). LBNS (LB with no NaCl) solid medium with 15% sucrose (Suc) for levansucrase (*sacB*) counterselection was used for the isolation of the *vacB* isogenic mutant. The antibiotics ampicillin (Ap), kanamycin (Km), gentamicin (Gm), streptomycin (Sm), spectinomycin (Sp), tetracycline (Tc), and rifampin (Rif) were used at concentrations of 100, 50, 20, 30, 30, 15, and 200 μg/ml, respectively.

DNA isolation and PCR assays. Plasmid DNA was isolated by using a QIAprep spin miniprep kit from Qiagen, Valencia, CA. *A. hydrophila* genomic DNA (gDNA) for sequencing was isolated by using the published protocol with some modifications (35) or by utilizing a DNeasy tissue kit (Qiagen) for PCR assays. DNA fragments were purified by using a PCR purification kit or gel extraction kit (Qiagen). The primers (Table 2) were synthesized by Integrated DNA Technologies, Inc., Coralville, IA.

Transformation of *E. coli* and electroporation of *A. hydrophila* strains. The preparation of competent cells for transformation and electroporation was de-

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Purpose
vacB-N	ATGATCCTCGACCTCATCAAGGGCCATGAA	PCR amplification of a portion (2.0 kb) of the *vacB* gene
vacB-C	TCACGAAGTCGATCTTGCGATCGTCCAGAT	
vacB-N1	TGCTGGTCTCGGCGAACAGGTGCCG	DNA sequencing of the PCR product to obtain middle part of the *vacB* gene
vacB-C1	CACCAGACCGTCGATGTGGATCTCG	
vacB-N/TGA	CTCACCAACGACTACTACCAGTTCGACCCG	DNA sequencing of the gDNA to obtain the ORF of the *vacB* gene
vacB-C/ATG	CAGTTGACCGTCGCGCTCCATGGCGCGCAG	
vacB-N/up	GTCTCATCATGACTGCAAACTGTGCATAGTATGAC	Cloning of the *vacB* gene into pCR2.1 vector
vacB-C/down	GGGGCCATCAGCCCCTTACGACCTCAGACCGGGTC	
vacB-N/g	GAACGCCTGCTTGAGATAGGCGGCGGATTGGGCGA	PCR verification of the *vacB* mutant
vacB-C/g	AGTAACAGGGTTCGGATTTTTTTCGTTTTCAGGAC	
vacB/HindIII^N	CCCAAGCTTGTCTCATCATGACTGCAAACTGTGCA	Cloning of the *vacB* gene into pBR322 vector
vacB/SalI^C	ACGCGTCGACGGGGCCATCAGCCCCTTACGACCTC	
vacB/AflIII^N	CCCACATGTCTCAAAAAGATCCTTTCCTCGAACGCGAGGC	Cloning of the *vacB* gene into pBAD/Thio-E vector
vacB/PmeI^C	AGCTTTGTTTAAACTTACCCCTTGGCCTTGTCGCGCGCTT	

[a] Underlining indicates restriction endonuclease site.

scribed previously (15, 47). Competent *E. coli* cells for transformation were prepared by using a Z-Competent transformation kit from Zymo Research, Orange, CA. The competent *A. hydrophila* cells in 300 mM sucrose were electroporated in 0.2-cm gap cuvettes (Bio-Rad, Hercules, CA) by using a Gene-Pulser Xcell.

Sequencing of the *A. hydrophila* chromosomal *vacB* gene and construction of its knockout mutant. To sequence the *vacB* gene of *A. hydrophila* SSU strain, we first PCR amplified a portion (~2.0 kb) of this gene using gDNA of the wild-type (WT) bacterium as the template and the *vacB*-N/*vacB*-C pair of primers (Table 2). To sequence the middle part of the *vacB* gene, the above PCR product was DNA sequenced with *vacB*-N1/*vacB*-C1 primers. To determine the open reading frame (ORF) of the *A. hydrophila* SSU *vacB* gene, gDNA was sequenced using primers *vacB*-N/TGA and *vacB*-C/ATG (Table 2).

