Functional Genomic Characterization of Virulence Factors from Necrotizing Fasciitis-Causing Strains of Aeromonas hydrophila

Christopher J. Grim, Food and Drug Administration
Elena V. Kozlova, University of Texas Medical Branch
Duraisamy Ponnusamy, University of Texas Medical Branch
Eric C. Fitts, University of Texas Medical Branch
Jian Sha, University of Texas Medical Branch
Michelle L. Kirtley, University of Texas Medical Branch
Christina J. van Lier, University of Texas Medical Branch
Bethany L. Tiner, University of Texas Medical Branch
Tatiana E. Erova, University of Texas Medical Branch
Sandeep J. Joseph, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: Applied and Environmental Microbiology
Volume: Volume 80, Number 14
Publisher: American Society for Microbiology | 2014-07-01, Pages 4162-4183
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/AEM.00486-14
Permanent URL: https://pid.emory.edu/ark:/25593/s2tp5

Final published version: http://dx.doi.org/10.1128/AEM.00486-14

Copyright information:
© 2014, American Society for Microbiology.

Accessed April 23, 2020 1:51 PM EDT
**Functional Genomic Characterization of Virulence Factors from Necrotizing Fasciitis-Causing Strains of Aeromonas hydrophila**

Christopher J. Grim, Elena V. Kozlova, Duraisamy Ponnusamy, Eric C. Fitts, Jian Sha, Michelle L. Kirtley, Christina J. van Lier, Bethany L. Tiner, Tatiana E. Erova, Sandeep J. Joseph, Timothy D. Read, Joshua R. Shak, Sam W. Joseph, Ed Singletary, Tracy Felland, Wallace B. Baze, Amy J. Horneman, Ashok K. Chopra

Food and Drug Administration, Laurel, Maryland, USA; Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, USA; Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine, Atlanta, Georgia, USA; Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland, USA; Maryland Institute of Applied Environmental Health, School of Public Health, University of Maryland, College Park, Maryland, USA; Doctors Hospital, Augusta, Georgia, USA; Mercy Hospital and Trauma Center, Janesville, Wisconsin, USA; Department of Veterinary Sciences, M. D. Anderson Cancer Center, Bastrop, Texas, USA; Pathology and Laboratory Medical Services, VA Maryland Health Care System, Baltimore, Maryland, USA

The genomes of 10 *Aeromonas* isolates identified and designated *Aeromonas hydrophila* WI, Riv3, and NF1 to NF4; *Aeromonas dhakensis* SSU; *A. jandaei* Riv2; and *A. caviae* NM22 and NM33 were sequenced and annotated. Isolates NF1 to NF4 were from a patient with necrotizing fasciitis (NF). Two environmental isolates (Riv2 and -3) were from the river water from which the NF patient acquired the infection. While isolates NF2 to NF4 were clonal, NF1 was genetically distinct. Outside the conserved core genomes of these 10 isolates, several unique genomic features were identified. The most virulent strains possessed one of the following four virulence factors or a combination of them: cytotoxic enterotoxin, exotoxin A, and type 3 and 6 secretion system effectors AexU and Hcp. In a septicemic-mouse model, SSU, NF1, and Riv2 were the most virulent, while NF2 was moderately virulent. These data correlated with high motility and biofilm formation by the former three isolates. Conversely, in a mouse model of intramuscular infection, NF2 was much more virulent than NF1. Isolates NF2, SSU, and Riv2 disseminated in high numbers from the muscular tissue to the visceral organs of mice, while NF1 reached the liver and spleen in relatively lower numbers on the basis of colony counting and tracking of bioluminescent strains in real time by *in vivo* imaging. Histopathologically, degeneration of myofibers with significant infiltration of polymorphonuclear cells due to the highly virulent strains was noted. Functional genomic analysis provided data that allowed us to correlate the highly infectious nature of *Aeromonas* pathotypes belonging to several different species with virulence signatures and their potential ability to cause NF.

A seminal paper regarding *Aeromonas* species as a cause of human infection was first published in 1968 (1). Since then, water-associated infections with aeromonads have been on the rise (2, 3). Further, the high resistance of *Aeromonas* species to both water chlorination, especially in biofilms, and multiple antibiotics (4) squarely resulted in the placement of this organism on the EPA’s Contaminant Candidate List 2 (5) and its categorization as an emerging human pathogen. Indeed, *Aeromonas* species were isolated as pure cultures from 22% of the wounds of infected patients during the 2004 tsunami in southern Thailand (6). In addition to causing severe wound infections, this pathogen is associated with hemolytic-uremic syndrome and necrotizing fasciitis (NF) (7–11). The two earliest human cases of NF with *Aeromonas* were published in 1997 (12, 13), while the latest reported case of NF resulted from trauma and freshwater contact following a motorcycle accident (14).

Over the past 2 decades, several virulence factors of *Aeromonas* have been extensively characterized. For example, an aerolysin-related cytotoxic enterotoxin (Act) is a type 2 secretion system (T2SS)-secreted exotoxin with the ability to cause both diarrhea and severe tissue damage in the host (15–19). The toxin leads to apoptosis or necrosis of host cells, depending upon the dose (16–18, 20, 21). A functional T3SS and two potent and diverged T3SS effectors, namely, AexT and AexU, have been identified in aeromonads (22), with GTPase-activating protein (GAP) and ADP-ribosyltransferase (ADP-RT) activities, leading to host cell death. Likewise, two T6SS effectors, hemolysin-coregulated protein (Hcp) and the valine-glycine repeat G (VgrG) family of proteins (VgrG1,-2, and -3) have been reported and characterized (23, 24). The biological effects of translocated VgrG proteins are associated with their specific C-terminal extensions. In *Aeromonas hydrophila* SSU (recently reclassified as *Aeromonas dhakensis* sp. nov. comb. nov. (25)), we have shown that VgrG1 carries a C-terminal vegetative insecticidal protein 2 (VIP-2) domain with ADP-RT activity, and its translocation into host cells leads to the ADP-ribosylation of actin, resulting in cytotoxic effects (24). Likewise, the T6SS-dependent translocation of Hcp was first shown by our group in *A. dhakensis* SSU-infected human colonic epithelial cells, which led to caspase 3 activation and subsequent apoptosis of host cells (23). In addition to its ability to be translocated into host cells, the secreted form of Hcp inhibited bacterial phagocytosis (26).

*Aeromonads* have various quorum-sensing (QS) systems...
that could modulate bacterial virulence genes (27, 28). Likewise, the ability of bacteria to swim and swarm contributes to the overall virulence of aeromonads (29, 30). It is unclear whether any of these virulence factors/mechanisms of Aeromonas species contribute to the pathogenesis of NF or whether NF is a host-mediated disease. During NF caused by group A streptococcus (GAS), superantigens produced by the bacteria interact with major histocompatibility complex II of the antigen-presenting cells, as well as the β-chain of the T-cell receptor and the receptor of the CD28 costimulatory molecule (31). However, such superantigens have not yet been identified in aeromonads. Clearly, individuals who are somehow predisposed to NF genetically, physiologically, or immunologically are at risk (32). With this variation in host susceptibility, exposure to a large number of Aeromonas bacteria of a particular pathotype in a unique environment could likely lead to a rapid, fulminant, and necrotic infection, requiring rapid surgical and chemotherapeutic intervention. To provide some answers, we performed genome-wide sequencing of NF-causing strains of A. hydrophila and compared them with other clinical and environmental isolates of aeromonads to identify any unique or pathotype-specific genes that could be implicated in NF. In addition, we developed an NF mouse model of infection and monitored the dissemination of bacteria in this model in real time by in vivo imaging.

### MATERIALS AND METHODS

#### Bacterial strains

The sources of the Aeromonas strains and plasmids used in this study are listed in Table 1. Two A. hydrophila isolates successively cultured from a patient with NF were designated NF1 and NF2. These NF strains were recovered from the patient during and immediately after amputation surgery (culture of stump tissue) and preserved in Trypticase soy broth with 30% glycerol at −80°C. Following extended cultivation, two additional colony types, NF3 and NF4, were isolated from the same sheep blood agar (SBA) plate as NF2. These two colonies appeared much smaller than the dominant isolate, NF2.

Since major pathogenic aeromonads form beta-hemolytic colonies relatively similar in colony size, morphology, and color on SBA plates, it is highly conceivable that the original culture from the amputated leg was a “mixed culture” of similar-looking Aeromonas strains, namely, A. hydrophila NF1 and NF2. No other Gram-positive or Gram-negative organisms were isolated, respectively, from well water (NM3) and from a child with diarrhea (NM22) who consumed that contaminated well water (33). Other Gram-positive or Gram-negative organisms were isolated from the cultures. During a second culture of tissue taken later from the amputation stump, a single colony of the persistent strain, NF1-Rifr, was chosen for identification. Two environmental isolates (A. jandaei Riv2 and A. hydrophila Riv3) were isolated from the river water from Dhakensi. These isolates were confirmed in this study to be strains of A. caviae on the basis of phylogenetic analysis of the rpoD gene (data not shown). A. hy-

