Independent Emergence of *Yersinia ruckeri* Biotype 2 in the United States and Europe[▽]

Timothy J. Welch,^{1*} David W. Verner-Jeffreys,² Inger Dalsgaard,³ Thomas Wiklund,⁴
Jason P. Evenhuis,¹ Jose A. Garcia Cabrera,⁵ Jeffrey M. Hinshaw,⁶
John D. Drennan,⁷ and Scott E. LaPatra⁸

National Center for Cool and Cold Water Aquaculture, Agricultural Research Service, U.S. Department of Agriculture, Kearneysville, West Virginia 25430¹; Cefas Weymouth Laboratory, Weymouth, Dorset DT4 8UB, United Kingdom²; National Veterinary Institute, Division of Veterinary Diagnostics and Research, Technical University of Denmark, 1790 Copenhagen V, Denmark³; Laboratory of Aquatic Pathobiology, Marine and Environmental Biology, Åbo Akademi University, BioCity, Artillerigatan 6, Åbo/Turku, Finland⁴; Universidad Complutense de Madrid, Madrid, Spain⁵; Department of Biology, North Carolina State University, Mills River, North Carolina 28759⁶; Intervet/Schering-Plough Animal Health, Elkhorn, Nebraska 68022⁷; and Clear Springs Foods, Inc., Research Division, Buhl, Idaho⁸

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Biotype 2 (BT2) variants of the bacterium *Yersinia ruckeri* are an increasing disease problem in U.S. and European aquaculture and have been characterized as serovar 1 isolates that lack both peritrichous flagella and secreted phospholipase activity. The emergence of this biotype has been associated with an increased frequency of enteric redmouth disease (ERM) outbreaks in previously vaccinated salmonid fish. In this study, four independent specific natural mutations that cause the loss of both motility and secreted lipase activity were identified in BT2 strains from the United States, United Kingdom, and mainland Europe. Each of these was a unique mutation in either *fliR*, *fliA*, or *fliB*, all of which are genes predicted to encode essential components of the flagellar secretion apparatus. Our results demonstrate the existence of independent mutations leading to the BT2 phenotype; thus, this phenotype has emerged separately at least four times. In addition, BT2 strains from the United Kingdom were shown to have the same mutant allele found in U.S. BT2 strains, suggesting a common origin of this BT2 lineage. This differentiation of distinct BT2 lineages is of critical importance for the development and validation of alternative vaccines or other treatment strategies intended for the control of BT2 strains.

Yersinia ruckeri, a Gram-negative enterobacterium, is the causative agent of enteric redmouth disease (ERM), a hemorrhagic septicemia that primarily affects farmed salmonid fish species (11, 16). *Y. ruckeri* was first recognized as a pathogen in the 1950s in Idaho-farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum) (29, 30), and is presently found throughout the world, in areas where salmonid fish species are intensively cultured (16). The majority of reported ERM outbreaks are caused by subgroups of serovar 1 (10). These strains are biochemically and genetically homogenous, defined by both shared lipopolysaccharide antigens (31, 42) and a heightened virulence phenotype (9). The highly clonal nature of this group suggests a relatively recent emergence and dissemination of this pathogen, possibly through the shipment of infected eggs or fish during the early development and global expansion of the trout aquaculture industry (16). Losses due to ERM can reach 10 to 25% in untreated immunologically naive populations and as a result can cause significant economic losses (5, 16). A vaccine against ERM was the first commercialized fish vaccine and characteristically consists of killed whole-cell preparations of motile serovar 1 *Y. ruckeri* strains (34). ERM vac-

cines are typically administered by brief (30-s) direct immersion when fish are approximately 3 to 10 g; this method allows the simultaneous vaccination of large numbers of fish with minimal handling stress. Immersion ERM vaccination has been used worldwide for approximately 2 decades and has proven an effective and economical means for the control of *Y. ruckeri*-caused disease. Despite the success of this vaccine, the mechanism(s) by which it elicits protective immunity has remained unclear.

