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Yersinia ruckeri Isolates Recovered from Diseased Atlantic Salmon (*Salmo salar*) in Scotland Are More Diverse than Those from Rainbow Trout (*Oncorhynchus mykiss*) and Represent Distinct Subpopulations

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ABSTRACT

***Yersinia ruckeri* is the etiological agent of enteric redmouth (ERM) disease of farmed salmonids. Enteric redmouth disease is traditionally associated with rainbow trout (*Oncorhynchus mykiss*, Walbaum), but its incidence in Atlantic salmon (*Salmo salar*) is increasing. *Yersinia ruckeri* isolates recovered from diseased Atlantic salmon have been poorly characterized, and very little is known about the relationship of the isolates associated with these two species. Phenotypic approaches were used to characterize 109 *Y. ruckeri* isolates recovered over a 14-year period from infected Atlantic salmon in Scotland; 26 isolates from infected rainbow trout were also characterized. Biotyping, serotyping, and comparison of outer membrane protein profiles identified 19 *Y. ruckeri* clones associated with Atlantic salmon but only five associated with rainbow trout; none of the Atlantic salmon clones occurred in rainbow trout and *vice versa*. These findings suggest that distinct subpopulations of *Y. ruckeri* are associated with each species. A new O serotype (designated O8) was identified in 56 biotype 1 Atlantic salmon isolates and was the most common serotype identified from 2006 to 2011 and in 2014, suggesting an increased prevalence during the time period sampled. Rainbow trout isolates were represented almost exclusively by the same biotype 2, serotype O1 clone that has been responsible for the majority of ERM outbreaks in this species within the United Kingdom since the 1980s. However, the identification of two biotype 2, serotype O8 isolates in rainbow trout suggests that vaccines containing serotypes O1 and O8 should be evaluated in both rainbow trout and Atlantic salmon for application in Scotland.**

IMPORTANCE

Vaccination plays an important role in protecting Atlantic salmon against the bacterial pathogen *Yersinia ruckeri*, but, in recent years, there has been an increasing incidence of vaccine breakdown in salmon. This is largely because current vaccines are aimed at rainbow trout and are based on serotypes specific for this species. A wider range of serotypes is responsible for infection in Atlantic salmon, but very little is known about the diversity of these strains and their relationships to those recovered from rainbow trout. In the present study, we demonstrate that *Y. ruckeri* isolates recovered from diseased Atlantic salmon in Scotland are more diverse than those from rainbow trout; furthermore, isolates from the two species represent distinct subpopulations. In addition, a new O serotype was identified that is responsible for a significant proportion of the disease in Atlantic salmon. Our findings are likely to have important implications for the development of improved vaccines against *Y. ruckeri*.

The Gram-negative bacterium *Yersinia ruckeri* is the etiological agent of enteric redmouth (ERM) disease of cultured salmonids and causes significant economic losses to the fish-farming industry. *Yersinia ruckeri* was first isolated in 1956 from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum) in the Hagerman Valley in Idaho (1, 2) but has since become widely disseminated and is present in fish populations in large areas of North and South America, Europe, Australia, and South Africa (3). Enteric redmouth disease is an acute infection primarily of rainbow trout and is typically characterized by a hemorrhagic septicemia, which manifests as subcutaneous hemorrhages in and around the oral cavity, the latter giving rise to the name "redmouth" disease (1, 4–6). Internally, petechial hemorrhages on the surfaces of the liver, pancreas, pyloric ceca, swim bladder, and lateral musculature may occur, and the spleen and lower intestine are often inflamed, with the lower intestine filled with an opaque yellowish fluid (4, 6). However, *Y. ruckeri* is also becoming increasingly responsible for infections in Atlantic salmon (*Salmo salar*), particularly in countries, such as Australia (7, 8), Chile (9, 10), Norway (11, 12), and Scotland, where salmon production is of significant economic importance. Indeed, a less severe form of the disease, known as yersiniosis or salmon blood spot disease, has been recognized in Atlantic salmon in Australia since 1980 (7, 8, 13). Yersiniosis is characterized by a marked unilateral or bilateral exoph-

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thalmos, often with patches (the blood spots) of hemorrhagic congestion on the iris of the eye (7). Histopathological features are those of a typical septicemia; bacteria are readily detected in the blood and in circulating macrophages and may also localize at sites of tissue hemorrhage (7).