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. dhakensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSU-Rifr</td>
<td>Rifr strain of A. dhakensis SSU</td>
<td>CDC</td>
</tr>
<tr>
<td>SSU-Rifr-luc</td>
<td>Strain with Tn7-luciferase operon</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>SSU ΔhyRI mutant</td>
<td>hyRI gene deletion mutant of A. dhakensis SSU</td>
<td>This study</td>
</tr>
<tr>
<td>SSU ΔluxS mutant</td>
<td>luxS gene deletion mutant of A. dhakensis SSU</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>SSU ΔqieB mutant</td>
<td>qieB gene deletion mutant of A. dhakensis SSU</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 7966T</td>
<td>Canned milk isolate</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 7966T-Rifr-luc</td>
<td>Rifr strain of A. hydrophila ATCC 7966T and Tn7-luciferase operon</td>
<td>This study</td>
</tr>
<tr>
<td>NF1</td>
<td>Initial isolate from patient wound site</td>
<td>This study</td>
</tr>
<tr>
<td>NF1-Rifr-luc</td>
<td>Strain with spontaneous rifampin resistance and Tn7-luciferase operon</td>
<td>This study</td>
</tr>
<tr>
<td>NF2-NF4</td>
<td>Morphologically distinct isolates obtained successively from NF1 wound site following amputation and surgical debridement</td>
<td>This study</td>
</tr>
<tr>
<td>NF2-Rifr-luc</td>
<td>Rifr strain of A. hydrophila NF2 with Tn7-luciferase operon</td>
<td>This study</td>
</tr>
<tr>
<td>WI</td>
<td>Tracheal aspirate</td>
<td>This study</td>
</tr>
<tr>
<td>Riv3</td>
<td>River water isolate</td>
<td>This study</td>
</tr>
<tr>
<td>A. jandaei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riv2</td>
<td>River water isolate</td>
<td>This study</td>
</tr>
<tr>
<td>Riv2-Rifr-luc</td>
<td>Rifr strain of A. jandaei Riv2 with Tn7-luciferase operon</td>
<td>This study</td>
</tr>
<tr>
<td>A. caviae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM22</td>
<td>Stool sample isolate</td>
<td>33°</td>
</tr>
<tr>
<td>NM33</td>
<td>Well water isolate</td>
<td>33°</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA103</td>
<td>ExoA-positive strain</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>ΔexoA mutant</td>
<td>exoA gene deletion mutant of P. aeruginosa PA103</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>Plasmid pUTmini-Tn5::luxKm2</td>
<td>Km'</td>
<td>Laboratory stock</td>
</tr>
</tbody>
</table>

* Origin, Philippines.
* ATCC, American Type Culture Collection.
* Origin, United States.
drophila strain WI, resistant to multiple antibiotics (ampicillin–subbac-
tam, amoxicillin–clavulanic acid, aztreonam, ceftazolin, cefoxime, cip-
rolloxacin, ertapenem, gentamicin, imipenem, and levofloxacin), was
from the tracheal aspirate of a patient with acute adult respiratory and
kidney failure. A. dhakensis SSU was obtained from the Centers for Dis-
ease Control and Prevention (CDC) and was originally obtained from a
patient with diarrhea during a cholera-like outbreak in the Philippines.

Biochemical identification of Aeromonas isolates. All of the bacterial
strains were identified by conventional methods, specifically, Aerokey II
(34) and additional biochemical tests (35). River water samples were col-
clected and transported in whirl–Pak bags (Nasco, Fort Atkinson, WI), and
10-ml aliquots were passed through 47-mm paper filters (pore size, 0.45
µm). The filters were placed on ampicillin–dextrin agar with vancomycin
to select for aeromonads (this medium detects all Aeromonas species ex-

Genome sequencing and annotation. Genome sequencing of A. hy-
drophila NFI and NF2 and A. dhakensis SSU was performed at the Emory
Genome Center with GS–FLX (454 Life Sequencing, Branford, CT). The
number of reads was 519,429 for strain NFI, 418,086 for NF2, and 261,221
for strain SSU. The estimated average coverage of the NFI, NF2, and SSU
genomes was 23, 19-, and 11-fold, respectively. Genomes were assembled
with Newbler software (37). The draft genome of strain NFI was
4,809,530 bp on 170 contigs, that of NF2 was 4,789,684 bp on 154 contigs,
and that of strain SSU was 4,879,288 bp on 281 contigs.

Genome sequencing of A. hydrophila Riv3 and WI, A. caviae NM22
and NM33, and A. jandaei Riv2 was performed at the University of Texas
Medical Branch (UTMB) Molecular Genomics Core Facility with a HiSeq
1000 (Illumina, San Diego, CA) by using the 2 × 50-cycle paired-end
protocol. Trimmed reads were assembled into contigs by using ABYSS
(38). The draft genome of strain WI was 5,172,023 bp on 112 contigs, that
of Riv2 was 4,478,089 bp on 43 contigs, and that of strain Riv3 was
4,861,432 bp on 58 contigs. The draft genomes of strains NM22 and
NM33 contained 4,419,058 bp on 250 contigs and 4,470,246 bp on 162
contigs, respectively.

Genome sequencing of A. hydrophila NF3 and NF4 was performed on a
MiSeq (Illumina, San Diego, CA) by using paired-end version 2 chem-
istry. The number of reads was 2,169,752 for strain NF3 and 6,762,904 for
strain NF4. Paired-end FASTQ data sets were trimmed and assembled
into contigs by using CLC Genomics Workbench, version 6.0.5 (CLC bio,
Aarhus, Denmark). A draft genome of strain NF3 was 4,757,900 bp on 197
contigs, and that of NF4 was 4,799,984 bp on 156 contigs. The estimated
average coverage of NF3 and NF4 genomes was 42- and 50-fold, respec-
tively. Genomic contigs were annotated by using the RAST annotation
server (39) to identify RNAs and protein–coding genes.

Comparative genomic analyses. Comparative genomic analysis was
performed to identify shared and dispensable genetic traits among the
Aeromonas strains that were sequenced. In addition to the 10 genomes
sequenced during this study, the closed genomes of A. hydrophila ATCC
7966T (GenBank accession no. CP000462.1), from a tin of milk with a fishy
odor (40), and A. salmonicida A449 (GenBank BioProject PRIN58631),
from a brown trout (41), as well as the draft genomes of A. hydrophila E1 and
E2 (SRA063950), from a patient with a wound infection (42), A. aquat-
orum AAK1 (GenBank accession no. BAFL00000000.1), recently reclassi-
fied as A. dhakensis sp. nov. comb. nov. (25) and from the blood of a
patient with a patient with sepsis and NF (43), A. veronii B565 (GenBank accession
no. CP002607.1), from aquaculture pond sediment (44), and A. caviae
Ac398 (GenBank Whole-Genome Shotgun project no. CACP00000000)
from a child with profuse diarrhea (45), were used.

To facilitate comparisons, the genomes of these other seven aeromon-
ads were also annotated by using the RAST annotation server. Genome-
to-genome comparisons were performed primarily with a SEED viewer
(46), which uses bidirectional protein–protein BLAST (blastp) sequence
comparison of translated open reading frames. For all draft genomes,
genomes at the end of a contig or interrupted by contig gaps were analyzed by
using bidirectional BLASTN analysis against all other genomes. Genomic
regions (GRs), defined as regions present in one (unique) or more
genomes and missing from at least one other genome (dispensable), were
identified as previously reported (42) for the genomes of A. hydrophila
ATCC 7966T, WI, Riv3, NF1 to NF4, E1, and E2; A. dhakensis SSU; and A. jandaei
Riv2 (47, 48). Average nucleotide identity (ANI) by BLAST was computed with
JSpecies (49). Evolutionary analyses were conducted in
MEGA5 (50). The act (cytotoxic enterotoxin) gene locus was compared
among different Aeromonas strains by using the Artemis Comparison
Tool (51).

Core cluster alignment and phylogenetic inference. The complete
predicted proteome from the 16 Aeromonas genomes was searched against
itself by using BLASTP with an E value cutoff of 1e–05. The best blast
scores were converted into a normalized similarity matrix with the
OrthoMCL (52) algorithm, which uses an additional step of Markov Clus-
tering algorithm (MCL) to improve the sensitivity and specificity of the
orthologous sequences identified. Core genes were identified as the pro-
tein–coding gene clusters that were shared by all of the 16 Aeromonas
isolates used in this study. Multiple-sequence alignments of the core pro-
tein–coding genes were generated with the program MUSCLE (53) by
using default settings. These core alignments were filtered for uninforma-
tive characters by GBlocks (54) by using default settings. Whole-gen-
ome phylogeny was determined with a concatenation of aligned individ-
ual core protein sequences, followed by neighbor joining (NJ) with
NEIGHBOR in the PHYLIP package (55, 56). Distances were identified with
the PROTDIST program within PHYLIP. The support of the data for
each of the internal node of the phylogeny was estimated by using 100
bootstraps.

In vitro characterization of Aeromonas strains for various virulence
traits. On the basis of the literature and our own earlier studies, biofilm
formation, swimming and swarming motility, protease production, and
toxin/effector secretion via different secretion systems are correlated with
the virulence of aeromonads in animal models. We have refined several of
these in vitro assays to correlate gene expression with the functionality of
the target gene to obtain reliable and quantitative data.

Crystal violet (CV) biofilm assay. To measure solid-surface-associated
biofilms, a modified biofilm ring assay (57, 58) was used. Biofilm
formation was then quantified (39), and the results were normalized to
1 × 107 CFU to account for any minor differences in the growth rates of
the various bacterial strains used. The CFU count was determined by serial
dilution of the samples, followed by plating (33, 58).

Motility assay. Luria–Bertani (LB) medium with 0.35% Difco Bacto
agar (Difco Laboratories, Detroit, MI) was used to characterize swimming
motility, while Difco nutrient broth with 0.5% Eiken agar (Eiken Chem-
ical Co., Ltd., Tokyo, Japan) was used to measure the swarming motility of
aeromonads (30, 42, 58). These methods provided better quantification of
the motility data.

Measurement of protease activity. Protease activity was measured in
culture filtrates of Aeromonas isolates grown overnight (60). The hide
powder azure substrate was used to measure protease activity (42). Pro-
tease activity was calculated per milliliter of culture filtrate per 108 CFU as
determined by serial dilution of the samples and colony counting.

Measurement of hemolytic activity. To measure hemolytic activity
associated with Act, the culture filtrates from various Aeromonas isolates
were first treated with trypsin (final concentration, 0.05%) at 37°C for 1 h
and then subjected to a hemolytic activity assay with rabbit erythrocytes
(61). Hemolytic activity titers were calculated as the absorbance at 540 nm of
the hemoglobin release multiplied by the dilution factor of the culture
filtrates. Units of hemolytic activity were reported per milliliter of cell
filtrate/1 × 108 CFU (58). Although Act is active against erythrocytes of
other animal and human species, rabbit red blood cells are more sensitive
to the action of Act and hence were used in this study.