Bacterial flagella are highly conserved cell surface structures responsible for swimming motility. Flagellar proteins are secreted via a dedicated flagellar export apparatus that is distinct from the general (type II) secretion pathway and is related to the type III export pathways known to secrete virulence proteins in a variety of pathogens (7, 20). Early descriptions of *Y. ruckeri* noted the presence of peritrichous flagella and swimming motility as distinctive traits of this pathogen, and motility has been a key diagnostic feature (5, 29). Recently, nonmotile serovar 1 strains have been increasingly isolated in Europe and the United States and have been associated with disease outbreaks in previously vaccinated fish (3, 4, 15). In all cases, strains isolated from these outbreaks have been characterized as lacking motility, flagella, and secreted phospholipase activity and have been classified as *Y. ruckeri* biotype 2 (BT2). This association between vaccine failure and emergence of BT2 *Y. ruckeri* has led to the hypothesis that the loss of the flagellum is necessary for resistance to immersion vaccination and that

* Corresponding author. Mailing address: National Center for Cool and Cold Water Aquaculture, 11861 Leetown Road, Kearneysville, WV 25430. Phone: (304) 724-8340 Fax: (304) 725-0351. E-mail: tim.welch@ars.usda.gov.

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Source or reference
Strains		
<i>Escherichia coli</i>		
DH5 α	λ^- ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> ($r_K^- m_K^-$) <i>supE44 thi-1 gyrA relA1</i>	BRL
JM109 λ pir	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> Δ (<i>lac-proAB</i> $^+$) [<i>F'</i> <i>traD36 proAB lacI</i> q Z Δ M15] λ pir	Lab collection
MM294	<i>endA hsdR pro</i>	14
<i>Yersinia ruckeri</i>		
CSF007-82	Serovar 1, biotype 1, Idaho 1982	13
YRNC10	Serovar 1, biotype 2, North Carolina 2003	41
970611-2/2	Serovar 1, biotype 2, Denmark 1997	This work
950720-4/4	Serovar 1, biotype 2, Denmark 1995	This work
06076	Serovar 1, biotype 2, Spain 2003	This work
BTf1	CSF007-82-derived mini-Tn5 insertion in <i>flhA</i>	13
YRNC102-1	YRNC10-derived <i>flhR</i> Δ 1 repaired by marker exchange	This work
970611-2/2.2-1	970611-2/2-derived <i>flhR</i> Δ 2 repaired by marker exchange	This work
Plasmids		
pCR2.1	PCR cloning vector	Invitrogen
pBAD-TOPO	PCR cloning vector	Invitrogen
pVK102	Cosmid cloning vector	17
pTW-MEV	Suicide vector for marker exchange	40
pRK2013	Conjugative helper plasmid	14
pBBR1MCS	Conjugative cloning vector	18
pTW10.1	pVK102 containing ~30-kb BT2-complementing fragment	This work
pTW10.2	pTW-MEV containing wild-type <i>flhR</i> allele	This work
pBADBAE	<i>flhBAE</i> cloned by PCR into pBAD-TOPO	This work

the prolonged use of this vaccine has provided the selective force driving the emergence of nonmotile variants (15). This would suggest that flagellar proteins may be an important protective component of immersion ERM vaccines, which is consistent with the recognized immune signaling properties of flagellin, the principal component of the Gram-negative bacterial flagellum (26, 33, 38, 39).

Recently, we demonstrated that mutation of a single essential flagellar secretion gene in a motile BT1 *Y. ruckeri* strain resulted in loss of both motility and lipase secretion, thus mimicking the BT2 phenotype and demonstrating a genetic link between motility and phospholipase secretion (13). Additionally, BT2 strains have been shown to be genetically similar to motile serovar 1 strains and contain intact, yet apparently nonfunctioning, flagellar secretion and biosynthesis genes (13, 42). These results suggest that the loss of motility and secreted lipase activity in BT2 strains could potentially result from limited damage to essential flagellar secretion gene(s) in a motile BT1 strain. In this study, we used genetic complementation to identify the molecular basis for the loss of motility and phospholipase activity in diverse BT2 strains of *Y. ruckeri*. We have identified four distinct BT2-causing mutations among U.S. and European strains of BT2 *Y. ruckeri*, indicating that this phenotype has emerged independently several times.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 shows the strains and plasmids used in this study. *Y. ruckeri* was routinely cultured at 28°C in Trypticase soy broth (TSB) or Trypticase soy agar (TSA). *Escherichia coli* strains were grown at 37°C in Luria broth (LB) or Luria agar (LA). When required, antibiotics (Sigma) were added at the following concentrations: ampicillin (100 μ g/ml) and kanamycin (100 μ g/ml).

Motility and phospholipase assays. Motility was assessed by inoculating bacterial strains onto TSA soft agar using the edge of a sterile coverslip. TSA soft

agar consisted of half-strength TSB (15 g/liter; Difco) solidified with 0.30% agar (Difco). Plates were examined for motility after 24 and 48 h of incubation at 28°C. Motility was confirmed by phase-contrast microscopy, and presence of the flagellum was assessed by direct staining using a commercial stain as directed by the manufacturer (Benton Dixon). Phospholipase activity was determined by calcium precipitation on Tween 80- and CaCl₂-amended TSA plates as described previously (13).