Serotyping is particularly important for strain differentiation of *Y. ruckeri*, and the O-serotyping scheme of Davies (14) is the most commonly used (12, 15–17). In this scheme, five O serotypes, O1, O2, O5, O6, and O7, are recognized by rapid slide agglutination assay using defined O antigens (14); the individual O serotypes correspond to discrete lipopolysaccharide (LPS) types as identified by SDS-PAGE and Western blotting (18). It is well established that serotype O1 strains of *Y. ruckeri* are responsible for the majority of disease outbreaks in rainbow trout worldwide (3, 14, 19–21). However, yersiniosis of Atlantic salmon in Australia (7, 8) and Chile (9) is associated with a serotype O1 subtype (O1b). Historically, serotype O1 isolates associated with ERM disease in North America and Europe were motile and classified as the Hagerman strain. However, in 1990, Davies and Frerichs (22) described, for the first time, a nonmotile serotype O1 variant that also lacked the ability to hydrolyze Tween 20 and 80; conversely, motile serotype O1 isolates were able to hydrolyze both substrates. The motile lipase-positive and nonmotile lipase-negative isolates were designated biotypes 1 and 2, respectively (22). Since the first identification of biotype 2 isolates in the United Kingdom, nonmotile variants have been described elsewhere in Europe (16, 17, 23–25), the United States (26), and Australia (7). Indeed, biotype 2 *Y. ruckeri* strains are causing increasing concern because they are responsible for disease in fish previously vaccinated against biotype 1 strains (17, 20, 23, 26, 27). A typing scheme based on the molecular mass variation of the major outer membrane proteins (OMPs) was developed to further differentiate between strains of *Y. ruckeri* (28). This approach allowed six distinct clonal groups to be distinguished among serotype O1 isolates and was able to differentiate between the classical Hagerman and the newly emerged biotype 2 strains (21). Subsequently, OMP analysis has played an important role in various epidemiological analyses of *Y. ruckeri* (9, 15, 24, 29–31).

Previous comparative studies of *Y. ruckeri* strain diversity have included only small numbers of isolates recovered from Atlantic salmon (19, 24, 30, 32–34). Surprisingly, few investigations have focused exclusively, or predominantly, on isolates associated with yersiniosis of this species (9, 12). There is concern that the emergence of novel strains may become a problem in Atlantic salmon. However, little information is currently available either about the diversity of strains infecting Atlantic salmon or about the relationship of these strains to those responsible for infections of rainbow trout. The present study was prompted by an increasing incidence of yersiniosis/ERM associated with cultured Atlantic salmon in Scotland between 2001 and 2014. The objective of the study was to characterize 109 *Y. ruckeri* isolates recovered from Atlantic salmon over this 14-year period by biotyping, serotyping, LPS profiling, and OMP typing. For reference, the Atlantic salmon isolates were also compared with a smaller number (26 isolates) of representative rainbow trout isolates recovered over the same period.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. The properties of 10 representative reference strains (28) and 135 field isolates (109 from Atlantic salmon and 26 from rainbow trout) recovered in the United Kingdom between 2001 and 2014 are presented in Tables S1 and S2 in the supplemental material, respectively. All isolates were recovered from individual moribund or dead fish that were showing clinical signs of ERM/yersiniosis; in some cases, isolates were recovered from two or more fish at a particular site. The geographic origins of the field isolates are shown in Fig. S1 in the supplemental material. Bacteria were stored at −80°C in 50% glycerol (vol/vol) in tryptone soya broth (TSB; Oxoid) and were routinely subcultured on tryptone soya agar (TSA; Oxoid) at 22°C for 48 h. Liquid cultures were prepared by inoculating three or four colonies into 10-ml volumes of TSB and incubating overnight at 22°C with shaking at 120 rpm. For the production of OMPs, 400-μl overnight cultures were inoculated into 400-ml volumes of TSB in 2-liter Erlenmeyer flasks. Cultures were grown aerobically at 22°C for ~16 h with shaking at 120 rpm or until a minimum optical density at 600 nm (OD_{600}) of 1.2 (mid-log phase) was achieved. Anaerobic conditions were created by growing bacteria statically in 1,000-ml volumes of TSB in 1-liter Erlenmeyer flasks overlaid with sterile mineral oil. For serotyping, isolates were grown as bacterial lawns on TSA at 22°C for 48 h.