For the neutralization assay, culture filtrates of the strains studied were
mixed with either preimmune (control) or hyperimmune rabbit serum
(laboratory stock, 1:10 dilution) containing antibodies to Act (42, 62) and
incubated at 37°C for 1 h before use.
Lactone production. N-Acyl-homoserine lactone (AHL) production was detected by cross-streaking various Aeromonas strains against the biosensor Chromobacterium violaceum CV026 on LB agar plates (27, 42). Violet pigment production from C. violaceum CV026 by AHLs produced by Aeromonas isolates was scored on the basis of color intensity after overnight incubation of the plates at 37°C. The result was recorded as no lactone production (−) or a low (+), moderate (++), or high (+++) level of AHLs. An isogenic shyRI mutant of A. dhakensis SSU (Table 1) was used as a negative control because of its inability to produce AHLs (62).

**Western blot analysis.** Western blot detection was used to detect the production and secretion of Hcp (a T6SS effector), AexU (a T3SS effector), and exotoxin A (ExoA) by various Aeromonas isolates. Briefly, the supernatants and cell pellets from cultures grown overnight were separated and the pellets were directly lysed in SDS-PAGE loading buffer. The proteins in the supernatants were first precipitated with 10% (vol/vol) trichloroacetic acid and then dissolved in the loading buffer before being subjected to SDS-PAGE. For Western blot analysis, specific antibodies to Hcp and AexU (available in the laboratory) (22, 26, 63) and to ExoA of Pseudomonas aeruginosa (LSBio, Inc., Seattle, WA) were used. At the amino acid level, ExoA of A. hydrophila is 65% homologous to that of P. aeruginosa (64). The bacterial strains were grown overnight in the LB medium at 37°C. The yield of ExoA produced by bacteria was influenced by the concentration of iron in the culture medium (65, 66).

**Generation of bioluminescent Aeromonas strains.** The reporter strains were generated following triparental conjugation of rifampin-resistant (Rif') Aeromonas isolates with Escherichia coli SM10 p pir carrying the pTNS2 plasmid and SM10 harboring the pUC18-mini-Tn7-T-Km2-lux plasmid (67). The minitransposon system contains a lux luminescence operon with the native promoter and a kanamycin resistance (Km') cassette for transposon selection. This system allows site-specific transposition downstream of the glmS gene, which encodes a conserved glucosamine-6-phosphate synthetase, with the helper plasmid pTNS2 providing the transposase complex (67). The mutants were screened for bioluminescence by using an ImageQuant LAS4000 bioluminescence and fluorescence imaging workstation (GE Healthcare Sciences, Pittsburgh, PA). The reporter strains that were oxidase positive and emitted bioluminescence were confirmed by PCR with primers PTn7R: 5'-CACAGCATAACCTGAGCTGAT TTC-3' and GlmSFwd: 5'-GCCGATATCATTGCCGAT-3', which, respectively, corresponded to the 5' end of the Tn7 minitransposon and the 3’ end of the glmS gene in Aeromonas, followed by DNA sequencing. Rif’ Aeromonas strains were generated by spontaneous mutation in response to antibiotic selection (200 µg/ml) to aid in the selection of transposon mutants.

**Animal experiments. (i) Septicemic-mouse model of infection.** In a first set of experiments, groups (n = 6 to 23) of healthy female Swiss Webster mice (Taconic Farms) were infected via the intraperitoneal (i.p.) route with various aeromonads. The animals were infected with a dose of 5 × 10^7 CFU, and deaths were recorded for 14 days postinfection (p.i.). Before each study, a pilot experiment was performed with three different doses of SSU (8 × 10^6, 2 × 10^6, and 5 × 10^5 CFU). The dose chosen was that which was 100% lethal. For infection studies, Aeromonas cultures grown overnight were centrifuged and the pellets were washed three times in sterile phosphate-buffered saline (PBS) before being suspended in 1/10 of the original culture volume of PBS. Subsequently, each culture was titrated and inocula were prepared such that a 50-µl volume contained the intended infectious dose of the organism.

(ii) Mouse model of i.m. infection to mimic NF. Mice (n = 5/group) were anesthetized with isoflurane, 50 µl of various Aeromonas cultures was injected intramuscularly (i.m.) into one of the legs at doses of 5 × 10^6 to 5 × 10^7 CFU, and deaths were recorded for 14 days p.i. The animals were also observed for the development of possible necrotic lesions around the injection site. The animals used for the time point studies were euthanized by carbon dioxide narcosis, followed by cervical dislocation.

**Evaluation of bacterial dissemination after i.m. infection.** Animals (n = 5) infected as described above were euthanized at 24 or 48 h p.i. From each animal, the spleen and liver were collected aseptically and transferred to disposable tissue grinders (Fisher Scientific, Pittsburgh, PA) containing 1 ml of PBS for the spleen and 2 ml for the liver. These tissues were homogenized and serially diluted, and aliquots were subjected to bacterial counting after incubation on LB agar plates at 37°C for 24 h.

**Gross pathological examination of lesions and processing of muscle tissues for histopathological analysis.** At the time of collection of internal organs for CFU determination, as well as from those mice that survived for longer times, the lesions associated with NF were grossly examined. In addition, leg muscle tissues adjacent to the injection site were collected and fixed in 10% buffered formalin. After 48 h of fixation, the tissues were processed and sectioned at 5 µm before staining with hematoxylin and eosin (H&E). The tissue sections were evaluated by light microscopy in a blinded fashion.

**IVBB in mice.** As we sought to characterize the i.m. model of mouse infection to mimic NF, we used in vivo imaging of bioluminescent bacteria (IVBB) to evaluate colonization and dissemination of bacteria to peripheral organs. Consequently, strains SSU, ATCC 7966T, NF1, and Riv2 harboring the Tn7 minitransposon system with a lux operon were used.

**Results.** All the experiments were performed in triplicate, and statistical significance was analyzed by one-way analysis of variance (ANOVA). All of the animal data were subjected to Kaplan-Meier survival estimates, and a P value of ≤0.05 was considered significant.

**Accession numbers.** The whole-genome shotgun projects and their associated short-read archive (SRA) files for the genomes described in this study have been deposited at NCBI under the following Biosample numbers: *A. hydrophila* WI, SAMN02597475; *A. hydrophila* NF1, SAMN02597476; *A. hydrophila* NF2, SAMN02597477; *A. hydrophila* NF3, SAMN02597478; *A. hydrophila* NF4, SAMN02597479; *A. hydrophila* Riv3, SAMN02597480; *A. dhakensis* SSU, SAMN02597481; *A. jandaei* Riv2, SAMN02597482; *A. caviae* NM22, SAMN02597483; and *A. caviae* NM33, SAMN02597484.

**RESULTS**

**Genome-wide comparisons of various Aeromonas species.** Four *A. hydrophila* isolates, NF1 to NF4, two environmental river water isolates, Riv2 and Riv3, tracheal aspirate isolate WI of *A. hydrophila*, and diarreal isolate SSU of *A. dhakensis* were sequenced, assembled, and annotated. Likewise, *A. caviae* NM22 (stool) and NM33 (water) isolates were sequenced and used for comparison analysis along with sequences from two recently described *A. hydrophila* strains, E1 and E2, from a case of human wound infection (42, 69). Additional comparisons were made with a number of previously published Aeromonas genomes (33, 40, 44, 45, 70).

**ANI comparisons.** Pairwise ANI comparisons of the NF-causing strains revealed that NF2 to NF4 were very similar to each other, compared to the general level of ANI between isolates the same species (Table 2). However, NF3 and NF4 appeared to be more similar to each other than to NF2 (Table 2), and in general, the values were below what we would expect for “true” clonal
relationships, i.e., identical strains. The ANI values are sensitive to differences in the sequencing and assembly technologies used, i.e., Roche 454 GS Junior versus Illumina MiSeq, as well as differences in coverage and the number of assembled contigs (which probably reflects differences in the number of repeat elements between strains). For example, the genome of strain NF3 was composed of 197 contigs, while those of NF2 and NF4 contained 154 and 156 contigs, respectively. Differences in gene content, measured by pairwise core genome size (Table 2), also suggested that the genomes of NF2 to NF4 were highly similar. Indeed, the core genome of these three strains (NF2 to NF4) contained 3,964 coding sequences (CDSs) at the 100% identity level, while the total core genome contains 4,230 CDSs. Visual inspection confirmed that many genes below 100% identity were the result of single-nucleotide polymorphisms (SNPs) altering the reading frame and truncating or lengthening the translated product, which is a common artifact found in pyrosequencing.

In order to correct for this problem, we built a robust tree by using only the 2,541 conserved full-length protein sequences (Table 2). This tree suggested that while isolates NF2 to NF4 were closely related, NF2 was a significantly more distant relative. In 696,126 positions in the concatenated protein alignment, there were 63 amino acid substitutions between NF3 and NF4, whereas there were 297 and 320 substitutions between NF2 and NF3 and between NF2 and NF4, respectively. The high numbers of substitutions may be, to some extent, an artifact of the use of different sequencing technologies (454 for NF2 and MiSeq for NF3 and NF4). However, this level of variation suggests that the three strains had a last ancestor in common well before they infected the patient or coinhabited the environmental niche directly before infection. For comparison, a recent simulation of within-host variation of the 2.9-Mbp *Staphylococcus aureus* genome showed that a diversity of four or five SNPs per genome should be expected from long-term clonal expansion with a constant population size of 5,000 (71).