Molecular biological methods. DNA purification, restriction endonuclease digestions, DNA ligations, and agarose gel electrophoresis were performed using standard protocols (21). DNA was PCR amplified using HotStarTaq (Qiagen) according to the manufacturer's instructions. DNA sequencing was carried out using a model 3730xl DNA analyzer (Applied Biosystems) and BigDye version 3.1 reagents.

Complementation experiments. The pVK102 cosmid library used for complementation of BT2 strain YRNC10 was generated by shotgun cloning of genomic DNA from BT1 strain CSF007-82 using the following procedure. Total genomic DNA was treated with the restriction endonuclease HindIII under conditions which resulted in partial digestion (1 min at 37°C) and subsequently ligated with pVK102 DNA which had been digested to completion with HindIII. The ligation mix was then packaged *in vitro* and transfected using a commercial kit (Giga-packIII Gold packaging extract; Agilent Technologies) as specified by the manufacturer. Approximately 2,000 cosmid clones were then pooled and transferred *en masse* to strain YRNC10 by conjugation using *E. coli* MM294(pRK2013) as a helper. The resulting YRNC10 exconjugants were then pooled into 50 groups of 40 clones, and the pooled clones were inoculated onto plates containing motility media. After 5 days at 28°C, a complementing cosmid clone (pTW10.1) was identified as a sector of motile cells observed emanating from the point of inoculation from one pool. To identify a minimal complementing cosmid clone, pTW10.1 was subcloned into the broad-host-range conjugative cloning vector pBBR1MCS (18) by shotgun cloning using the following method. Purified pTW10.1 DNA was mechanically sheared to an average size of 2 to 5 kb using a nebulizer (Invitrogen). Sheared DNA was then prepared for cloning using the DNA Terminator end repair kit (Lucigen) and ligated into pBBR1MCS. Subclones were then transferred to strain YRNC10 by *en masse* conjugation and screened for complementation as described above. The plasmid used to complement Danish BT2 strain 950720-4/4 and Spanish strain 06076 was constructed by PCR amplifying the *flhBAE* region (shown in Fig. 2B) using primers FlhBFwd (5'-GCA GCT TTG TTC CGC CCA TAA C-3') and FlhERev (5'-TCA CTG ATA ATT CAC TAT TAC C-3'). The resulting PCR product was cloned directly using the pBAD TOPO TA kit (Invitrogen), and the construct was verified by

FIG. 1. Transcomplementation of flagellar motility and lipase secretion in BT2 strain YRNC10. Strains tested were as follows: row 1, CSF007-82 (BT1); row 2, YRNC10 (BT2); row 3, YRNC10 (BT2)/pTW10.1. Motility and lipase phenotypes were determined as described in Materials and Methods after 24 h (motility) or 48 h (lipase) of incubation at 28°C. Flagella were stained using a commercial stain as directed by the manufacturer (Benton Dixon reference number 261206) and observed by phase-contrast microscopy at a magnification of $\times 2,500$. Flagellum and lipase images shown were representative of at least 10 cells/colony examined.

DNA sequencing. The resulting plasmid (pBADBAE) was transferred to *Y. ruckeri* strains by electroporation (8).

Construction of strains YRNC102-1 and 970611-2/2.2-1. The *fliR* mutations in strains YRNC10 (*fliR* Δ 1) and 970611-2/2 (*fliR* Δ 2) were repaired by marker exchange-eviction mutagenesis essentially as described by Reid and Collmer (28), employing a *sacB*-containing R6K-based suicide vector pTW-MEV (40). First, the *fliR* gene was amplified from the wild-type BT1 strain CSF007-82 by PCR using primers *fliQF* (5'-TTA AGC TGA TGT TGT TTG TTC TGG-3') and *fliIR* (5'-GGG ATT TAT CCG GCC ATG-3'). This PCR product was then cloned directly using the TA cloning reagents (Invitrogen) into vector pCR2.1 and then subcloned as an *Xba*I-*Not*I fragment into the *Spe*I-*Not*I sites of plasmid pTW-MEV, resulting in plasmid pTW10.2. Plasmid pTW10.2 was then mobilized to *Y. ruckeri* strains YRNC10 and 970611-2/2 by conjugation, and selection with ampicillin was used to identify plasmid cointegrants. These clones were then grown overnight in the absence of selection and subsequently plated onto TSA containing 10% sucrose in order to identify sucrose-resistant clones that had undergone a second recombination event leading to *sacB* excision. A portion of these sucrose-resistant and ampicillin-sensitive clones had undergone a second recombination event leading to the replacement of the relevant mutant allele with the wild-type *fliR* allele. Successful gene replacement clones were identified by PCR amplification and sequencing of the *fliR* allele.