Biotyping. Isolates grown overnight as liquid cultures were viewed for motility by the hanging drop method using phase-contrast microscopy. The presence or absence of flagella was confirmed in selected isolates by transmission electron microscopy (TEM) as previously described (22). To detect lipase activity, isolates were grown on TSA supplemented with 0.1% (vol/vol) Tween 20 and 80 at 22°C for 48 h as previously described (22, 35). A positive result was recorded when clear zones were observed around the colonies.

O serotyping. O serotyping of isolates was conducted by slide agglutination as previously described (14). Cross-absorbed anti-O1 and anti-O5 antisera were prepared against the cross-agglutinating isolate RD426 as previously described (14).

Isolation of outer membrane proteins. Outer membranes (OMs) were isolated by Sarkosyl extraction as previously described (28, 36). Protein concentrations were determined by the modified Lowry procedure (37). One hundred-microliter aliquots of the OM suspensions were adjusted to 2 mg/ml in 20 mM Tris-HCl (pH 7.2), and the samples were stored at −80°C.

SDS-PAGE. OMPs were adjusted to 1 mg/ml in 2× sample buffer (0.125 M Tris-HCl [pH 6.8], 20% [vol/vol] glycerol, 4% [wt/vol] SDS, 10% [vol/vol] β-mercaptoethanol, and 0.0025% [wt/vol] bromophenol blue) and heated at 100°C for 5 min. Twenty micrograms of protein for each sample was separated by SDS-PAGE and stained with Coomassie brilliant blue R250 as previously described (28, 36). The apparent molecular masses of the OMPs were estimated using a low-molecular-mass SDS marker kit (GE Healthcare). OMP types were assigned as previously described (18, 28). To distinguish between the major OMPs (OmpA, OmpC, and OmpF), selected OM samples were heated at 50, 60, 70, 80, 90, and 100°C for 5 min prior to separation by SDS-PAGE (28).

Lipopolysaccharide analysis. Lipopolysaccharide was prepared by proteinase K digestion of the OM samples as previously described (18, 36, 38). Briefly, 25 μg of proteinase K (P6556; Sigma) was added to 100 μl of a 1 mg/ml OM sample (previously heated at 100°C for 5 min) and incubated at 60°C for 1 h. Ten microliters of each digested LPS sample was separated by SDS-PAGE as described above. The gels were fixed overnight in a 40% ethanol and 5% acetic acid solution, and the LPS was visualized by silver staining using the method of Tsai and Frasch (39).

Western blotting. The LPSs of paired representative isolates were analyzed by Western blotting using anti-O1, -O2, and -O5 antisera, as well as with cross-absorbed anti-O1 and anti-O5 antisera, essentially as previously described (18, 40, 41). The membranes were incubated in primary rabbit antiserum diluted 1:250 in antibody buffer (1% gelatin in 0.05% Tween 20 in Tris-buffered saline [TBS]) for 2 h at room temperature and in secondary horseradish peroxidase-conjugated anti-rabbit antibody diluted 1:3,000 in antibody buffer for 2 h at room temperature. The membranes were developed in a substrate solution containing 0.05% (wt/

