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First Report of *Yersinia ruckeri* Biotype 2 in the USA

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[Communication]

First Report of *Yersinia ruckeri* Biotype 2 in the USA

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Abstract.—A polyphasic characterization of atypical isolates of *Yersinia ruckeri* (causative agent of enteric redmouth disease in trout) obtained from hatchery-reared brown trout *Salmo trutta* in South Carolina was performed. The *Y. ruckeri* isolates were biochemically and genetically distinct from reference cultures, including the type strain, but were unequivocally ascribed to the species *Y. ruckeri*, based on API 20E, VITEK, fatty acid methyl ester profiles, and 16S rRNA gene sequencing analysis. These isolates were nonmotile and unable to hydrolyze Tween 20/80 and were therefore classified as *Y. ruckeri* biotype 2. Genetic fingerprint typing of the isolates via enterobacterial repetitive intergenic consensus (amplified by polymerase chain reaction) and fragment length polymorphism showed biotype 2 as a homogeneous group distinguishable from other *Y. ruckeri* isolates. This is the first report of *Y. ruckeri* biotype 2 in the USA.

Enteric redmouth disease (ERM) is one of the most serious diseases affecting freshwater trout aquaculture. The causal agent, *Yersinia ruckeri,* was first identified from diseased trout in Idaho (USA) during the 1950s (Ross et al. 1966). Later, *Y. ruckeri* was isolated throughout other parts of North and South America, Europe, and even Australia (Austin and Austin 1999). Initially considered a phenotypically homogeneous species (Ewing et al. 1978), *Y. ruckeri* is characterized as a motile Gram-negative rod; oxidase negative; lysine decarboxylase and ornithine decarboxylase positive; able to ferment glucose, mannitol, maltose, and trehalose; and negative for indole, salicin, and esculin tests (Brenner et al. 2005).

Davies and Frerichs (1989) extensively characterized 147 isolates of *Y. ruckeri* from different origins and found that 18% of the isolates were nonmotile and negative for Tween hydrolysis. They proposed these atypical strains to be recognized as *Y. ruckeri* biotype 2. In recent years, atypical, nonmotile isolates of *Y. ruckeri* have been reported as responsible for ERM outbreaks in rainbow trout *Oncorhynchus mykiss* in England (Austin et al. 2003) and Spain (Fouz and Amaro 2006). Interestingly, both outbreaks affected fish that had been vaccinated with commercial ERM vaccines (biotype 1).

During the summers of 2003–2005, a state-operated trout hatchery in South Carolina suffered a series of high-mortality infectious episodes affecting brown trout *Salmo trutta*. Diseased fish showing typical signs of ERM were examined and necropsied at the Fish Disease Diagnostic Laboratory (Auburn University, Alabama). Gram-negative, oxidase-negative, fermentative, nonmotile rods were isolated from the fish and partially identified following blue book protocols (FHS 2004). In this study, we fully characterized these atypical *Y. ruckeri* isolates and confirmed the presence of *Y. ruckeri* biotype 2 in the USA.

Methods

Case study.—Over a 3-year period, there were five ERM outbreaks investigated in brown trout at the Walhalla State Fish Hatchery, Mountain Rest, South Carolina. These outbreaks occurred in June, September, and January (January isolate not included in this report). The brown trout examined were Walhalla strain and had been hatched on premises from eggs collected from broodfish maintained at this hatchery. The affected fish were 86–225 mm in total length. Fish affected in 2005 had been previously vaccinated against ERM with Ermogen (following manufacturer's

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TABLE 1.—Historical and discriminatory data related to isolates of *Yersinia ruckeri* examined in an investigation of enteric redmouth disease in brown trout at Walhalla State Fish Hatchery, South Carolina. Blank cells indicate that the test was not conducted; positive (+) and negative (–) results are also shown. The type strain (ATCC 29473) and three previously confirmed isolates were included as a reference.