Strain NF1 was cultured from the initial surgical site and was distinctly different from the NF2 to NF4 group, supported by pairwise ANI values, gene content comparisons (Table 2), and the core protein phylogeny (Fig. 1). Interestingly, all four isolates (NF1 to NF4) were of the same molecular serotype comparisons of the gene content and similarity of CDSs located between the *waaL* and *rmlA* genes. Further, the O-antigen region of *A. dhakensis* SSU was identical in terms of gene content to NF1 to NF4 but divergent in nucleotide identity at a level comparable to that of the overall genome nucleotide identity between these

<table>
<thead>
<tr>
<th>Strain (total no. of CDSs)</th>
<th>ANI (%) or no. of CDSs shared with strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila ATCC 7966</strong> (4,279)</td>
<td>96.78</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila WI (4,660)</strong></td>
<td>96.31</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila Riv3 (4,358)</strong></td>
<td>96.11</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila NF2 (4,366)</strong></td>
<td>96.48</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila NF3 (4,373)</strong></td>
<td>96.76</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila NF4 (4,375)</strong></td>
<td>96.91</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila NF1 (4,361)</strong></td>
<td>96.83</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila E2 (4,241)</strong></td>
<td>96.88</td>
</tr>
<tr>
<td><strong>Astrazeneca hydrophila E1 (4,373)</strong></td>
<td>96.84</td>
</tr>
<tr>
<td><strong>A. dhakensis SSU (4,527)</strong></td>
<td>96.87</td>
</tr>
<tr>
<td><strong>A. salmonicida A449 (4,570)</strong></td>
<td>96.86</td>
</tr>
<tr>
<td><strong>A. jandaei Riv2 (4,107)</strong></td>
<td>96.88</td>
</tr>
<tr>
<td><strong>A. veronii B565 (4,045)</strong></td>
<td>96.89</td>
</tr>
<tr>
<td><strong>A. caviae Ac398 (4,043)</strong></td>
<td>96.9</td>
</tr>
<tr>
<td><strong>A. caviae NM33 (4,172)</strong></td>
<td>96.91</td>
</tr>
<tr>
<td><strong>A. caviae NM22 (4,134)</strong></td>
<td>96.92</td>
</tr>
</tbody>
</table>

*ANIs (upper right) and shared gene contents (lower left) revealed by pairwise comparisons are shown. Values in bold indicate that the strains are of the same species on the basis of a threshold of 95.0%.*
two groups of organisms (see Fig. S1 in the supplemental material).

Pairwise ANI comparisons of all of the *A. hydrophila* genomes examined in this study revealed a heterogeneous collection of isolates of this species (Table 2). This is not surprising, given that some strains were from environmental sources, while clinical isolates were from different patients, sites, and sources. However, it is interesting that clinical isolates from different patients and disease syndromes were just as divergent from environmental isolates as clinical isolates were, according to ANI. Genomic comparison of gene contents (Table 2), on the other hand, revealed that the genomes of clinical strains had more genes in common than did a clinical isolate and an environmental isolate (see below).

**Core and pangenome.** In terms of a core genome, among seven *A. hydrophila* strains (NF2 was used to represent NF2 to NF4); we estimated a core genome of 3,617 CDSs. In terms of core genome stability, on average, 110 CDSs were subtracted from the core genome upon the addition of each *A. hydrophila* genome (see Fig. S2 in the supplemental material). Variation from this mean was high because of a large number of dispensable genes, as well as the geotemporal relatedness of strains from the same patient, such as NF1 to NF2 and E1 to E2. As expected, the core genome size decreased sharply when a genome from a different species was included in the analysis (see Fig. S2). To identify genetic features related to or indicative of virulent pathotypes, we identified dispensable and unique GRs from 11 sequenced genomes (9 of *A. hydrophila*, 1 of *A. dhakensis*, and 1 of *A. jandaei*) and determined the distribution of these features (Table 3; see Table S1 in the supplemental material).

**Surface appendages.** Like those of other Gram-negative bacteria, *Aeromonas* genomes harbored genes for an assortment of fimbriae and pili. All of the genomes harbored the Tap type IV pilus (TFP) locus, whose pilin and subunit gene clusters were under considerably stronger evolutionary pressure than those from other Tap TFP gene clusters or the overall genomes (see Fig. S3 in the supplemental material). All of the genomes also contained genes for the mannose-sensitive hemagglutinin (MSHA) TFP locus (Table 3). The genome of *A. salmonicida* A449 was found to be missing a seven-gene internal cluster, *mshNEGF2BAC*. The genomes of both *A. caviae* NM22 and NM33 revealed a deletion of the majority of the *msh* locus genes because of the insertion of a mobile element, resulting in a truncated gene arrangement of *mshH-acd*-(mobile element)-*mshDOPQ* (Table 3).

The tight adherence (TAD)/fimbrial low-molecular-weight protein (Hp) pilus was present in *A. hydrophila* ATCC 7966T, as well as in *A. salmonicida* A449 and *A. veronii* B565. Three fimbriae were widely distributed among *Aeromonas* genomes, two (class 5) alternate chaperone-usher fimbriae and one (P) chaperone-usher fimbria. Additionally, the genome of *A. hydrophila* Riv3 contained a second (P) fimbria that was encoded on a prophage element (Table 3).

All *Aeromonas* genomes contained five genetic loci encoding a single polar, unsheathed flagellum, with one exception. The genomes of *A. caviae* NM22 and NM33 did not contain the *flaABG fliDS* locus, which encodes flagellins (*flaABG*), the cap protein (*fliD*), and the essential *fliS* gene product (Table 3). Accordingly, these two strains were nonmotile (data not shown). A 35-kb lateral flagellar gene cluster was present in the genomes of NF1 to
TABLE 3 Distribution of GRs of *A. hydrophila* and *A. dhakensis* strains among *Aeromonas* species genomes

<table>
<thead>
<tr>
<th>Virulence trait(s)</th>
<th>Presence in or absence from strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 79666™</td>
</tr>
<tr>
<td>Surface appendages and features</td>
<td></td>
</tr>
<tr>
<td>CFA/I (α C/U) fimbriae, AHA_0060</td>
<td>+</td>
</tr>
<tr>
<td>CFA/I (α C/U) fimbriae, AHA_1021</td>
<td>+</td>
</tr>
<tr>
<td>P (α C/U) fimbriae, AHA_0521</td>
<td>+</td>
</tr>
<tr>
<td>Phage-encoded P (α C/U) fimbriae</td>
<td>-</td>
</tr>
<tr>
<td>Tap type IV pilus</td>
<td>+</td>
</tr>
<tr>
<td>MSHA BFP type IV pilus, mshaH acd</td>
<td>+</td>
</tr>
<tr>
<td>mshaHJK3LM_DOPQ</td>
<td>-</td>
</tr>
<tr>
<td>yopT and chaperone (T3SS)</td>
<td>-</td>
</tr>
<tr>
<td>aexTU and chaperone (T3SS)</td>
<td>-</td>
</tr>
<tr>
<td>T3SS</td>
<td>-</td>
</tr>
<tr>
<td>T6SSs</td>
<td>-</td>
</tr>
<tr>
<td>Phospholipase/lecithinase/hemolysin (FHA family, RTX toxin)</td>
<td></td>
</tr>
<tr>
<td>Ferric hydroxamate uptake (flu) operon</td>
<td></td>
</tr>
<tr>
<td>Ferrichrome iron uptake cluster, AHA_1931–AHA_1934</td>
<td></td>
</tr>
<tr>
<td>Ferric siderophore cluster, AHA_4368–AHA_4378</td>
<td></td>
</tr>
<tr>
<td>Ferric siderophore-TonB cluster, AHA_3433–AHA_3439</td>
<td></td>
</tr>
<tr>
<td>Extracellular protease precursor, AHA_2712–AHA_2714</td>
<td></td>
</tr>
<tr>
<td>ExoA homolog</td>
<td>-</td>
</tr>
<tr>
<td>syp exopolysaccharide</td>
<td>-</td>
</tr>
<tr>
<td>Group II capsule</td>
<td>-</td>
</tr>
<tr>
<td>RTX toxin, AHA_1359, transporter cluster</td>
<td>+</td>
</tr>
<tr>
<td>FHA family, RTX toxin</td>
<td>+</td>
</tr>
<tr>
<td>Ferrichrome iron uptake cluster, AHA_1931–AHA_1934</td>
<td>+</td>
</tr>
<tr>
<td>Ferric siderophore operon, ASA_4368–ASA_4378</td>
<td>-</td>
</tr>
<tr>
<td>Ferric siderophore-TonB cluster, AHA_3433–AHA_3439</td>
<td>+</td>
</tr>
<tr>
<td>T3SSs and T6SSs</td>
<td></td>
</tr>
<tr>
<td>lcp (T6SS)</td>
<td>+</td>
</tr>
<tr>
<td>T3SS</td>
<td>-</td>
</tr>
<tr>
<td>yopH and chaperone (T3SS)</td>
<td>-</td>
</tr>
<tr>
<td>actTU (T3SS)</td>
<td>+</td>
</tr>
<tr>
<td>yopT and chaperone (T3SS)</td>
<td>-</td>
</tr>
<tr>
<td>Resistance, including antimicrobials</td>
<td></td>
</tr>
<tr>
<td>ABC-type multidrug transport, AHA_0484–AHA_0486</td>
<td>+</td>
</tr>
<tr>
<td>MATE pump, AHA_3203–AHA_3204</td>
<td>+</td>
</tr>
<tr>
<td>DMT efflux, AHA_3820–AHA_3821</td>
<td>+</td>
</tr>
<tr>
<td>Multidrug resistance protein B, AHA_4116–AHA_4117</td>
<td>+</td>
</tr>
<tr>
<td>acrB, AHA_1320–AHA_1323</td>
<td>+</td>
</tr>
<tr>
<td>NodT family RND efflux pump</td>
<td>-</td>
</tr>
<tr>
<td>RND efflux pump, AHA_2959–AHA_2960</td>
<td>+</td>
</tr>
<tr>
<td>Macrolide efflux pump, macAB, AHA_0738–AHA_0761</td>
<td>+</td>
</tr>
<tr>
<td>Triparente MRS</td>
<td>-</td>
</tr>
<tr>
<td>Polymyxin B resistance (arm)</td>
<td>+</td>
</tr>
<tr>
<td>cepS, cephalosporinase</td>
<td>+</td>
</tr>
</tbody>
</table>

(Continued on following page)
NF4, E1, SSU, A449, and A. jandaei Riv2, all of which were highly pathogenic species or strains (Table 3). The gene content of this locus was highly conserved among *Aeromonas* species, and the gene cluster had the same chromosomal location in all of the strains; downstream from the gene encoding rRNA small-subunit methyltransferase I (RsmI, AHA_3894). Phylogenetic reconstruction of the evolutionary history of this locus argues for vertical transmission (see Fig. S4 in the supplemental material).