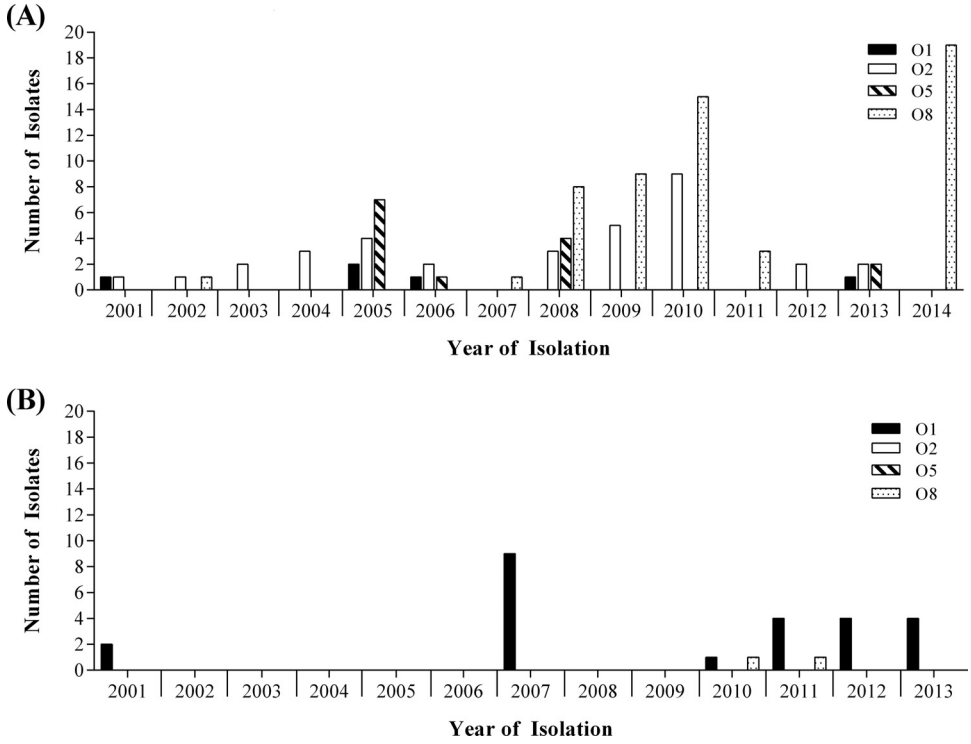

FIG 1 Distribution of O serotypes (O1, O2, O5, and O8) among *Y. ruckeri* isolates in Atlantic salmon (A) and rainbow trout (B) over the 14-year period of 2001 to 2014.

vol) 4-chloro-1-naphthol (dissolved in 20 ml of ice-cold methanol) and 0.05% (vol/vol) hydrogen peroxide in 100 ml of TBS. Development was stopped by washing the membranes in distilled water for 10 min. The membranes were dried on filter paper and subsequently photographed.

RESULTS

Biotyping. All 109 isolates recovered from Atlantic salmon belonged to biotype 1 (motile, lipase positive). In contrast, 24 of 26 isolates recovered from rainbow trout belonged to biotype 2 (nonmotile, lipase negative), and two isolates belonged to biotype 1 (see Table S2 in the supplemental material). Transmission electron microscopy confirmed the presence of flagella on motile, biotype 1 isolates and the absence of flagella on nonmotile, biotype 2 isolates (see Fig. S2 in the supplemental material). In motile isolates, one or two flagella were normally associated with each bacterial cell, although, in some cases, the flagella had become detached.

Serotyping. Of the 135 field isolates included in the study, 29 belonged to serotype O1, 34 belonged to serotype O2, and 14 belonged to serotype O5 (see Table S2 in the supplemental material). However, 58 isolates gave a strong positive agglutination reaction with type O1 antiserum but also cross-agglutinated weakly with serotype O5 antiserum. This cross-reactivity was removed when anti-O5 antiserum was cross-absorbed with the cross-agglutinating isolate RD426, but, critically, the cross-absorbed antiserum still caused agglutination of serotype O5 isolates. These cross-agglutinating isolates were designated serotype O8 (see Table S2). The 109 isolates recovered from Atlantic salmon exhibited substantial serotypic diversity, with isolates of serotypes O1, O2, O5, and O8 recovered (Fig. 1A). Serotype O8 was the most commonly recovered serotype (56 isolates), and serotype O2 (34 isolates) was the second most abundant; serotypes O1 (5 isolates) and O5 (14 isolates) were recovered less frequently. Serotype O2 represented the most frequently recovered serotype from 2001 to 2006 (with the exception of 2005). Conversely, only a single serotype O8 isolate was recovered during this period (in 2002), whereas this was the most frequently recovered serotype in each year between 2007 and 2014, with the exception of 2012 and 2013 (Fig. 1A); notably, serotype O8 isolates successively increased in number in years 2007, 2008, 2009, 2010, and 2014. Serotype O5 isolates were recovered mainly between 2005 and 2008 and represented the most common serotype in 2005. Of the 26 isolates recovered from rainbow trout, 24 belonged to serotype O1, whereas two isolates represented the novel O8 serotype (Fig. 1B). Notably, these serotype O8 isolates were nonmotile, biotype 2, and appeared for the first time in rainbow trout in 2010.