Isolate	Date collected	Organ	Motility[a]	Tween 20/80	Voges–Proskauer[b]	API 20E (22°C/48h)	API 20E (22°C/48h)	VITEK GNI+
From rainbow trout, USA								
ATCC 29473			+	+	+	5107100	5105100	6000300023
From goldfish *Carassius auratus*, Arkansas								
PB98–25	1998	Internal	+	+	+	5107100	5105100	6000300023
From channel catfish, Arkansas								
LV-1	1996	Internal	+	+	+	5107100	5105100	6000300023
From unknown fish species, southeastern USA								
U1	1994	Unknown	+	–	–	5104100	5105100	6000300023
From brown trout, South Carolina								
SC03–05#3	26 Jun 2003	Spleen	–	–	–			
SC03–05#6	26 Jun 2003	Liver	–	–	–			
SC04–07#1	8 Jun 2004	Liver	–	–	–			
SC04–07#4	8 Jun 2004	Kidney	–	–	–	5107100	5105100	
SC04–15#1	14 Sep 2004	Kidney	–	–	–	5107100	5105100	6000300023
SC04–15#2	14 Sep 2004	Liver	–	–	–	5107100	5105100	6000300023
SC04–15#3	14 Sep 2004	Kidney	–	–	–	5107100	5105100	
SC04–15#4	14 Sep 2004	Liver	–	–	–	5107100	5105100	
SC05–12#1	20 Jun 2005	Kidney	–	–	–	5107100	5105100	6000300023
SC05–14#1	20 Jun 2005	Kidney	–	–	–	5107100	5105100	6000300023

[a] Motility was assayed at 30°C.
[b] Assayed in MRVP broth at 30°C.

instructions; Aqua Health, Ltd., Prince Edward Island, Canada) at approximately 7 months of age. Moribund fish exhibiting clinical signs of disease were submitted to the Fish Disease Diagnostic Laboratory at Auburn University for diagnosis. Four to seven fish were necropsied during each episode. The observed gross lesions varied but generally included hyperemia of the skin and intestine, exophthalmia, and pale liver. Initial bacterial isolates were cultured from trunk kidney and liver on brain heart infusion agar (BHIA; Becton Dickinson, Sparks, Maryland). The BHIA plates were incubated at 30°C for 18–24 h, and pure cultures were obtained after 24 h incubation by streaking single colonies for isolation.

Characterization of the isolates.—All isolates (Table 1) were examined following standard methods (FHS 2004) for Gram-negative staining, catalase production, cytochrome oxidase, H_2S and indole, motility, oxidative-fermentative metabolism of glucose, Voges–Proskauer reaction, and Tween 20/80 hydrolysis. In addition, cultures were subjected to API 20E and VITEK (Gram-negative identification plus [GNI+] cards V1311) (bioMerieux, Inc., Durham, North Carolina) rapid identification systems; we followed the manufacturer's instructions, except that incubation temperatures and times were modified to 48 h at 22°C and 24 h at 28°C for API 20E.

Fatty acid methyl ester analysis.—Fatty acid methyl ester (FAME) profiles were obtained from all isolates cultured on sheep blood agar plates for 24 h at 28°C (Shoemaker et al. 2005).

Gene sequencing.—Cells were grown on tryptone soy agar (Becton Dickinson) and were incubated at 28°C for 24 h. Total bacterial DNA was extracted using the Qiagen DNeasy Tissue kit (following manufacturer's instructions; Qiagen, Valencia, California). Universal primers 20F (5′-AGAGTTTGATC[AC]TGGCTCAG-3′; positions 8–27 of *Escherichia coli* numbering) and 1500R (5′-CGATCCTACTTGCGTAG-3′; positions 1,510–1,492 of *E. coli* numbering) were used to amplify the 16S rDNA gene. The polymerase chain reaction (PCR) conditions and cycles were performed according to Arias et al. (1995). Expected size amplicons (about 1.5 kilobase pairs) were resolved through standard agarose gel electrophoresis. Amplified products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation, Indianapolis, Indiana) and submitted for sequencing to the Auburn University Sequencing Core. Sequences were cleaned up, and their homology to 16S rRNA genes was confirmed by nucleotide–nucleotide BLAST (Basic Local Alignment Search Tool) search (http://www.ncbi.nlm.nih.gov/BLAST/).