**Toxins.** Several other virulence factor-encoding genes were identified in the genomes analyzed and included lipases, hemolysins, siderophore clusters, elastase, cytolethal distending toxins, T3SS, and the capsule polysaccharide-encoding loci (Table 3). The distribution of three of these factors, *act* (cytotoxic enterotoxin), *exoA* (ExoA), and a T3SS, correlated with virulent pathotypes. The *act* gene was present in ATCC 7966<sup>T</sup>, WI, NF1 to NF4, E1, SSU, A449, Riv2, and B565 but absent from all three of the *A. caviae* genomes and Riv3 (Table 3).

Closer inspection of the *act* genetic locus revealed variability in terms of gene content (Fig. 2A). For example, in the genomes of NF2 to NF4, a gene encoding a putative lipoprotein and a group I glutathionylspermidine synthase (EC 6.3.1.8) was inserted downstream of the *act* gene. Additionally, the *act* gene was truncated in *A. hydrophila* NF2 to NF4 (567 bp), as opposed to other *A. hydrophila* and *A. dhakensis* *act* gene sequences, which were 1,482 bp in length (Fig. 2A). A putative arylsulfate sulfotransferase-encoding gene was inserted in this locus in the genomes of *A. hydrophila* Riv3, WI, and E2, of which only *A. hydrophila* WI possessed the *act* gene (Fig. 2A). Additionally, the *act* locus of Riv2 was approximately 600 bp longer than that of strain ATCC 7966<sup>T</sup> because of the insertion of a 516-bp MerR family transcriptional regulator-encoding gene between the *act* and *thiC* genes (data not shown). These alterations in the *act* gene locus might affect the expression of the gene in various *Aeromonas* isolates. The sizes of the *act* genes from other species of *Aeromonas* were 1,479 bp for *A. salmonicida* and 1,464 bp for *A. jandaei* Riv2 and *A. veronii* B565. Despite these findings, phylogenetic analysis of the *act* locus supported the hypothesis that this gene was vertically transmitted in *Aeromonas* genomes (Fig. 2B and C).

Five genomes (*A. hydrophila* NF2 to NF4 and E1 and *A. dhakensis* SSU) contained a gene encoding an ExoA homologue, which is a NAD-dependent ADP-RT commonly associated with pathogenic *Pseudomonas* species (Table 3). The toxin leads to ADP-riboseylation of host elongation factor 2, resulting in the shutdown of protein synthesis and cell death (72). Importantly, this feature is a distinguishing trait among the three NF isolates, namely, NF2, NF3, and NF4. All of these isolates had this gene (100% identity among the isolates); however, this gene was absent from the genome of NF1. The *exoA* gene was also present in the genome of a highly virulent E1 strain isolated from a case of wound infection and a diarrheal *A. dhakensis* isolate, SSU (Table 3).

**T3SSs, T6SSs, and their effectors.** The genomes of eight *Aeromonas* strains harbored a T3SS and included *A. hydrophila* NF1 to NF4 and E1, *A. dhakensis* SSU, *A. salmonicida* A449, and *A. jandaei* Riv2 (Table 3). The T3SS was inserted at the same locus, a serine ribosylation factor 2, in all of the genomes of *A. hydrophila* and *A. dhakensis*. The T3SS is located on plasmid pAsA5 in *A. salmonicida* A449 and is flanked by transposases, indicating horizontal acquisition, while the T3SS was inserted between genes encoding a short-chain-specific acyl coenzyme A dehydrogenase (EC

<table>
<thead>
<tr>
<th>Virulence trait(s)</th>
<th>Presence in or absence from strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ampS(H)</em>, class D beta-lactamase</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td><em>ompK-ampG</em></td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td><em>imiS(H)</em>, class 3 metallo-beta-lactamase</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>LPS modification, cephalosporin hydroxylase, AHA_4152–AHA_4169</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>LPS modification, aminoglycoside 3'-N-acetyltransferase, AHA_4170–AHA_4182</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Aminoglycoside 6'-N-acetyltransferase</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Mercury, arsenic, heavy metal resistance</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Heavy metal resistance trp, TEM-1 beta-lactamase</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Aminoglycoside 3'-PTS</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Chloramphenicol acetyltransferase, mercury resistance, OXA-1 beta-lactamase transposon</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Spectinomycin 9-O-adenyltransferase, sulfonamide, OXA beta-lactamase</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Macrolide 2'-PTS</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Integrative conjugative element (46 kb), organic solvents</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Quaternary ammonium compound, ethanolamine, propanediol</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Organic hydroperoxide resistance, AHA_3609–AHA_3611</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Accessory arsenic resistance operon</td>
<td>+ + + + + + + + + + + + + + + + +</td>
</tr>
</tbody>
</table>
1.3.99.2) involved in isoleucine degradation and a cystathionine beta-lyase (EC 4.4.1.8) in the genome of *A. jandaei* Riv2.

In terms of gene content, the 26.7-kb T3SSs (hypothetical ascUTSRQPO ascN aopN acr12 ascXY ascV acrRGVH aopBD axsCBAD ascCDEFGHIJKL) of the *A. hydrophila* strains analyzed in this study were identical (Table 3). The T3SS of *A. dhakensis* SSU was slightly larger, approximately 27.5 kb, because of the insertion of an *aopX* effector protein-encoding gene downstream of *ascU* (which is transcribed in the opposite direction). This gene was also present in the T3SS of *A. salmonicida* A449. The T3SS of *A. jandaei* Riv2 was considerably larger, at 30 kb, having accessory genes at both ends of the gene cluster. There was a 1,182-bp zinc metalloprotease-encoding gene located downstream of *ascU* and an *exoU* effector protein homologue and chaperone located downstream of the *asl* gene.

Three putative T3SS effector protein gene clusters were identified outside the T3SS locus (Table 3). The *aexTU* effector-chaperone pair was inserted between a tryptophan-specific-permease-encoding gene and a phenylalanyl-tRNA synthetase beta subunit-encoding gene (AHA_0123 and AHA_0124, respectively). This effector protein was the most widely distributed of the three. Also present in this three-gene cluster was a homologue of the gene for Txp40, a 40-kDa insecticidal toxin. Interestingly, this gene cluster was present in the genome of *A. hydrophila* Riv3, even though the main T3SS gene locus was absent.

The *yopH sycH* effector-chaperone pair (Table 3) was located between a hexose phosphate transport protein (*UhpT*)-encoding gene and a phosphotransferase system (PTS) fructose-specific IIABC component-encoding gene. Its distribution was more restricted; it was present in the five strains of *A. hydrophila* with a T3SS locus and *A. salmonicida* A449 (Table 3). The genome of *A. jandaei* Riv2 also contained a *yopT sycT* effector-chaperone gene pair (Table 3) inserted between a siroheme synthase-encoding gene and a magnesium transporter-encoding gene (homologues of AHA_4121 and AHA_4122, respectively). Additionally, the genome of *A. salmonicida* A449 contained a *yopO* effector homologue and a putative chaperone-encoding gene pair.

In contrast to the T3SS, a gene cluster encoding a T6SS was more widely distributed; it was present in 12 of the 16 *Aeromonas* genomes that were compared (Table 3). These included the genomes of all nine *A. hydrophila* isolates, *A. dhakensis* SSU, *A. salmonicida* A449, and *A. jandaei* Riv2. All of the genomes that har-
bored the cluster had an 18-CDS region, impB to vgrG, in common. The 5’ region of the T6SS cluster was more variable, with most of the genomes containing accessory genes in comparison to ATCC 7966\(^3\), WI, and Riv3. Among the \textit{A. hydrophila} and \textit{A. dhakensis} genomes, the cluster was located between two tRNA genes, tRNA-Ser-GGA (AHA_1825) and tRNA-His-GTG (AHA_1851). The T6SS cluster was also adjacent to tRNA-His-GTG in the genome of A449, but there was a rearrangement in the 5’ region adjacent to the cluster. On the contrary, the cluster was inserted at a different chromosomal location in the genome of \textit{A. jandaei} Riv2; however, its exact location could not be deduced because of a repetitive DNA (vgrG) sequence present at both ends of the gene cluster, which led to its assembly into a distinct contig.

In the genome of \textit{A. hydrophila} ATCC 7966\(^7\), a single \textit{hcp} gene and two vgrG genes were present in this main cluster. A smaller accessory T6SS-related gene cluster, at a separate chromosomal locus, contained a second \textit{hcp} gene and one additional vgrG gene. This number of T6SS effector protein-encoding genes was apparently conserved among other \textit{Aeromonas} genomes; however, because of the high homology among paralogues of each type of effector-encoding gene, the exact gene content could not always be concluded. The genomes of \textit{A. veronii} B565 and \textit{A. caviae} Ae398, NM22, and NM33 did not contain an effector-encoding gene, \textit{hcp} or \textit{vgrG}.

**Resistance to antimicrobials.** The \textit{cepS(H)} cephalosporinase gene was absent only from the genome of \textit{A. veronii} B565, and the \textit{imiS(H)} gene was missing only from the three \textit{A. caviae} genomes (NM22, NM33, and Ae398) (Table 3). Two different aminoglycoside-modifying enzymes were found in some genomes of \textit{A. hydrophila}, namely, ATCC 7966\(^7\), Riv3, E1, and E2, while several \textit{Aeromonas} genomes contained at least one of these. The genome of \textit{A. hydrophila} strain WI also possessed a number of modifying enzymes that contributed to antibiotic resistance (Table 3). Among these were genes that imparted resistance to aminoglycosides, chloramphenicol, streptomycin, sulfonamides, macrolides, tetracycline, ampicillin, and oxacillin. At least one of the oxacillin \(\beta\)-lactamases appeared to be an extended-spectrum \(\beta\)-lactamase (Table 3). Most of these genes were flanked by mobile-element genes.

In addition, several clusters of genes that impart resistance to heavy metals, quaternary ammonium compounds, organic hydroperoxide, and organic solvents were also identified, as well as genes for several putative multidrug efflux systems, including members of the RND, ABC, MATE, and MFS superfamilies (Table 3).