Detection of *Y. ruckeri* flagellar proteins by Western SDS-PAGE. For antiserum production, flagellar filaments were collected from *Y. ruckeri* strain CSF007-82 as follows. Cells were grown to late log phase for 48 h in TSB at 28°C, harvested by centrifugation, washed, and resuspended in phosphate-buffered saline (PBS) to an optical density at 600 nm (OD_{600}) equal to 100 units. Flagellar filaments were then removed from the cells by shearing in a rotating-blade blender (Oster model 4096-009) at maximum speed for 60 s. Deflagellated cells were then removed by centrifugation ($10,000 \times g$ for 15 min), and the flagellar filaments were subsequently concentrated by centrifugation at $100,000 \times g$ for 90 min. Polyclonal antiserum was produced in a New Zealand White rabbit by injection with the concentrated flagellar filaments described above using the following injection schedule. Priming was accomplished by injection with 0.5 ml of flagella (2 mg/ml) mixed 1:1 with complete Freund's adjuvant. This was followed by injection with 0.5 ml of flagella (2 mg/ml) mixed 1:1 with incomplete Freund's adjuvant 7, 14, 28, and 56 days later. Antiserum was collected 70 days after immunization and was preabsorbed to remove nonflagellar antibodies using

FIG. 2. Schematic representation of the flagellar secretion gene clusters found to contain BT2-causing mutations. Allele designations and geographic origins for each allele are indicated.

acetone powder extracts prepared from cells of strain BTF1, an *fliA* mutant derivative of strain CSF007-82, previously shown to be defective in the production of flagella (13). For immunoblot experiments, protein separation was performed as described by Laemmli (19) by using a resolving gel consisting of 12.5% acrylamide (30:0.8 acrylamide-bisacrylamide) and electroblotting to nitrocellulose membranes (Bio-Rad). Membranes were then blocked for 1 h at room temperature, washed in PBS, and then incubated for 2 h at room temperature with a 1/5,000 dilution of the anti-flagellum antiserum described above. Flagellin antibodies were then detected using the protein detector Western blot kit (KPL) as described by the manufacturer.

Nucleotide sequence accession number. The sequences determined in this study have been deposited in the GenBank database under accession number JF330168.

RESULTS

Complementation of the biotype 2 phenotype in strain YRNC10. The hypothesis that BT2 strains are recent mutant derivatives of motile BT1 strains was tested by the use of transcomplementation, utilizing DNA from a motile BT1 strain, to restore motility to a BT2 strain and thereby identify the genetic defect(s) causing the BT2 phenotype. For this, a cosmid library containing total DNA from BT1 strain CSF007-82 was constructed by shotgun cloning into the conjugative cosmid vector pVK102. Cosmid clones were then pooled and mobilized *en masse* to BT2 strain YRNC10, and the resulting transformants were screened for motility and lipase secretion. Using this approach, a single cosmid clone was identified (designated pTW10.1) that induced both motility and lipase secretion in YRNC10 (Fig. 1). In control experiments, the pVK102 vector alone did not restore flagellar secretion. Additionally, to ensure that complementation was due to pTW10.1 and not to spontaneous mutation(s) in the recipient clone, pTW10.1 cosmid DNA was purified and shown to complement an independent YRNC10 clone when introduced by conjugation. Since pTW10.1 contained a relatively large insert (approximately 30 kb), shotgun subcloning was used to identify several small clones that were sufficient for complementation (results not shown). DNA sequencing revealed that all complementing clones had in common the 2,176-bp region containing the three open reading frames (ORFs) shown in Fig. 2A. The *fliP*, *fliQ*, and *fliR* genes encode putative conserved integral membrane components of the flagellar secretion apparatus that are each essential for flagellar secretion in *Salmonella* (20, 22). The predicted primary amino acid sequences encoded by the *fliP*, *fliQ*, and *fliR* genes are 86, 77, and 63% identical, respectively, to analogous proteins in *Salmonella enterica*. This region was 100% identical to that of the