Genotyping.—Two different genotyping techniques were used to characterize the isolates. First, enterobacterial repetitive intergenic consensus (ERIC) sequences from all *Y. ruckeri* isolates were amplified by PCR (Cubero and Graham 2004). Second, amplified fragment length polymorphism (AFLP) fingerprints were determined (Arias et al. 1997, 2004).

Polyphasic data analysis.—Phenotypic (API 20E, VITEK, and FAME) and genotypic (ERIC-PCR and AFLP) data were introduced into a computerized database and analyzed using the software package BioNumerics version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Phenotypic data based on limited character sets (API 20E and VITEK) were analyzed using a binary coefficient (Dice) based on positive–negative results. The FAME profile cluster analysis was carried out using Pearson's product-moment correlation coefficient (r). Genotyping data were analyzed according to Arias et al. (2003). Levels of similarity between fingerprints were calculated with the Dice coefficient based on presence–absence of bands. Similarity matrices of all data described above were analyzed by the unweighted pair-group method to generate dendrograms based on average linkages. Composite data sets, character tables containing data from more than one typing method, were used to create a polyphasic profile for all isolates of *Y. ruckeri*.

Results

Case Study

Cultures from necropsied fish produced a dominant culture type of 1-mm, circular, convex, opaque, grayish-white colonies on BHIA plates incubated at 30°C for 24 h. Growth at 20°C was also noted but required an additional 24 h before comparable colonies appeared. Isolates were subjected to the classical microbiology tests previously discussed. Cultures showed the key characteristics of the *Enterobacteriaceae*, namely the presence of Gram-negative, fermentative rods that produce catalase but not oxidase. These results and the lesions observed in the necropsied fish were consistent with characteristics of *Y. ruckeri*.

Additional biochemical tests (motility at 30°C, H_2S and indole production, Tween 20/80 hydrolysis, and Voges–Proskauer) were performed according to established identification methods (FHS 2004). The *Y. ruckeri* type strain and three previously confirmed *Y. ruckeri* isolates were included as a reference. No H_2S or indole was produced by any of the isolates examined. However, motility, Tween 20/80 hydrolysis, and Voges–Proskauer tests presented variable results among the isolates tested (Table 1). All South Carolina isolates were negative for motility, acetoin production (Voges–Proskauer), and Tween 20/80 hydrolysis, whereas the type strain (American Type Culture Collection [ATCC] 229473) and reference cultures LV-1 and PB98–25 were positive for these tests. Isolate U1 was motile but negative for Tween 20/80 hydrolysis and Voges–Proskauer reaction.

Confirmatory Identification Tests

Putative South Carolina isolates of *Y. ruckeri* were subjected to a complete polyphasic characterization. Biochemical profiles were obtained using two commercial miniaturized systems used for Enterobacteria identification: API 20E and VITEK GNI+ cards (see Table 1 for bionumbers). South Carolina isolates were indistinguishable from reference cultures examined via API 20E, regardless of incubation temperature. Note that all isolates except U1 gave a Voges–Proskauer-positive reaction when assayed by API 20E. The API 20E profiles from strips incubated at 22°C for 48 h differed in gelatin hydrolysis results from those incubated at 28°C for 24 h. No gelatin hydrolysis was observed at 28°C after 24 h of incubation; therefore, the test was recorded as negative. However, after 48 h at 22°C, all *Y. ruckeri* isolates except U1 were positive for gelatin hydrolysis. When the VITEK system was used to identify the isolates, all isolates presented the same bionumber (6000300023) as the type strain. This bionumber was different from the ones previously reported for *Y. ruckeri* (Linde et al. 1999), although no strain information was provided in that study.

All South Carolina isolates shared a very similar FAME profile that although slightly different from the one exhibited by the type strain ATCC 29473 (Table 2), resulted in a good identification. Finally, partial sequencing of approximately 1,500 base pairs (bp) of the 16S rRNA genes from all South Carolina isolates perfectly matched the 16S rRNA gene sequence from the *Y. ruckeri* type strain (GenBank accession number X75275). Based on these data, and despite unique biochemical properties, ascription of South Carolina isolates to the species *Y. ruckeri* was unequivocal.