**Other dispensable GRs.** In addition to the genomic features associated with virulence or survival in the clinical setting, there were a number of other dispensable GRs in \textit{A. hydrophila} and \textit{A. dhakensis} genomes (see Table S1 in the supplemental material). A number of these features were known operons involved in the catabolism or transport of specific substrates, such as N-acetylmuramic acid, N-acetylgalactosamine, thiamine, malate, arabinose, DL-lactate, \(\alpha\)-cystine, taurine, sucrose, tungstate, \(\alpha\)-ribosylxanthinosamine, tetrathionate, phosphonate, and \(\beta\)-fucose (Table S1). However, many of the dispensable features contained mostly hypothetical-protein-encoding genes, with perhaps one or two otherwise annotated genes. In general, the distribution of many of these dispensable genomic features correlated with species delineation. For example, many GRs were not present in the three \textit{A. caviae} strains. Additionally, each genome contained a number of unique and, in rare cases, homologous prophage and prophage-like elements (data not shown). Of interest was the presence of a small integron, 8 to 13 kb, in the genomes of the four NF \textit{A. hydrophila} strains, E1, and \textit{A. jandaei} Riv2, of the zona occludens toxin prophage type (see Table S1).

**In vitro evaluation of virulence characteristics.** We focused our studies on NF-causing strains (NF1 and NF2) of \textit{Aeromonas}, as the mechanisms associated with the pathogenesis of NF are poorly understood. Among the environmental isolates, we studied two that were from river water (Riv2 and Riv3) through which the NF patient acquired the infection. These strains were compared to an environmental isolate (ATCC 7966\(^7\)) of \textit{A. hydrophila} (as a negative control), the genome of which we first sequenced and annotated (40), and that of highly virulent \textit{A. dhakensis} SSU, whose sequence was annotated during this study. Our purpose was to discern if there were any pathogenic features unique to these strains, especially those associated with NF. \textit{A. hydrophila} WI was chosen because of its resistance to multiple antibiotics.

**Biofilm formation.** As shown in Fig. 3, the NF1 and SSU isolates formed comparable and significant biofilms. However, all of the other isolates produced statistically significantly lesser biofilms. Films, which were minimal with the ATCC 7966T and Riv3 isolates.

**Swimming and swarming motility.** All of the isolates tested exhibited swimming motility (Fig. 4), albeit to various degrees. Among these isolates, NF1 and Riv2 showed a hypermotility phenotype compared to SSU. Both ATCC 7966\(^7\) and WI were minimally motile. To more accurately measure the migration zone sizes of hypermotile strains (NF1 and Riv2), we used larger (100-mm instead of 60-mm) swimming agar plates. Our data indicated that while the swimming motility of SSU remained unaltered, one could clearly visualize the hypermotility phenotype of the NF1 and Riv2 isolates because of the larger surface area available to them for swimming (data not shown).

As noted in Fig. 5, NF1 and Riv2 swarmed to a higher degree than SSU, a difference that became statistically significant when 100-mm agar plates were used (data not shown). While ATCC 7966\(^7\), NF2, and Riv3 had moderate levels of swarming motility, WI did not swarm.

**Protease and hemolytic activities.** As shown in Fig. 5.5 in the...
supplemental material, all of the *Aeromonas* strains studied produced a protease(s) to various degrees; however, no statistically significant difference between them and SSU was found. In terms of hemolytic activity associated with Act, we noted that the hemoglobin release from rabbit erythrocytes caused by NF2, WI, Riv2, Riv3, and ATCC 7966T was at a level much lower than that of SSU and NF1 (see Table S2 in the supplemental material). To demonstrate that most this hemolytic activity was associated with Act, we neutralized the toxin by using anti-Act polyclonal antibodies. Indeed, 75 to 98% of the Act-associated hemolytic activity of the bacterial strains tested was neutralized by these antibodies (see Table S2).

**AHL production.** As shown in Fig. S6A in the supplemental material, all of the *Aeromonas* isolates (clinical versus water) produced similar levels of AHLs within 24 h, except for ATCC 7966T, as judged by the production of purple pigment by *C. violaceum* CV026 (73) (see Fig. S6B). We used an AHL-negative mutant of SSU (ΔahyRI) (62) as a negative control in the assay.

**Production and secretion of Hcp and AexU.** Hcp secretion is T6SS dependent and a reliable indicator of the presence of a functional T6SS in bacteria (74). On the basis of Western blot analysis, Hcp production and secretion were noted in *A. jandaei* Riv2, as well as in *A. dhakensis* SSU (Fig. 6A). In contrast to ATCC 7966T, which did not synthesize Hcp, isolates NF1, NF2, Riv3, and WI synthesized Hcp but were unable to secrete it into the medium (Fig. 6A). As another positive control, we used the production and secretion of Hcp by *Versinia pestis*, which also suggested the functionality of the T6SS in the plague bacterium.

The AexU toxin is a T3SS effector identified in SSU, which possesses GAP and ADP-RT activities (63, 75). Both isolates NF1 and NF2 produced AexU, as was also noted in SSU (Fig. 6B). None of the other strains tested produced AexU.

**Existence of ExoA homolog.** On the basis of genome-wide sequencing, a homolog of *P. aeruginosa* ExoA was found in SSU and NF2 isolates. Comparison of the DNA sequence of the *exoA* gene of *P. aeruginosa* with that of *Aeromonas* species revealed 78% homology. At the amino acid level, the sequence identity ranged from 64 to 65%. A sequence identity of 97 to 98% was noted for isolates SSU, NF2, and E1. To demonstrate that the *exoA* gene in the *Aeromonas* strains tested was expressed, we performed Western blot analysis with antibodies to *P. aeruginosa* ExoA. As noted in Fig. 6C, a secreted version of ExoA was seen in SSU and NF2 isolates. As a positive control, we used *P. aeruginosa* PA103, which also demonstrated a correct-size ExoA band of 66 kDa (76).

By analyzing the ExoA protein sequences at the NCBI conserved-domain database, a Pfam protein sequence search (http://pfam.sanger.ac.uk/), and SMART analysis (http://smart.embl-heidelberg.de/), it was revealed that the ExoA sequence from aeromonads possessed three domains similar to that found in *P. aeruginosa*. The ExoA sequences of SSU, NF2, and E1 had 639 amino acid residues. Interestingly also, the amino acid sequences of ExoA homologs in more recently sequenced *A. hydrophila* genomes, according to the NCBI database, were shorter than that of ExoA of SSU. Prediction of signal peptides in *Archaea* with the hidden Markov model revealed that the first 25 amino acid residues represent the leader peptide in SSU, NF2, and E1, similar to that seen in ExoA of *P. aeruginosa* PA103 (76).

**Virulence associated with clinical and environmental aeromonads in a mouse model of infection.** (i) Septicemic-mouse model. To test the virulence of aeromonads, animals were injected with various *A. hydrophila* strains (SSU, ATCC 7966T, NF1, NF2, WI, and Riv3) or *A. jandaei* Riv2 via the i.p. route at a dose of 5 × 10⁷ CFU. As shown in Fig. 7, 100% of the animals infected with *A. dhakensis* SSU and 90% of the mice challenged with NF1 or Riv2 died within 2 days p.i. The mortality rates were 35 and 45%, respectively, when the mice were infected with NF2 and Riv3 at the same infectious dose. Both strains ATCC 7966T and WI of *A. hydrophila* were the least virulent in a septicemic-mouse model, allowing the survival of 90 to 100% of the animals.

(ii) Developing NF by *Aeromonas* species in a mouse model. Previously, the subcutaneous injection route was used on the footpads of humanized CD46 transgenic mice to induce *Streptococcus pyogenes*-associated NF (77). However, *Aeromonas*-related NF has...
always been linked to infections acquired through nonintact skin and exposure of wounds or skin lacerations to contaminated soil or water (9, 78, 79). To mimic this infection route, we challenged mice i.m. with strains SSU, NF1, NF2, and Riv2 or a mixture of NF1 and NF2 and used strain ATCC 7966T as a negative control. As depicted in Fig. 8A, *A. jandaei* Riv2 was highly virulent, resulting in 100% mortality in mice within 24 h p.i. at a dose of 5 × 10⁷ CFU. Isolates SSU and NF2 also resulted in 100% mortality but at 48 h p.i.

Interesting were our findings that while NF2 was still 100% lethal to mice at a lower dose of 5 × 10⁶ CFU by the i.m. route (data not shown), 65% of the animals survived when injected by the i.p. route with NF2 (Fig. 7). On the other hand, strain NF1, which was relatively more virulent than strain NF2 in a septicemic-mouse model of infection (Fig. 7), with 10% versus 65% survival, exhibited greatly reduced virulence (100% survival) when injected via the i.m. route at the same dose of 5 × 10⁷ CFU (data not shown). At a higher dose of 5 × 10⁸ CFU, animals infected with NF1 also succumbed to infection by day 6 (Fig. 8A). As expected, none of the animals infected with strain ATCC 7966T died.

As shown in Fig. 8B, while NF2 was still highly virulent (100% mortality) at doses of 1 × 10⁸ to 2 × 10⁸ CFU when given by the i.m. route, NF1 resulted in death in only 40% of the mice infected with a dose of 2 × 10⁸ CFU. Since the NF1 and NF2 isolates were obtained at different times (NF1 from the initial wound site and

FIG 6 Production and secretion of Hcp, AexU, and ExoA by various strains of *Aeromonas* and *P. aeruginosa* as determined by Western blotting. While only SSU and Riv2 secreted Hcp into the supernatant, the Hcp protein was identified in the cell pellets of all of the other strains studied, except for *A. hydrophila* ATCC 7966T (A). Isolates SSU, NF1, and NF2 produced AexU (B), while ExoA was secreted by isolates SSU and NF2, as well as by *P. aeruginosa* PA103 (C). *Y. pestis* (YP) served as a control. The molecular sizes of Hcp, AexU, and ExoA are indicated.