To clone the *vacB* gene (2394-bp) into pCR2.1 vector (Invitrogen, Carlsbad, CA), the target gene with up- and downstream DNA flanking sequences was PCR amplified using gDNA and the *vacB*-N/up and *vacB*-C/down primers, and the ligation mixture was transformed into chemically competent *E. coli* TOP10 cells (Table 1). To generate the *vacB* knockout mutant, an Sm/Sp cassette (the Ω element) from a pHP45Ω plasmid (33) was introduced into a unique SmaI restriction enzyme site of the *vacB* gene in the pCR2.1/*vacB* recombinant plasmid. Then, the *vacB*-Ω fragment from a pCR2.1/*vacB*-Ω plasmid was cloned into a suicide pJQ200SK vector (36) at the XbaI and SpeI restriction enzymes sites and transformed into *E. coli* SM10 (with λ*pir*) strain (14). After conjugation between *A. hydrophila* SSU and the *E. coli* SM10 strain harboring the pJQ200SK/*vacB*-Ω plasmid, the knockout *vacB* mutant was screened on LBNS plates containing Rif, Sp, Sm, and sucrose. From single *A. hydrophila* SSU colonies with a Rif-, Sp-, Sm-, and sucrose-resistant but Gm-sensitive-phenotype, we isolated gDNA and performed PCR to confirm the disruption of the *vacB* gene using *vacB*-N/g and *vacB*-C/g primers (Table 2). The mutant colonies were identified as *Aeromonas* by a positive oxidase test to differentiate them from *E. coli*, which is oxidase negative (26).

Complementation of the *A. hydrophila vacB* mutation. The *vacB* gene was PCR amplified by using the *vacB*/HindIII^{N} and *vacB*/SalI^{C} primers (Table 2) and *A. hydrophila* gDNA as the template. We included 125-bp of the upstream flanking sequence containing the potential promoter region of the *vacB* gene for complementation studies. The PCR product was purified by a PCR purification kit, digested with appropriate enzymes, and ligated to the pBR322 vector at the HindIII-SalI restriction-enzymes sites. This ligation mixture was then transformed into *E. coli* JM109 cells, and plasmid DNA was isolated from Ap^{r} and Tc^{s} transformants. The presence of pBR322/*vacB* plasmid (Table 1) was confirmed by restriction endonuclease mapping of the recombinant plasmid. Then, pBR322/*vacB* plasmid DNA was electroporated into the *vacB* isogenic mutant of the *A. hydrophila* strain. As a negative control, the *A. hydrophila vacB* mutant strain, containing the pBR322 vector alone, was also generated (Table 1).

Cloning of the *vacB* gene under the *araBAD* promoter. To regulate the expression of the *vacB* gene, the recombinant plasmid pBAD/*vacB* was generated by using the *vacB*/AflIII^{N} and *vacB*/PmeI^{C} primers (Table 2) by replacing the NcoI-PmeI fragment of the pBAD/Thio-E vector (Invitrogen) under its arabinose P_{BAD} promoter. To induce expression of the gene from the plasmid, arabinose (0.2%) was added to the medium (24). To construct the pBAD plasmid for use as a control, pBAD/Thio-E vector was digested with NcoI and PmeI restriction endonucleases, treated with DNA polymerase I (Klenow fragment), and ligated. Both plasmids (pBAD and pBAD/*vacB*) were then electroporated in *E. coli* K-12 strain CAN20-12ER^{-} (RNases I^{-}, II^{-}, D^{-}, BN^{-}, and RNase R^{-}) (Table 1) for measuring RNase R activity.