FIG. 3. Genetic complementation of flagellar production (A) and phospholipase secretion (B) phenotypes of the following strains: column 1, CSF007-82 (BT1); column 2, YRNC10 (BT2); column 3, YRNC102-1 (BT2), *fliRΔ1* repaired; column 4, 970611-2/2 (BT2); column 5, 970611-2/2.2-1 (BT2), *fliRΔ2* repaired; column 6, 950720-4/4 (BT2)/pBAD; column 7, 950720-4/4 (BT2)/pBADBAE; column 8, 06076 (BT2)/pBAD; column 9, 06076 (BT2)/pBADBAE. Lipase phenotypes were determined as described in Materials and Methods, and flagella were visualized as described in the legend to Fig. 1. Flagella and lipase images shown were representative of at least 10 cells/colony examined, except for strain 950720-4/4 (BT2)/pBADBAE (column 7), where a low incidence of flagella was observed (approximately 1/200 cells were flagellated).

corresponding region recently sequenced as part of a draft genome sequence of the *Y. ruckeri* ATCC 29473 strain, which, like CSF007-82, is a motile serovar I strain (6). The predicted *Y. ruckeri* FliP, FliQ, and FliR proteins were greater than 93, 95, and 79% identical, respectively, to analogous proteins found in other members of the *Yersinia* genus (not shown).

Identification of the BT2-causing mutation in strain YRNC10. Complementation of the BT2 phenotype in strain YRNC10 with the *fliP*, *fliQ*, and *fliR* genes suggests that this region may be absent or damaged in BT2 strain YRNC10. To confirm this, the *fliPQR* gene cluster was amplified and sequenced from strain YRNC10, revealing a single-residue deletion at nucleotide 90 within the *fliR* ORF at codon 30 of 260 (denoted as *fliRΔ1* allele; see Fig. 2). This mutation caused a predicted frameshift followed by a stop codon, resulting in premature truncation just after amino acid 32. The *fliPQR* region was otherwise identical between these strains. These results are consistent with the hypothesis that motility and secreted lipase phenotypes in BT2 strain YRNC10 are due to damage to a single essential flagellar secretion gene, specifically the *fliR* gene. However, in this complementation analysis, it remained possible that expression of the *fliPQR* region from a multiple-copy plasmid could compensate for damage to other essential flagellar secretion genes outside this region. To investigate this possibility, the single-nucleotide deletion present in BT2 strain YRNC10 was repaired by exchanging the *fliRΔ1* allele with a wild-type copy of *fliR* using allelic exchange and thus creating an isogenic repaired derivative referred to as YRNC102-1. Repair of the *fliRΔ1* mutation resulted in observed motility and production of both flagella and secreted lipase activity (Fig. 3, column 3, and Fig. 4, lane 3) at levels similar to that of BT1 strain CSF007-82. These results provide unambiguous evidence that this mutation is responsible for the lack of flagellar motility and secreted phospholipase production in BT2 strain YRNC10.

Identification of additional BT2-causing mutations in European strains. DNA sequencing of the *fliR* allele of additional *Y. ruckeri* BT2 strains from the southeastern United States revealed that all 17 strains examined contained the *fliRΔ1* mutant allele (Table 2). Interestingly, BT2 strains examined from the United Kingdom were also uniformly positive for this *fliRΔ1* mutant allele (29 strains examined; see Table 2). In contrast, none of the BT2 strains examined from Denmark, Finland, or Spain contained the *fliRΔ1* mutation. To identify

potential BT2-causing mutations in Danish, Finnish, and Spanish BT2 strains, the *fliPQR* region, as well as a previously identified flagellar secretion region encoding *flhBAE* (13), was PCR amplified and sequenced from several strains. These analyses led to the identification of three additional flagellar secretion mutations shown in Fig. 2. Danish BT2 strain 970611-2/2 contained a 10-bp deletion within the *fliR* coding region, 350 bp downstream of the *fliRΔ1* deletion, extending from nucleotide 440 to 449 within the ORF. This mutation, designated *fliRΔ2*, caused a predicted frameshift at codon 147 of 260 and premature truncation 22 amino acids downstream (Fig. 2). The *fliRΔ2* mutant allele was prevalent among Danish BT2 strains (30 of 31 examined; Table 2) as well as Finnish BT2 strains (18 of 18 examined; Table 2). Danish BT2 strain 950720-4/4 was the same as the wild type at the *fliR* allele yet contained a mutation within the *flhA* gene. This mutation, referred to as *flhA*(G256D), caused a predicted nonsynonymous substitution (G for D) at codon 256 of 692 within the predicted FlhA protein (Fig. 2). A final mutation was identified in the Spanish strain 06076. This mutation consisted of a 10-bp