FIG 7 Virulence of various *Aeromonas* strains in a septicemic-mouse model of infection. Swiss-Webster mice (*n* = 6 to 23) were infected with a dose of 5 × 10⁷ CFU of the strains shown by the i.p. route. The animals were monitored for death for 14 days. The data were statistically analyzed by using the Kaplan-Meier survival estimate, and the actual *P* values are presented. The number of animals used for each strain is shown in parentheses.
NF2 after amputation) from the same patient, we determined whether mixing these two cultures in equal numbers could augment their virulence in a mouse model. Our data indicated that the virulence of NF2 predominated in the mixed culture, as 80% of the animals died by day 2 (Fig. 8B).

**Dissemination of Aeromonas species after i.m. injection in a mouse model.** To evaluate the dissemination pattern of various Aeromonas isolates, infected mice (n = 5) were sacrificed at 24 or 48 h p.i. and their spleens and livers were subjected to CFU determination. In agreement with the animal survival data, greater bacterial loads were noted in the livers and spleens of mice in those groups that were infected with SSU (1 × 10^8 CFU), Riv2 (1 × 10^8 CFU), and NF2 (2 × 10^8 CFU) (Fig. 9A to C). The number of bacteria in these organs increased over the course of infection from 24 to 48 h. Because of the highly lethal nature of the Riv2 isolate, some of the animals died between 24 to 48 h after infection (Fig. 9B). Strain NF1 disseminated to the liver and spleen in lower numbers, even at a slightly higher infective dose (5 × 10^8 CFU) (Fig. 9D), than strains SSU, NF2, and Riv2 (Fig. 9A to C), with strain ATCC 7966^T^ disseminating minimally to the livers and spleens.

**FIG 8** Percent survival of mice after infection with various Aeromonas isolates by the i.m. route. (A) Animals were infected with isolate SSU, NF1, NF2, Riv2, or ATCC 7966^T^ at 5 × 10^8 CFU/mouse and monitored for death for 14 days. (B) Animals were infected with NF1 and NF2 at the lower doses indicated or with a mixture of these two isolates at 1 × 10^8 CFU each per mouse. Isolates SSU, NF1, NF2, and Riv2 were statistically significantly more virulent than avirulent A. hydrophila strain ATCC 7966^T^ (A). Likewise, NF2 at both the doses was statistically significantly more virulent than NF1 (B). The data were statistically analyzed by using Kaplan-Meier survival estimates, and the actual P values are presented.
spleens of mice (1/5) over the course of 48 h of infection (Fig. 9E).

Gross and histopathological evaluations of tissues from *Aeromonas* species-associated NF. We initially focused on two isolates, namely, SSU and NF1. At 24 h p.i. with isolate SSU (5 × 10^8 CFU), muscle tissues around the injection site revealed severe edematous and hemorrhagic swelling, unlike those of animals injected with PBS or lipopolysaccharide (20 μg) (Fig. 10A). These inflammatory changes were more progressive in nature at 48 h p.i. (Fig. 10B1 and B2). In addition, the leg tissues of mice infected

FIG 9 Bacterial dissemination patterns for various *Aeromonas* isolates in a mouse model of NF. Mice (n = 5) were infected by the i.m. route with the dose of isolate SSU (A), Riv2 (B), NF2 (C), NF1 (D), or ATCC 7966^T^ (E) indicated. At 24 or 48 h p.i., the spleen and liver were aseptically removed from each mouse, homogenized, and subjected to bacterial colony counting. M1 to M5 represent individual mice, and some groups have fewer animals because of deaths p.i.
with the NF1 isolate (2 × 10⁸ CFU) were liquefied and showed gangrenous necrosis after 7 days, as shown in Fig. 10C.

Histopathological examination of skeletal muscle tissues from thigh regions adjacent to the injection site revealed various levels of multifocal and diffuse necrotic changes in striated muscle bundles, accompanied by edematous exudation and infiltration of polymorphonuclear cells (PMNs), specifically, neutrophils (Fig. 11A). Specifically, tissues collected from animals infected with strains ATCC 7966T and NF1 showed more PMN infiltration and edematic fluid but limited multifocal ne-
crotic changes (Fig. 11A). On the other hand, tissues from animals infected with strains SSU and Riv2 exhibited extensive and diffuse necrotic changes with moderate levels of edema and infiltration of PMNs (Fig. 11A). Since strain NF2 was highly lethal to mice by the i.m. route, we reduced the infectious dose to $5 \times 10^6$ to $5 \times 10^7$ CFU. These tissues also revealed various levels of multifocal and local, as well as diffuse, necrotic changes in the skeletal muscle. In addition, infiltration of PMNs (specifically, neutrophils) and various severities of edematous fluid accumulation were noticed. As depicted in Fig. 11B, necrotic and degenerated myofibers could be seen (arrow) with significant inflammatory and edematous responses (asterisk) in animals infected with NF2.

**Dissemination of bioluminescent bacteria in a mouse model of NF.** We infected mice ($n = 10$) by the i.m. route in the right hind leg with a challenge dose of $5 \times 10^6$ CFU of each of the above-mentioned strains, except Riv2. Since Riv2 exhibited higher virulence than the other strains tested, two lower infectious doses ($5 \times 10^5$ and $1 \times 10^5$ CFU) were used. After infection, we measured bioluminescence in the legs, liver, and spleen. In addition, we determined bacterial counts in the livers and spleens of five animals per time point at 24 and 48 h p.i.

At 24 h p.i. for no strain SSU bioluminescence was observed beyond the baseline level, while two animals were positive for bacterial colonies in the liver and one was positive for bacterial colonies in the spleen (Fig. 12A). It should be noted that for animals to show bioluminescence, the number of organisms should be $\sim 10^5$ CFU (68). At 48 h p.i., three mice were positive for bioluminescence in the leg at the injection site and two had luminescence extending into the peritoneal cavity. The bioluminescence observed also corresponded to the bacterial counts in the liver and spleen (Fig. 12A). Both of the animals that had strong luminescence in the peritoneal cavity showed bacterial counts in the range of $10^9$ to $10^7$ CFU in the liver and spleen. For each of the other strains (NF1, ATCC 7966T, and Riv2), strong bioluminescence was observed in the infected leg at 24 h p.i. (Fig. 12B to E), with the exception of one mouse in the ATCC 7966T group (Fig. 12C).

At 48 h p.i., a decrease in luminescence was observed in the legs across all of the mice infected with strain ATCC 7966T, with a majority of the animals (4/5) having dissemination into the liver but remaining below $10^7$ CFU. No bacteria were detected in the spleen (Fig. 12C). While strain NF1, luminescence was observed in all of the mice at both the 24- and 48-h time points, with dissemination of bacteria to the spleen and liver; however, luminescence was absent from the peritoneal cavity (Fig. 12B). In mice infected with strain Riv2 at a dose of $5 \times 10^7$ CFU, luminescence was observed at the injection site in the hind leg, as well as extending into the peritoneal cavity of one mouse, corresponding to viable plate counts of $\sim 10^7$ CFU in the liver and spleen at 48 h (Fig. 12D). In mice infected with $1 \times 10^7$ CFU of Riv2, strong luminescence was observed at the injection site, as well as extending into the peritoneal cavities of two mice at 48 h, representing disseminating infection even at the lower dose (Fig. 12E). At both doses of Riv2, one mouse died by 24 h and two additional animals died at 48 h and were not included for imaging.

**DISCUSSION**

In this study, genetically diverse isolates of *Aeromonas* (belonging to *A. hydrophila*, *A. jandaei*, and *A. caviae*) were sequenced and analyzed to identify virulence factors associated with their distinct pathotypes. Our purpose was to discern if there were any pathogenic features unique to these strains, especially those associated with NF. NF is part of a family of diseases referred to as necrotizing soft tissue infections (NSTIs). Even milder NSTIs often require several surgical debridement (80). There are three major classifications of NSTIs, type I, involving mixed infections of anaerobes and aerobic bacteria (polymicrobial), type II, involving *S. pyogenes*, and type III, gas gangrene or clostridial myonecrosis, with all three requiring intensive care and rapid treatment. A variant of type I is salt water NF, in which an apparently minor skin wound is contaminated with salt water containing *Vibrio* species. Even with rapid surgical intervention (81, 82), case fatalities can be as high as 50% (83) and leave surviving patients with lifelong disabilities and disfigurement (81, 82, 84).

Whole-genome sequencing of isolates NF1 to NF4 from a patient revealed that NF1 was genetically distinct and that strains NF2 to NF4 were not true clones in the sense that they likely did not derive from a last common ancestor either during the course of infection or in the environment directly prior to infection. Comparative genomic analysis, coupled with functional assays, of strains NF1 and NF2 and other clinical and environmental *Aeromonas* strains allowed us to investigate pathovar-specific virulence factors or markers.

Although isolates Riv2 and Riv3 were cultured from the same water source through which the patient with NF contracted the infection, they were genetically different (e.g., Riv3) from isolates NF1 to NF4 of *A. hydrophila*. We believe that since these water samples were collected at a later time subsequent to the infection of the patient, water microbiota changed because of fluctuations in the environmental conditions (85, 86). Nevertheless, our studies reinforce the idea that both benign and highly virulent strains can be present at all times in fresh and brackish waters, where aeromonads flourish. In fact, the advent of *Aeromonas* species as a cause of NF leads us to suggest that this condition is another variant of type 1, which can be termed “estuarine fasciitis.”

In the same vein, recent studies of Skwore et al. (85) recovered tetracycline- and ciprofloxacin-resistant *Aeromonas* strains from Lake Erie in Pennsylvania. Importantly, the prevalence of aerosynin and serine protease-encoding genes and their corresponding hemolytic and proteolytic/cytotoxic activities was also high in antibiotic-resistant strains of some *Aeromonas* species (e.g., *A. hydrophila* and *A. veronii*), suggesting that they contribute to bacterial virulence (85). Since the antibiotics of choice for the treatment of aeromonads are sulfamethoxazole-trimethoprim and ciprofloxacin (87), the presence of antibiotic-resistant *Aeromonas* strains in natural water becomes even more significant in the management of serious infections such as NF.