Isolate Characterization

Phenotypic characterization was carried out by comparing the different FAME profiles. Main fatty acids exhibited by all isolates are listed in Table 2. Cluster analysis of FAME divided the isolates in two groups (data not shown). The main cluster contained all South Carolina isolates and PB98–25. Type strain and isolate LV-1 presented an almost identical profile, grouping with the South Carolina isolates at 95% similarity. The most dissimilar isolate was U1, sharing 92% similarity with the rest of the isolates.

Genotyping by ERIC-PCR further supports the

TABLE 2.—Fatty acids of *Yersinia ruckeri* detected by the CLIN40 method as applied to our investigation of enteric red mouth disease in brown trout in Walhalla State Fish Hatchery, South Carolina. The type strain (ATCC 29473) and three other previously confirmed isolates were included in the analysis.

	Feature name (%)								
Isolate	12:0	14:0	15:0	12:0 aldehyde/ unknown	16:1 w7c/15 iso 2OH	16:0	17:0 CYCLO	17:0	18:1 w7c
ATCC 29473	5.280	1.000	1.630	9.470	10.900	29.270	23.610	1.190	12.050
SC03–05#3	4.980	1.030	0.740	8.200	21.200	30.860	16.240	0.750	12.520
SC03–05#6	5.050	0.950	1.130	8.560	19.240	30.040	16.920	1.090	12.740
SC04–07#1	4.990	0.970	1.140	8.260	17.970	30.130	19.180	1.070	12.340
SC04–07#4	4.990	0.960	1.120	8.190	18.790	29.980	18.580	1.100	12.450
SC04–15#1	4.990	0.990	1.600	7.820	17.770	28.930	20.000	1.500	12.410
SC04–15#2	4.970	1.020	1.160	8.540	20.420	30.240	15.510	1.110	12.380
SC04–15#3	5.060	0.990	0.710	8.280	20.150	31.190	16.750	0.770	12.780
SC04–15#4	4.820	0.950	1.190	7.990	18.680	30.000	18.820	1.120	12.570
SC05–12#1	5.100	0.980	2.070	9.140	15.890	28.950	19.220	1.820	12.380
SC05–14#1	5.010	1.010	1.640	8.760	18.300	29.180	17.940	1.500	12.170
PB98–25	5.040	0.970	2.010	9.050	15.790	28.770	19.090	1.800	12.220
LV-1	5.430	1.070	1.500	9.870	11.910	29.660	21.630	1.100	11.870
U1	4.770	1.040	5.540	8.150	23.540	21.890	14.560	2.130	11.580
Mean (± SD)	5.034 (± 0.16)	0.995 (± 0.03)	1.655 (± 1.19)	8.591 (± 0.59)	17.896 (± 3.40)	29.220 (± 2.22)	18.432 (± 2.40)	1.289 (± 0.40)	12.318 (± 0.32)

homogeneity of the South Carolina isolates because all of them exhibited the same ERIC-PCR profile. All *Y. ruckeri* isolates yielded an ERIC-PCR pattern consisting of 5–8 bands ranging from 250 to 750 bp. Cluster analysis (data not shown) of selected bands (5% minimum profiling relative to maximum value of lane) showed that all South Carolina isolates shared an identical ERIC-PCR profile, whereas the type strain (ATCC 29473) clustered along with PB98–25 and LV-1 at 90% similarity. Finally, U1 proved again to be a unique isolate, clustering apart at 78%.

The AFLP typing of *Y. ruckeri* isolates yielded an average of 80 bands per profile, ranging in size from 50 to 700 bp. However, out of about 80 bands, only 15 band positions were polymorphic, denoting a low degree of genetic variability among the isolates. Figure 1 shows the dendrogram generated after analysis of AFLP patterns. As was found by FAME and ERIC-PCR, three distinct groups were inferred at 97% similarity (standard cutoff point for this technique in our laboratory; Arias et al. 2003). The main cluster grouped all South Carolina isolates regardless of year of isolation. The type strain along with the isolates from Arkansas clustered together at 96% similarity and joined the main group at 85% similarity. The U1 isolate once more showed its uniqueness and was not ascribed to either cluster.