Preparation of cell extracts. Overnight-grown *E. coli* K-12 strains (CAN20-12E [RNases I^{-}, II^{-}, D^{-}, BN^{-}, but RNase R^{+}], CAN20-12ER^{-} with pBAD and CAN20-12ER^{-} with pBAD/*vacB*) were reinoculated in the morning, and at an optical density at 600 nm of 0.4 arabinose, at a final concentration of 0.2%, was added. After 4 h of growth, the pellets from 10-ml cultures were resuspended in 1 ml of solution containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 300 mM KCl, and 0.1 mM phenylmethylsulfonyl fluoride (7). After incubation for 1 h on ice with lysozyme (300 μg/ml) and 0.1% Triton X-100, the cells were disrupted by sonication on ice by using two 10-s pulses. The cell extracts were recovered by centrifugation at 13,000 × *g* for 10 min at 4°C to remove cell debris, and the protein concentration of the extracts was determined by a Bradford assay (4). Bovine serum albumin was used as the standard. *E. coli* K-12 strain CAN20-12E was used as the positive control, and *E. coli* strain CAN20-12ER^{-} containing pBAD vector alone was used as the negative control.

Assay for RNase R. The assay for RNase R activity was conducted according to the described procedure (7). Reaction mixtures (100 μl) contained 20 mM Tris-HCl (pH 8.0), 0.25 mM $MgCl_2$, 180 mM KCl, 40 μg of [^{3}H]poly(A) (50 to 100 cpm/nmol; GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and 30 μg of the indicated cell extracts. Reaction mixtures were incubated at 37°C for 1 h. After precipitation with 10% cold trichloroacetic acid, the release of acid-soluble radioactivity was determined by using a Beckman scintillation counter LS 6500.

Cold growth assay. For the cold-growth experiment, *A. hydrophila* overnight cultures (WT, *vacB* mutant, and complemented strains) were reinoculated in LB broth with the appropriate antibiotics and grown at 37°C up to an optical density at 600 nm of 0.4, and then the diluted (10^{-5}) strains were plated (50 μl) on LB agar plates with antibiotics. The plates were incubated for 7 days at 4°C. As a control, plates inoculated similarly were incubated at 37°C.

Motility assay. The equal numbers of CFU of tested *Aeromonas* cultures were stabbed into 0.3% LB agar plates and incubated at 37°C overnight. Their motility was assayed by examining the migration of bacteria through the agar from the center toward the plate's periphery (15).

Cytotoxicity assay. RAW 264.7 murine macrophages were seeded onto 96-well plates (10^{5} cells/well) and infected with either the live WT *A. hydrophila* SSU strain or its *vacB* knockout mutant at an multiplicity of infection of 10 for 4 h (43). In another set of experiment, host cells were treated with 5 μl of filter-sterilized, overnight-grown (at 37°C with shaking [180 rpm] in selected LB medium) bacterial culture supernatants. After incubation at 37°C for 2 h, the tissue culture medium was examined for the release of lactate dehydrogenase enzyme by using a CytoTox96 kit (Promega, Madison, WI). The percentage of lactate dehydrogenase released by RAW 264.7 macrophages infected with bacterial cells was determined according to the manufacturer's instructions. In macrophages treated with culture supernatants, cytotoxicity was reported per milliliter of the culture filtrate per 10^{8} CFU and was expressed as a fold change compared between WT *A. hydrophila* SSU and the *vacB* mutant strains. The purpose of these experiments was to show whether VacB directly or indirectly contributed to host cell toxicity by altering functionality of type III and type VI secretion systems (17, 44–46, 48). The culture filtrates were used to demonstrate whether the isogenic mutant of the *vacB* gene affected cytotoxic and hemolytic activities associated with a potent cytotoxic enterotoxin (Act) or other secreted cytotoxins or hemolysins produced by *A. hydrophila* SSU (17, 21, 44).

Hemolytic activity. *A. hydrophila* SSU strains (WT, *vacB* knockout, and complemented) were grown at 37°C overnight with shaking (180 rpm) in selected LB medium. The cultures were spun down, and supernatants were added to the first well in each row of a 96-well microtiter plate, followed by serial twofold dilution (before 100 μl of 1× Dulbecco phosphate-buffered saline [DPBS] was added to each of the wells of the microtiter plate). Then, 100 μl of 3% rabbit erythrocytes (Colorado Serum Co., Denver, CO) was added, and the plate was incubated at 37°C for 1 h. A control included 1× DPBS alone. The supernatants were taken from wells that showed partial lysis of red blood cells, and the hemoglobin release was recorded at 540 nm by using a microplate reader. The hemolytic units were reported per ml/10^{8} CFU of culture supernatants.