FIG. 4. Western SDS-PAGE detection of flagellin from whole-cell extracts. The following strains were analyzed: column 1, CSF007-82 (BT1); column 2, YRNC10 (BT2); column 3, YRNC102-1 (BT2), *fliRΔ1* repaired; column 4, 970611-2/2 (BT2); column 5, 970611-2/2.2-1 (BT2), *fliRΔ2* repaired; column 6, 950720-4/4 (BT2)/pBAD; column 7, 950720-4/4 (BT2)/pBADBAE; column 8, 06076 (BT2)/pBAD; column 9, 06076 (BT2)/pBADBAE. The strains analyzed in this figure correspond to those shown in Fig. 3.

TABLE 2. Frequency of biotype 2-causing alleles among U.S. and European isolates

Country	Year(s) collected	Allele	Fraction of isolates positive for allele
United States	1993, 1995–1997, 2003	<i>fliRΔ1</i>	17/17
United Kingdom	1981, 1986, 1987, 1990, 1993, 1999, 2002–2004	<i>fliRΔ1</i>	29/29
Denmark	1995, 1997, 1998, 2001–2006, 2008, 2009	<i>fliRΔ2</i>	30/31
Denmark	1995	<i>flhA(G256D)</i>	1/31
Finland	2005–2008	<i>fliRΔ2</i>	18/18
Spain	2003	<i>flhBΔ1</i>	1/1

deletion spanning from nucleotides 769 to 778 within the *flhB* gene, causing a predicted frameshift after codon 256 of 383 and a premature translational stop six codons downstream (Fig. 2). The *flhA* and *flhB* genes are also predicted to encode membrane-associated components of the flagellar biosynthesis system in *Salmonella* (20, 22). In addition, *flhA* has been previously shown to be necessary for flagellar biosynthesis in *Y. ruckeri* (13).

Genetic complementation of European BT2 strains. Complementation analysis was used to verify that the flagellar secretion mutations identified in Danish and Spanish BT2 strains are responsible for the BT2 phenotype observed in these strains. For the *fliRΔ2* mutation in Danish BT2 strain 970611-2/2, allelic exchange was used to repair the mutant allele. The resulting strain (970611-2/2.2-1) was motile and capable of producing both flagella and phospholipase activity (Fig. 3, column 5, and Fig. 4, lane 5) at levels that were similar to that of BT1 strain CSF007-82. Danish BT2 strain 950720-4/4 and Spanish strain 06076, containing mutations in *flhA* and *flhB*, respectively, were tested by the addition of a recombinant plasmid containing the *flhBAE* region (schematic in Fig. 2B). Transfer of this plasmid (pBADBAE) to strain 06076 (Spanish) resulted in robust complementation of the flagellar secretion defect (Fig. 3, column 9, and Fig. 4, lane 9). However, transfer of pBADBAE to strain 950720-4/4 (Danish) resulted in limited complementation, as indicated by a low occurrence of flagellated cells (flagella detected in less than 1/200 cells), as well as a lack of detectable lipase secretion (Fig. 3, column 7) and limited production of flagellin as determined by Western blotting (Fig. 4, lane 7). There are several possible explanations for this failure to completely complement, the most likely of which is that the *flhA(D256G)* mutation may exert a dominant negative influence interfering with the wild-type FlhA protein. Alternatively, Danish BT2 strain 950720-4/4 might have additional genetic differences which explain the reduced expression of the flagellar secretion phenotype.

DISCUSSION

Herein, we report the molecular-genetic basis for the concurrent loss of motility and secreted lipase activity in multiple BT2 *Y. ruckeri* strains. Four types of BT2-causing mutations were identified among 96 strains from the United States,

United Kingdom, and mainland Europe, each found in three separate genes encoding critical components of the flagellar secretion apparatus (Fig. 2). Three of the four mutations cause a frameshift that renders the respective proteins incomplete and presumably nonfunctional. The fact that flagellar motility and lipase secretion in the BT2 strains carrying these mutant alleles were restored by supplying a wild-type copy of the damaged gene indicates that these mutations alone are responsible for the BT2 phenotype observed and shows that these strains have otherwise intact flagellar secretion and motility systems, as well as the genetic elements necessary for phospholipase secretion. This study also provides further evidence that *Y. ruckeri* production of secreted lipase activity requires an intact flagellar secretion system (13). While it is difficult to predict the effect of the nonsynonymous *flhA(G256D)* mutation observed in Danish strain 950720-4/4, the incomplete complementation phenotype observed in this strain when the *flhA* gene was provided in *trans* suggests that this mutation is partially responsible for the loss of motility seen in this strain. These findings clearly demonstrate that BT2 strains have evolved from related motile strains through mutations in the flagellar secretion apparatus.