Agreement between all typing methods used was satisfactory, so data from API 20E, VITEK, and FAME were combined in a single similarity matrix that was then used to generate a dendrogram (Figure 2). Our data confirmed the South Carolina isolates as a different biotype within the species, genetically homogeneous and distinct from the type strain and other typical isolates of *Y. ruckeri*.

Discussion

During the summers of 2003–2005, Walhalla State Fish Hatchery experienced a series of infectious outbreaks. Moribund fish presented typical signs of ERM, and microbial examination resulted in the isolation of pure cultures of a Gram-negative bacterium from the infected fish. Preliminary bacteriological tests confirmed the isolates as enterobacteria, and putative identification indicated *Y. ruckeri* as the causal agent of the outbreaks. However, all isolates recovered were nonmotile, an unusual characteristic for *Y. ruckeri*. An extensive polyphasic characterization of these atypical isolates ascribed them to *Y. ruckeri* biotype 2 (Davies and Frerichs 1989). Our study reports the first isolation of *Y. ruckeri* biotype 2 in the USA.

Genetic characterization of South Carolina biotype 2 isolates was performed using two genotyping methods that have been successfully used to fingerprint fish pathogenic enterobacteria (Coquet et al. 2002; Arias et al. 2004). Attempts to genetically characterize *Y. ruckeri* have proven to be difficult in the past because of the low intraspecific diversity found within the species (Schill et al. 1984; Sousa et al. 2001). This homogeneity is a drawback for epidemiological studies that could help us to better understand ERM epizootiology. Only a few studies (Romalde et al. 1993; Sousa et al. 2001) have been able to show genetic variability in *Y. ruckeri*. By using ERIC-PCR, we were able to correlate South Carolina biotype 2 isolates with a specific ERIC-PCR profile. Our AFLP fingerprinting

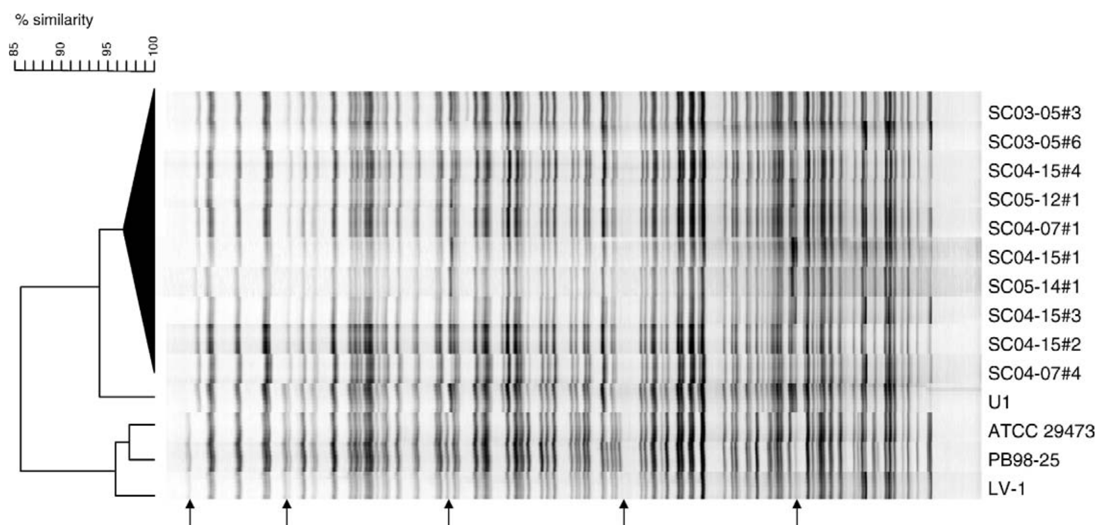

FIGURE 1.—Amplified fragment length polymorphism (AFLP) patterns of *Yersinia ruckeri*. The dendrogram was derived by the unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis of AFLP profiles of 14 *Yersinia ruckeri* strains. The tracks show the processed band patterns after conversion, normalization, and background subtraction. Levels of linkage are expressed as percent of similarity based on the Dice coefficient. Arrows note some of the polyphasic bands between South Carolina (SC) isolates (grouped under the same cluster) and the reference strains.