Animal experiments. Eight-week-old female Swiss-Webster mice were inoculated intraperitoneally with a lethal dose (3×10^{7} CFU, representing two 50% lethal doses) of WT, *vacB* knockout mutant, and *vacB*-complemented *Aeromonas* strains in groups of 10 mice each. One mouse group was inoculated with DPBS and served as a control. Mice were observed daily for signs of distress and mortality for up 2 weeks.

Statistics. Wherever applicable, at least three independent experiments were performed. The data were analyzed by using Student *t* test or the Fisher exact test, and *P* values of ≤0.05 were considered significant.

Nucleotide sequence accession number. The nucleic acid and translated amino acid sequences of the *A. hydrophila* SSU strain *vacB* gene were deposited in the GenBank database under accession number EU380595.

RESULTS

Identification and cloning of the *A. hydrophila* SSU *vacB* gene. To define additional genetic determinants that could control virulence gene expression in a pathogenic diarrheal *A. hydrophila* SSU isolate, the nucleotide sequence of the *vacB* gene in *S. flexneri* (50) was compared to that of the genome sequence of *A. hydrophila* ATCC 7966^{T} (42) from the TIGR database, which we recently annotated. We noted that the gDNA of *A. hydrophila* ATCC strain exhibited a 66% identity (from nucleotide position 84493 to 86780) with the *S. flexneri vacB* gene. Based upon the ORF sequence of the *vacB* gene of *A. hydrophila* ATCC 7966^{T}, the *vacB*-N and *vacB*-C primers were designed (Table 2) to obtain a PCR product, using gDNA of *A. hydrophila* SSU strain as the template. One major band

TABLE 3. RNase R activity of VacB from the *A. hydrophila* SSU strain[a]

E. coli K-12 strain	Mean cpm of [^{3}H]poly(A) ± SEM	Statistical significance (*P*)	
CAN20-12E	49,348.35 ± 78.77	<0.001	>0.05
CAN20-12ER$^-$(pBAD)	19,049.68 ± 72.37		
CAN20-12ER$^-$(pBAD/*vacB*)	48,438.36 ± 94.34	<0.001	

[a] For the RNase R activity assay, we used the same total protein concentration (30 μg) for each indicated *E. coli* K-12 strain cell extract.

(~2.0 kb) was PCR amplified and then sequenced using the "internal" primers *vacB*-N1 and *vacB*-C1 to obtain the complete sequence of this DNA product (a portion of *vacB* gene). Finally, to identify the coding region of the *vacB* gene of *A. hydrophila* SSU, we used chromosomal DNA sequencing with the *vacB*-N/TGA and *vacB*-C/ATG primers (Table 2). For further studies, the *vacB* gene was cloned into the EcoRI site of a pCR2.1 plasmid vector using PCR products from gDNA and primers without any sites for restriction endonucleases. The inserted *vacB* gene was verified by DNA sequencing of the recombinant plasmid pCR2.1/*vacB* (Table 1) with M13 forward and reverse primers.

The *vacB* gene encoded a protein (RNase R) of 798 amino acid residues with a molecular mass of 90.5 kDa. The overall identity of the *A. hydrophila* SSU *vacB* gene with that of *A. hydrophila* ATCC 7966^T was 94%. At the protein level, RNase R (VacB) of the *A. hydrophila* SSU strain showed a 97% homology with *A. hydrophila* ATCC 7966^T RNase R, 94% homology with *A. salmonicida* A449 RNase R (GenBank accession no. YP_001140610), 65% homology with *S. flexneri* VacB (GenBank accession no. D11024), 65% homology with *E. coli* CFT073 RNase R (GenBank accession no. NP_757111) (53), and 62% homology with RNase R of *Y. pestis* CO92 (GenBank accession no. NP_404028) (31).