Aeromonads readily form biofilms; however, it is unclear whether there are any variations in biofilm formation among clonally diverse clinical and environmental isolates. The dynamics of biofilm formation depend on the environmental niches where the microbes hide during an infection and dictate the pathogenic nature of a given organism by modulating virulence gene expression (88). In this vein, we described the dynamics of mature, three-dimensional biofilm formation by *A. dhakensis* strain SSU (89). Indeed, highly virulent isolates NF1 and Riv2 formed biofilms comparable to that of SSU, and these likewise were better swimmers and swarvers, which overall contributed to their virulence (Fig. 3 to 5).

The AI-1-based QS system consists of the *ahyRI* genes, with
FIG 12 Bioluminescent imaging and corresponding bacterial loads of harvested livers and spleens infected with luminescent *Aeromonas* strains SSU (A), NF1 (B), ATCC 7966\(^T\) (C) Riv2 (5 \(\times\) 10\(^7\) CFU) (D), and Riv2 (1 \(\times\) 10\(^8\) CFU) (E). Infected mice (n = 5) were imaged at 24 and 48 h p.i. and used for colony counting. For isolates SSU, NF1, and ATCC 7966\(^T\), the challenge dose was 5 \(\times\) 10\(^7\) CFU/mouse by the i.m. route. The CFU are reported for whole organs derived from colony plate counts.
ahyR encoding a transcription activator and the ahyI gene synthesizing an autoinducer AHL. The role of this system in biofilm regulation has been well established (27, 62, 90). However, in the present study, no direct correlation between AHL production and biofilm formation in the strains studied was noted (Fig. 3; see Fig. S6 in the supplemental material). It is plausible that in some *Aeromonas* strains, the regulation of biofilm formation is very tightly regulated, as dictated by specific environmental and nutritional niches found only under *in vivo* conditions and needing further exploration. Indeed, the expression of the locus that codes for lateral flagella and allows bacteria to swarm is linked to biofilm formation (91). Further, overlapping sets of regulators govern swimming motility and biofilm formation in a reciprocal fashion, depending upon the environmental cues that challenge microbes during an infection (92, 93).

In *P. aeruginosa*, it was noted that swimming isolates had diminished biofilm formation capabilities and that swimming motility occurred in the absence of either twitching or swimming motility. Further, the best swarmer secreted increased levels of proteases (94). On the contrary, the best *E. coli* biofilm formers displayed the greatest swimming motility (95). The above-mentioned scenario with *E. coli* seemed to fit with the SSU, NF1, and Riv2 isolates, as they exhibited greater swimming and swimming motilities (Fig. 4 and 5), formed better biofilms (Fig. 3), and were highly virulent in mouse models of infection (Fig. 7 and 8). However, on the basis of this study, no correlation seemed to exist between swimming motility (Fig. 5) and protease production (see Fig. S5 in the supplemental material).

The high hemolytic activity associated with Act, as well as the presence of the T3SS effector AexU also correlated with increased virulence of SSU and NF1 in mouse models of infection (Fig. 6 to 8; see Table S2 in the supplemental material). These results were corroborated by our earlier findings indicating that deletion of the *act* and *aexU* genes from *A. dhakensis* SSU significantly reduced their respective virulence in the septicemic-mouse model of infection (61, 63, 75).

Interestingly, however, were our findings that although Riv2 and NF2 were highly virulent in one or both *in vivo* models of bacterial infection (Fig. 7 and 8), they produced low levels of Act (see Table S2 in the supplemental material). For NF2, it is likely related to the truncated nature of the *act* gene, while in the Riv2 isolate, the toxin gene might be regulated differently from that of isolates SSU and NF1. These isolates were still highly virulent in an NF mouse model (Fig. 8), despite low Act-associated hemolytic activity. This could be explained by the presence of a functional ExoA in NF2 and secretion of T6SS-associated Hcp or differences in T3SS effector proteins in Riv2, which could compensate for low Act-associated hemolytic activity (Fig. 6). The reverse seemed to be true for the NF1 isolate, as it had high hemolytic activity and produced AexU but did not contain the *exoA* gene or secrete Hcp. In this regard, isolate SSU had all of the weapons (e.g., the presence of Act, ExoA, AexU, and Hcp) (Fig. 6) necessary to inflict serious damage on the host.

The reduced hemolytic activity of Act in the W1 isolate could be the result of an insertion of a putative arylsulfate-encoding gene upstream of the *act* gene that might have affected the expression of the gene (Fig. 2A). Finally, it is not clear why Riv3 had Act toxin-neutralizable hemolytic activity (see Table S2 in the supplemental material), as the genome of this isolate did not harbor the *act* gene. One plausible explanation is that the hemolytic activity seen in this isolate is contributed by another hemolysin for which we might have antibodies in our Act antitoxin preparation. Another possibility is that there might be a neutralizable cross-reactive epitope(s) among different hemolysins. Since the W1 isolate did not secrete Hcp or produce AexU and ExoA, it was the least virulent in a mouse model of infection. The same logic applies to strain ATCC 7966T (Fig. 6 and 7).

Our earlier study indicated the presence of an *exoA* gene in the genome of wound isolate E1 (42); however, the functionality of the gene was not confirmed. Indeed, ExoA was secreted by the SSU, NF2, and PA103 isolates (Fig. 6), as well as by E1 (data not shown). While secretion of ExoA was demonstrated in the SSU and NF2 isolates when they were grown in LB medium at 37°C, ExoA was secreted from the E1 isolate at a low iron concentration of 0.05 µg/ml at 32°C (65). The low level of ExoA secretion detected in PA103 was probably related to the complex nutritional conditions needed for its synthesis (96).

In a mouse model of NF, the pathodynamics of lesion development by isolates SSU, NF1, NF2, and Riv2 (Fig. 11) closely mimicked the time course events reported for human NF. When NSTIs were first characterized, an overabundance of neutrophils at the infection site was recognized as a hallmark of the disease (97). In the mouse model of NF, we also noted abundant neutrophils at the infection site (Fig. 11). Therefore, it is plausible that neutrophils might have a role in the pathogenesis of NSTIs and in the extensive tissue damage that is characteristic of the disease.

The mechanism and significance of enhanced neutrophil recruitment observed in NSTIs are unknown. However, increases in the level of resistin, a proinflammatory peptide derived from neutrophils, have been correlated with disease severity in both NSTIs and septic shock (98). It is known that degranulation events in neutrophils lead to the release of azurophilic and specific granules into the extracellular space, exposing the surrounding tissue to toxic enzymes and antimicrobial peptides (99).

Strain NF2 was less lethal to mice when given by the i.p. route but was highly virulent when injected by the i.m. route (Fig. 7 and 8). These data raised the possibility that the microenvironment prevailing at the i.m. injection site would favor efficient adaptation of the bacteria to the host immune system and thus later led to systemic infection because of the presence of unique virulence factors. On the other hand, *A. hydrophila* strain ATCC 7966T, which lacks a T3SS and many other virulence factors (e.g., Hcp, ExoA) (40), remained avirulent in mice although the site of i.m. injection showed infiltration of PMNs (Fig. 11).

Substantiating the above-mentioned paradigm, the animal mortality pattern caused by isolates SSU, NF1, NF2, and Riv2 (Fig. 8) closely matched their dissemination profiles (Fig. 9) and correlated with the presence of key virulence factors. As alluded to above, a hallmark characteristic of NF is the dissemination of bacterial infection along fascial planes of the limbs, followed by, in a setting of severe infection, sepsis (81). To study this phenomenon in more detail, we used various biofilm-excreting reporter *Aeromonas* strains. In most of the strains tested, we observed that infection was limited to the hind leg, the site of bacterial injection, although dissemination and colonization of that region were extensive, with further dissemination of some strains (e.g., SSU, Riv2) to other organs occurring by 48 h. Unfortunately, since NF2 was not highly virulent in a septicemic-mouse model of infection, we did not construct this reporter strain. However, significant dissemination...
of this isolate to other organs was revealed by plate counting (Fig. 9C).

A. hydrophila ATCC 7966T had no lethality in this mouse model, and the infection was confined to the injection site, with little or no dissemination, even within the region of infection (Fig. 12C). In both strains SSU and Riv2, dissemination above the threshold for imaging was noted, which was confirmed by viable plate counts (Fig. 12A, B, D, and E).

In contrast, mice infected with NF1 showed more uniform dissemination, albeit at lower levels at 24 and 48 h (Fig. 12B), but this strain exhibited larger necrotic lesions in the injected legs of mice. Despite being isolated directly from the initial wound site from a patient with NF, the NF1 isolate seemed to show a lower propensity to disseminate and grow rapidly outside the fascial tissue of the infected leg (Fig. 12B). In fact, infection seemed to be confined to the leg, and only after an aggressive necrotic lesion was formed did the infection spread significantly (data not shown). One week p.i., several of the mice infected with NF1 developed lesions that encompassed the entire leg (Fig. 10C) before advancing across the peritoneum. Histologically, strain NF1 also seemed to localize at the injection site and thus to favor the infiltration of PMNs, eventually leading to localized necrotic changes rather than causing systemic dissemination (Fig. 11).

On the basis of the genomic and functional data presented in this paper, the role of virulence factors such as Act, ExoA, and T3SS and T6SS and their effectors and the functionality of the lateral flagella in causing NF are evident. However, one should be cautious in data interpretation, as the unique environment cues the aeromonads are exposed to during NF may trigger bacterial gene expression differently to contribute to the severity of the disease. While this hypothesis remains to be proven, future studies on Aeromonas NF cases will provide information regarding the molecular and genetic features of these organisms. Studies such as those performed with GAS should be instrumental in revealing the intricacies of Aeromonas NF in vulnerable hosts.

ACKNOWLEDGMENTS

The financial support provided to A.K.C. through Leon Bromberg and Robert E. Shope and John S. Dunn Distinguished Chair in Global Health endowments, UTMB, is gratefully acknowledged.

We thank the Genomics Core (specifically, Tom Wood and Steven Widen), UTMB, for sequencing several of the aeromonad genomes.

REFERENCES


Tsai YH, Huang KC, Huang TJ, Hsu RW.

Suarez G, Sierra JC, Erova TE, Sha J, Horneman AJ, Chopra AK.

Stemper ME, Joseph SW, Moyer NP, Sha J, Chopra AK.