suggested *Y. ruckeri* biotype 2 could also constitute a genomovar. Even though AFLP can achieve resolution at the clone level, all biotype 2 isolates shared a very similar AFLP profile that, under our current cutoff value of 97%, had to be considered identical. Although some minor differences were observed between South Carolina isolates, no association between specific AFLP patterns and outbreak was possible. This suggests a clonal origin from South Carolina biotype 2 isolates.

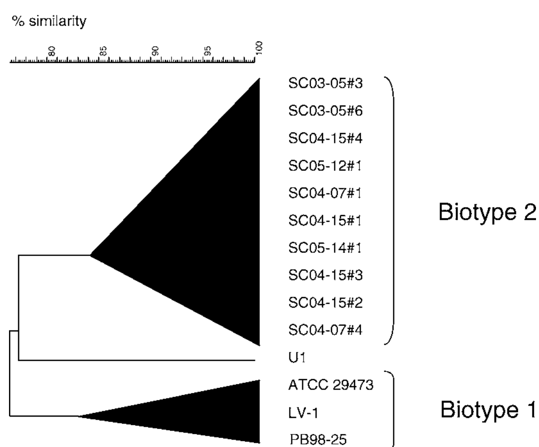

FIGURE 2.—Dendrogram based on polyphasic analysis of composite data sets (discriminative biochemical tests, API 20E profiles, and fatty acid methyl ester analysis) of *Yersinia ruckeri* isolates. Clusters corresponding to biotypes 1 and 2 are highlighted.

The combined analysis of all phenotypic data obtained from reference strains and South Carolina isolates supports the existence of at least two biotypes within *Y. ruckeri*, both biotypes being phenotypically homogeneous. Interestingly, isolate U1 shared phenotypic properties with both biotypes and could not be ascribed to either of them. The U1 isolate was motile but Voges–Proskauer-negative and Tween 20/80-negative. Additionally, it did not hydrolyze gelatin when incubated at 22°C for 48 h. This strain was isolated at the Fish Disease Diagnostic Laboratory at Auburn University a few years ago. Unfortunately, the case record was lost, and no information about source or specific geographic origin is available. However, U1 uniqueness warrants its inclusion in this study and highlights the intrinsic phenotypic diversity present in the species.

Although rapid and accurate molecular-based techniques are available for *Y. ruckeri* detection (Altinok et al. 2001), most fish disease diagnostic laboratories perform routine bacterial isolation followed by identification based on biochemical tests. Until now, *Y. ruckeri* has been considered a phenotypically homogeneous group that is identified based on typical enterobacteria characteristics (oxidase-negative, catalase-positive, fermentative, Gram-negative rods) and is differentiated from other enterobacterial fish pathogens

(e.g., *Edwardsiella ictaluri* or *E. tarda*) by key tests such as motility, H_2S production, and different patterns of sugar utilization. Fish pathologists and fish diagnostic laboratories should be aware of this atypical *Y. ruckeri* group. To avoid confusion and identification delays, biochemical properties of *Y. ruckeri* biotype 2 should be included in bacterial fish pathogen identification charts. Fish hatchery and farm managers should also be aware of this biotype. Standard vaccination protocols might not be effective against atypical nonmotile *Y. ruckeri* (Austin et al. 2003; Fouz and Amaro 2006). In fact, one of the outbreaks documented in our study affected ERM-vaccinated fish. Finally, more molecular epidemiology studies will be needed to track the origin of biotype 2 in the USA and to assess the prevalence of this biotype in wild and culture fish populations.

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