VacB of *A. hydrophila* SSU strain possesses RNase R activity. To assess the ability of *A. hydrophila* VacB to show RNase R activity, cell extracts were tested from the *E. coli* K-12 strain CAN20-12ER$^-$ that lacks RNases I, II, D, BN, and RNase R activities but carries the recombinant pBAD/*vacB* plasmid (Table 1). *E. coli* K-12 strain CAN20-12ER$^-$ containing pBAD vector alone was used as a negative control (RNase R$^-$ cell extract), and *E. coli* K-12 CAN20-12E (10) deficient in RNases I, II, D, and BN activities, but not in RNase R activity, was used as a positive control strain for this assay (RNase R$^+$ cell extract). As noted from Table 3, cell extract from the *E. coli* K-12 strain CAN20-12ER$^-$ with the pBAD/*vacB* plasmid showed an RNase R activity comparable to that of the positive control. In contrast, the RNase R activity in the cell extracts of control *E. coli* CAN20-12ER$^-$ (with the pBAD vector alone) strain was 61% lower than that of the positive control. The *E. coli* K-12 strains indicated above were grown in the presence of 0.2% arabinose to regulate the *vacB* gene expression from the *araBAD* promoter and to allow similar growth conditions for all bacterial cultures. We could not directly measure RNase R activity in the cell extracts of the WT versus the *vacB* mutant of *A. hydrophila* SSU since other RNases masked the effect of RNase R.

RNase R of *A. hydrophila* SSU is a cold-shock protein. To address whether the *A. hydrophila* SSU RNase R is a cold-shock protein, *A. hydrophila* WT, *vacB* knockout, and complemented strains were tested for their ability to grow at low temperatures (Fig. 1). The *vacB* mutant strain was unable to grow at low temperatures (Fig. 1B), whereas the WT strain was able to grow at 4°C (Fig. 1A). The complemented strain harboring the recombinant plasmid that encoded RNase R showed the recovery of this strain to grow at a low temperature (Fig. 1C). In order to delineate whether the *vacB* mutant of *A. hydrophila* died at 4°C or was dormant, we shifted the temperature of growth from 4 to 37°C after an initial incubation period of 7 days. We observed no growth of the *vacB* mutant after the temperature shift for up to 48 h. The above-mentioned *A. hydrophila* WT, mutant, and the complemented cultures grew similarly at 37°C (data not shown). These results indicated that the *vacB* gene is required for the *A. hydrophila* SSU strain to grow at a low temperature.

Effect of RNase R on *A. hydrophila* SSU virulence. To evaluate the effect of RNase R activity associated with the *vacB* gene on the pathogenesis of the *A. hydrophila* SSU strain, we

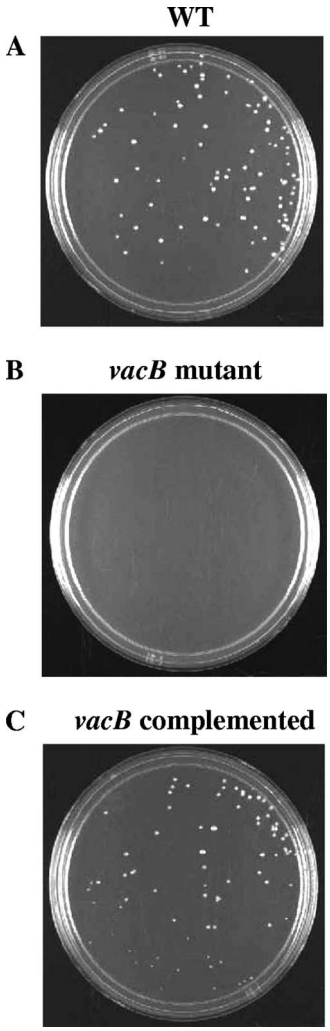

FIG. 1. Effect of RNase R on cold growth of the bacterium. *A. hydrophila* WT (A), *vacB* knockout mutant (B), and complemented (C) strains were plated (same CFU) on LB agar plates with appropriate antibiotics and incubated at 4°C for 7 days.