BT2 *Y. ruckeri* was recognized in the United Kingdom prior to 1989, with the earliest strains isolated in 1981 (11), while in Spain, Denmark, and Finland, BT2 strains have been identified more recently (15, 36, 42). Moreover, pulsed-field gel electrophoresis (PFGE) analysis established that the BT2 strains in Denmark and Spain are more closely related to the BT1 strains present in these countries than to BT2 strains from the United Kingdom, thus suggesting that the BT2 phenotype has arisen independently in mainland Europe from local BT1 strains (42). In this work, we have demonstrated that BT2 strains examined from the United Kingdom all contained the same BT2-causing mutation (*fliRΔ1*), while differing BT2 mutations were detected in strains from Denmark [*fliRΔ2* and *flhA(G256D)*] and Spain (*flhBΔ1*). These results demonstrate that the BT2 phenotype has arisen independently at least four times within the more virulent serovar 1 *Y. ruckeri* subgroups and that the distribution of BT2 strains throughout Europe is not simply the result of expansion and dissemination of a single highly successful BT2 clonal group.

This study provides strong evidence for cross-border BT2 strain dissemination. Our analysis has shown that BT2 strains from the southeastern United States uniformly contained the *fliRΔ1* mutant allele found also in United Kingdom strains. Similarly, PFGE analysis of strain YRNC10, a North Carolina BT2 strain, revealed a PFGE pattern identical to the majority of BT2 strains circulating in the United Kingdom (42). These results suggest that BT2 strains in the United States and United Kingdom originated from a common source. The mechanism of this cross-continent dissemination is unclear. Although the *fliRΔ1* lineage of BT2 *Y. ruckeri* has been prevalent in the United Kingdom since at least 1981, it apparently has not spread to mainland Europe. Similarly, we have found no evidence of the mainland European BT2 lineages [*fliRΔ2*, *flhA(G256D)*, and *flhBΔ1* strains] in the United Kingdom. This suggests that cross-border strain movement between the United Kingdom and mainland Europe is infrequent, confirming earlier reports that ERM-causing *Y. ruckeri* strains circulating in Europe and the United Kingdom represent distinct,

nonoverlapping subpopulations (9, 42). Finnish strains of BT2 *Y. ruckeri* also appear to have been imported. Work by Ström-Bestor et al. (36) documented the recent isolation of BT2 *Y. ruckeri* in Finland and demonstrated a high degree of genetic relatedness between Finnish and Danish BT2 strains as assessed by PFGE. Our results show that the prevailing BT2-causing allele in Danish BT2 strains (*fliR* Δ 2) was also found in all BT2 strains examined from Finland, further implying that BT2 *Y. ruckeri* strains in Denmark and Finland are clonal and suggesting that Finnish BT2 strains were recently introduced, as opposed to having arisen from a native Finnish strain. While the mechanism behind the spread of BT2 *Y. ruckeri* to Finland is unknown, it has been speculated that the introduction of this pathogen may have been caused by the importation of infected rainbow trout fry and fingerlings from Denmark (36).

Our results demonstrate that BT2 *Y. ruckeri* has been present in the southeastern United States for much longer than previously realized (at least 17 years), a finding which may explain why standard immersion vaccination protocols have never provided sufficient protection in this area (3) (Charles Carson, personal communication; Charles Zeigler, personal communication). This failure of immersion vaccination has led commercial trout producers in North Carolina to implement an injection vaccination procedure which has proven sufficiently efficacious but adds significantly to labor costs (Skipper Thompson, NC Cooperative Extension Service; unpublished data). An immersion vaccine designed to protect fish from both BT1 and BT2 strains has recently been developed and licensed for use in many European countries under the trade name AquaVac RELERA (4) (Chris Gould [Intervet/Schering-Plough Animal Health, United Kingdom], personal communication). The BT2 component of this vaccine consists of a United Kingdom BT2 strain which we have demonstrated contains the *fliR* Δ 1 mutant allele. Our results showing that U.S. strains of BT2 *Y. ruckeri* contain the same BT2-causing mutant allele present in United Kingdom strains (Table 2) suggests that these strains share an origin. If these strains remain antigenically similar, then the immersion vaccine produced for use in Europe may also be effective against U.S. BT2 strains.