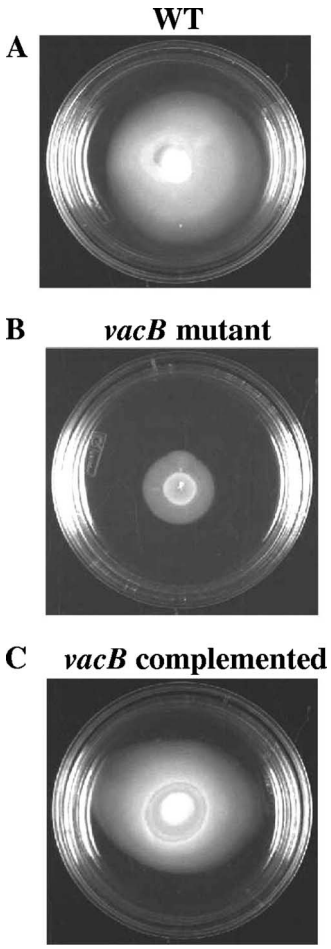

FIG. 2. *A. hydrophila* SSU with knockout mutant of the *vacB* gene shows a reduced motility (B) compared to those of the WT and complemented *A. hydrophila* strains (A and C).

assessed the biological activities of various virulence factors of WT *A. hydrophila* and its isogenic *vacB* mutant. The motility of *A. hydrophila* is an important factor for bacteria to reach the host target tissue, to colonize, and then to cause disease (22). We noted that the *vacB A. hydrophila* mutant strain had 57% less motility (Fig. 2B) than the WT strain (Fig. 2A). The motility phenotype was restored in the strain that was complemented with the pBR322/*vacB* recombinant plasmid (Fig. 2C).

The cytotoxicity on macrophages essentially remained unaltered when infected with the WT and the *vacB* knockout mutant strain of *A. hydrophila* SSU, indicating that the RNase activity was not essential for modulating cytotoxicity associated with various effectors of type III and type VI secretion systems (data not shown). Also, we did not observe any differences in the hemolytic and/or cytotoxic activities in culture supernatants of *Aeromonas* WT and its *vacB* mutant strain, indicating that VacB did not modulate the activities of Act and the other hemolysins (e.g., HlyA) or cytotoxins that we recently described in *A. hydrophila* SSU (16).

Subsequently, we examined the effect of RNase R of *A. hydrophila* in contributing to mouse mortality. We injected mice intraperitoneally with the isogenic v*acB* gene mutant strain, the *vacB* complemented strain (pBR322/*vacB*), and the WT control strain with pBR322 plasmid at a lethal dose of 3×10^7 CFU (Fig. 3). The animals infected with the *A. hydrophila* control strain or the complemented strain showed 80 to 90% mortality within 3 days. However, mice infected with the *vacB* mutant strain exhibited significant lower mortality (only 30%) over a tested period of 15 days, indicating bacterial attenuation as a result of a loss in the RNase R activity. We chose to use two 50% lethal doses for these studies since higher doses would not be appropriate because of other virulence factors (e.g., Act, AexU [T3SS effector], and hemolysin-coregulated

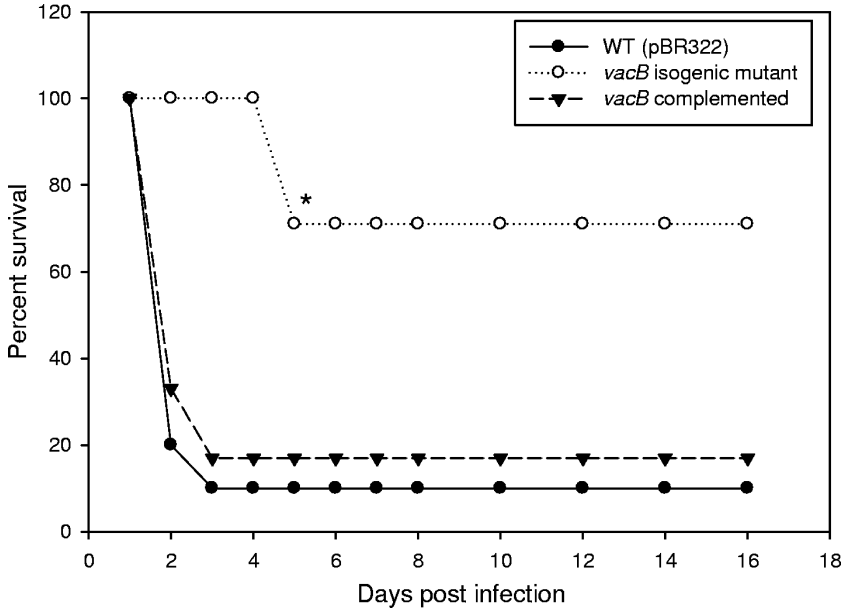

FIG. 3. The *A. hydrophila vacB* knockout mutant strain was less virulent in a mouse model. The complemented strain (pBR322/*vacB*) led to mouse mortality within 48 h, similar to the findings with the WT *A. hydrophila* strain harboring pBR322 plasmid. An asterisk denotes statistically significant values as determined by the Fisher exact test.

protein [type VI secretion system effector]) (45, 46, 48) produced by *A. hydrophila* that also lead to mouse lethality.

DISCUSSION

A majority of the studies concerned with exoribonucleases indicate their prominent role in quality control of rRNA (9), cell cycle-regulated degradation of tmRNA (small stable RNA) (25), and mRNA decay (8), which is a significant determinant of gene expression. Since publication of the report regarding the role of the *vacB* gene in the pathogenesis of serotypes of *Shigella* spp. and enteroinvasive *E. coli* strains (50), only recently did studies emerge that indicated a potential contribution of RNases, in particular PNPase, in regulating bacterial virulence (12, 39, 55). However, the role of RNase R in bacterial virulence is not fully explored. Interestingly, the *vacB* gene of *Brucella abortus* has no impact on bacterial virulence (29). Recent studies did indicate that the expression of RNase R is tightly regulated by temperature (6) and that it is essential for growth of the organism at low temperatures (e.g., 4°C) (34). These investigators demonstrated that the cold-sensitive phenotype of the *rnr* mutant was due to the cell death of the mutant strain and that defective protein synthesis was one of the important factors responsible for killing of *Pseudomonas syringae rnr* mutants at low temperature (34). These data confirmed our results indicating that RNase R from *A. hydrophila* SSU strain is a cold-shock protein. We did not observe growth of the *vacB* (*rnr*) mutant strain at a low temperature, in contrast to the findings with *Aeromonas* WT and complemented strains (Fig. 1). Our data also indicated that the *vacB* mutant died when grown at 4°C. It is plausible that VacB might have a regulatory role and could modulate the translation of some proteins in *A. hydrophila* SSU that are required for bacterial growth. We will investigate this possibility as well as the ability of the *vacB* mutant to inhibit protein synthesis in our future study.

As with RNase R, the exoribonuclease PNPase is also clearly required for *Y. pseudotuberculosis* and *Y. pestis* to grow at low temperatures (e.g., 5°C), and PNPase plays a multifaceted role in enhancing yersiniae survival in response to stressful conditions (39). Likewise, the role of PNPase in the proper configuration of the T3SS effector proteins was also reported (39). For the *rnr* mutant of *E. coli*, a growth defect characterized by the formation of colonies that were considerably smaller than that of the WT strain was noted (6); however, these investigators used 10°C temperature as the cold shock conditions. Interestingly, PNPase-deficient mutants of *P. putida* did not exhibit a cold-sensitive phenotype at 15 and 5°C (19). It is possible that the expression of the RNase gene and adaptation to the cold may be different in various bacteria.

Motility is an important virulence factor of gram-negative bacterial pathogens and is a very complex process that depends on the cooperation of many gene products (23, 28). We explored the role of RNase R in the pathogenesis of *A. hydrophila* and showed the decreased motility of the *vacB* mutant strain (Fig. 2). The experiments with some *rnc* (RNase III$^-$) mutants supported the notion that RNase III is involved in the motility of *E. coli* (2). These researchers noted that the mutants of *E. coli* deficient in RNase III were nonmotile. Further, these investigators also showed that all transductants and revertants that regained RNase III showed motility-positive phenotypes and that all transductants that remained or became *rnc*$^-$ were nonmotile.