What is driving the loss of flagellar motility in *Y. ruckeri*? Mutational loss of flagellar motility is not uncommon among bacterial pathogens. *Yersinia pestis*, the causative agent of plague, is thought to have evolved relatively recently (1,500 to 20,000 years) from *Y. pseudotuberculosis* (1, 12, 24, 44), and this transition from food-borne to insect-borne pathogen has involved both the gain and loss of critical functions, including the loss of flagellar motility (44). Similarly, the evolution of *Shigella* from *E. coli* included the loss of flagellar motility, and like BT2 *Y. ruckeri*, *Shigella* appears to have lost flagellar motility independently several times, resulting in distinct lineages (25, 37). Finally, genome sequencing of *Aeromonas salmonicida* subsp. *salmonicida*, a fish pathogen, revealed cryptic flagellar motility genes, suggesting that this pathogen also evolved from a motile ancestor (27). In all of these cases, it is thought that flagellar motility became obsolete or deleterious as these species evolved to a new mode of transmission or host niche (23, 37).

In several motile pathogens, the repression of flagellar gene expression inside the host is critical, as *in vivo* expression results in flagellin-mediated host recognition and attenuation (2,

23, 32, 35). Since expression of flagella can be disruptive to the infection process, mutational loss of flagellar motility may occur in pathogens that can tolerate the loss of motility, thereby ensuring that inappropriate flagellin expression does not occur (23). It is possible that these selective forces may be driving the loss of flagellar secretion and motility in *Y. ruckeri*. We have previously shown that a defined mutation in *flhA*, which encodes a critical component of the flagellar secretion apparatus, mimicked the BT2 phenotype and had no discernible effect on virulence in a motile BT1 strain when using a natural infection route and an immunologically naive host (13). Loss of motility and lipase secretion also has not affected the success of natural BT2 strains, as evidenced by the recent reports of BT2-caused disease outbreaks and laboratory virulence testing of BT2 strains (3, 4, 15, 36, 41). While these results suggest that mutational loss of flagellar secretion can be tolerated by this pathogen with no measurable effect on virulence, currently there is no evidence that loss of flagellar secretion provides a selective advantage in avoiding the immune response. A recent study found that naive rainbow trout produce a robust proinflammatory and acute-phase response to injection challenge with BT2 *Y. ruckeri* strain YRNC10 (43). While a selective advantage was not identified, the authors do not rule out the possibility that flagellin may induce immune gene expression in tissues that were not sampled or that flagellin may induce expression of immune genes not included in their analyses.

Another possibility is that the BT2 phenotype may confer a selective advantage in only vaccinated fish. This would suggest that flagellar antigens, and/or other antigens secreted through the flagellar secretion apparatus, are important protective components of immersion-applied ERM vaccines, and loss of these antigens in a BT2 strain allows the evasion of a vaccine-mediated response (15). This may explain the correlation between vaccine failure and the emergence of the BT2 phenotype. However, it should be noted that the link between flagellar secretion and vaccine failure has not been directly established. Our current efforts are directed toward determining if the BT2 phenotype is responsible for ERM vaccine failure.

In conclusion, it is likely that serovar 1 *Y. ruckeri* is an example of a recently emerged and disseminated pathogen, and the loss of flagellar motility, indicative of BT2 strains, is the first major phenotypic divergence within this clonal group. We speculate that the amplification and dissemination of *Y. ruckeri* occurred when this pathogen found an alternative niche (intensively cultured rainbow trout) and that loss of flagellar motility represents the early stages of dispensing with unnecessary or deleterious phenotypic traits through genome decay. This model is consistent with the epidemiology of *Y. ruckeri*, which suggests that ERM started as a geographically isolated disease which relatively quickly became widely disseminated (5, 16). While the selective pressure(s) driving the evolution of the BT2 phenotype remains unclear, the independent emergence of the BT2 phenotype in different geographic locations argues that this phenotype is likely important for the success of these strains. The correlation between the presence of BT2 strains and immersion vaccination failure is intriguing; however, it remains possible that the BT2 phenotype could alternatively confer an advantage in the growth or persistence of *Y. ruckeri* when outside the host in the aquaculture environment

or perhaps when associated with an alternative host or vector (43). Regardless, the identification of specific BT2-causing mutations and the creation of the isogenic-repaired derivatives reported here will now make it possible to determine the role that these mutations play in vaccine failure using a vaccination-challenge model. The differentiation of distinct BT2 lineages is also of critical importance for the development and validation of vaccination or other treatment strategies intended for the control of emergent BT2 strains.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

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