Based on our observations, RNase R from *A. hydrophila* SSU did not alter other biological activities associated with this pathogen, e.g., the ability to exhibit hemolytic and cytotoxic activities. However, in a future study, we will explore the possibility whether disruption of the *vacB* gene from *A. hydrophila* alters expression of the *act* and/or the hemolysin gene. Earlier it was shown that a cationic protein secreted by human eosinophils which exhibited structural homology to other RNases that had a RNase activity was not essential for cytotoxicity (38). On the contrary, a recent study showed that the exoribonuclease PNPase was necessary for *Yersinia* to affect the morphology of HeLa cells and that the PNPase-dependent cytotoxicity of *Yersinia*-infected host cells was delayed relative to the WT-infected HeLa cells (39). It was also reported that a *Burkholderia* strain living inside the arbuscular mycorrhizal fungus *Gigaspora margarita* possessed the *vacB* gene, which was involved in host cell colonization by this pathogenic bacteria (40).

Most significantly, we observed that RNase R mutant of the *A. hydrophila* strain led to the attenuation of virulence in a mouse lethality model. A similar pattern of mouse mortality was shown for the SmpB-SsrA system (small protein B-small stable RNA A, also known as tmRNA) in *Y. pseudotuberculosis*. Deletion of *smpB-ssrA* genes from *Y. pseudotuberculosis* rendered the bacterium avirulent in a mouse model of lethality (30). Previous studies also indicated that the *ssrA* gene played a role in the full virulence of *S. enterica* serovar Typhimurium. A >200-fold virulence defect was observed in BALB/c mice and was attributed to the altered expression of several genes induced during infection with the *ssrA* mutant (13, 27). Recently, it was reported that RNase R is required for the selective degradation of SsrA RNA in stalked cells and that SmpB binding controls the timing of SsrA RNA degradation by RNase R (25). *E. coli* has an enabling mechanism that requires RNase R activity and is dependent on the presence of SmpB protein and tmRNA, suggesting a requirement for active *trans*-translation in facilitating RNase R engagement and promoting nonstop mRNA decay (37). Most surprisingly, RNase II and PNPase do not play a significant role in the tmRNA-facilitated disposal of aberrant mRNAs (37). Whether VacB regulates RNA decay in *A. hydrophila* SSU is unknown and will be explored in a future study.

Thus, on one hand, *vacB* was first defined as a virulence factor (50), but on the other hand, VacB is known to be an exoribonuclease RNase R involved in mRNA posttranscriptional processing, ribosome rescue, and rRNA methylation (9, 10, 51). The process of mRNA decay is integral to the posttranscriptional control of gene expression, and mRNA turnover is a means of coordinating this process, first through integration with control of transcription and export and translation of mRNAs and second through enabling mRNAs involved in similar processes to decay at similar rates (54). Very recently, a study indicated that the *Salmonella* mutants deficient in several RNases (RNase E, RNase G, RNase III, and PNPase) that affected sRNA and mRNA turnover were very important for posttranscriptional studies in this bacterial model of pathogenesis (52).

In nature, bacteria remain mostly in the stationary phase of

the life cycle, and RNase R can be a modulator of gene expression in this phase of cell growth (1). The impressive spectrum of bacterial diseases in the world probably can be explained by the ability of pathogens to modify adequately the gene expression in response to environmental stimuli and to control virulence factors by RNA-mediated transcriptional regulators. RNase R could be one such regulator that could modulate bacterial virulence and, hence, future studies will focus on a global assessment of host responses after infection of animals with the WT and *vacB* mutant of *A. hydrophila*.

ACKNOWLEDGMENTS

We thank Murray Deutscher (University of Miami School of Medicine, Miami, FL) for providing *E. coli* K-12 CAN20-12E and CAN20-12ER$^-$ strains. We thank Mardelle Susman for editorial assistance.

This study was supported by a grant from the NIH/NIAID (AI41611) and from the Environmental Protection Agency. The NSF grant (EF-0334247) that was conduit for getting the ATCC 7966 *A. hydrophila* genome sequenced is also acknowledged.

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