Lipopolysaccharide analysis. The LPS profiles of *Y. ruckeri* exhibited the characteristic ladder-like pattern (representing the O-antigen repeats) of the smooth-type LPS that occurs in enteric bacteria (Fig. 2). Variation occurred in both the O-antigen (ladder patterns) and core polysaccharide regions of the LPS, representing each of the four serotypes. The LPSs of serotype O1 (Fig. 2, lanes 1 and 2) and O8 (Fig. 2, lanes 3 and 4) isolates share a core polysaccharide region but can be distinguished by having slightly different O-antigen ladder patterns. In particular, bands in the center of the gel migrated with different molecular masses (Fig. 2, arrows). The core polysaccharide and O-antigen regions of serotype O2 (Fig. 2, lanes 5 and 6) and O5 (Fig. 2, lanes 7 and 8) isolates were distinct from each other and from those of serotype O1 and O8 isolates. The LPS types identified by SDS-PAGE corresponded

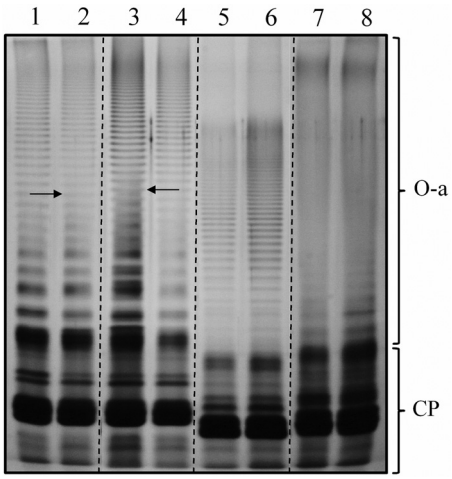

FIG 2 LPS profiles of serotype O1 (lanes 1 and 2), O8 (lanes 3 and 4), O2 (lanes 5 and 6), and O5 (lanes 7 and 8) *Y. ruckeri* isolates. Lanes 1 to 8 represent isolates RD332, RD358, RD428, RD474, RD464, RD486, RD362, and RD372, respectively. Arrows indicate the differing mobilities of O-antigen units in serotype O1 and O8 LPSs. O-a indicates the O-antigen component; CP indicates the core polysaccharide region.

with the O serotypes determined by slide agglutination and were designated LPS types O1, O2, O5, and O8.

Western blotting analysis of LPSs. Anti-O1 antiserum reacted strongly with the core polysaccharide and O-antigen side chains of the serotype O1 LPS (Fig. 3A, lanes 1 and 2) but not with either component of serotype O2 (Fig. 3A, lanes 3 and 4) or O5 (Fig. 3A, lanes 5 and 6) LPSs. However, this antiserum reacted strongly with the core polysaccharide region, and weakly with the O-antigen side chains, of the serotype O8 LPS (Fig. 3A, lanes 7 and 8). When anti-O1 antiserum was cross-absorbed with an O8 isolate (Fig. 3B), reactivity against the core polysaccharide regions of both the O1 (Fig. 3B, lanes 1 and 2) and O8 (Fig. 3B, lanes 7 and 8) LPSs was completely eliminated. The cross-absorbed anti-O1 antiserum gave a moderate reaction with the O-antigen side chains of the serotype O1 LPS (Fig. 3B, lanes 1 and 2), but no reaction occurred with the O-antigen region of the serotype O8 LPS (Fig. 3B, lanes 7 and 8). These observations indicated that serotype O1 and O8 LPS types possess a common core polysaccharide region but have different O-antigen side chains, albeit with some cross-reactive epitopes. Anti-O2 antiserum reacted strongly with both the O-antigen side chains and core polysaccharide regions of the serotype O2 LPS (Fig. 3C, lanes 3 and 4); minor cross-reactivity occurred against a component of the core polysaccharide region of the serotype O8 LPS in one isolate (Fig. 3C, lane 7, arrow). Anti-O5 antiserum reacted strongly with both the O-antigen side chains and core polysaccharide regions of the serotype O5 LPS (Fig. 3D, lanes 5 and 6). Weak reactions occurred against the core polysaccharide regions of both serotype O1 (Fig. 3D, lanes 1 and 2) and serotype O8 (Fig. 3D, lanes 7 and 8) LPSs; there was also a very weak reaction against O2 O-antigen side chains (Fig. 3D, lanes 3 and 4). When anti-O5 antiserum was cross-absorbed with an O8 isolate, cross-reactivity against the O1 and O8 core polysaccharide regions was mostly removed (Fig. 3E, lanes 1 and 2 and 7 and 8); there remained some cross-reactivity against a core polysaccharide component of one of the O8 isolates (Fig. 3E, lane 7, arrow). Overall, the results indicate that the O8 LPS possesses a novel O-antigen that has not previously been recognized in *Y. ruckeri* isolates recovered in the United Kingdom, although the core polysaccharide region is identical to that of the O1 LPS.

Outer membrane protein analysis. Having initially grown

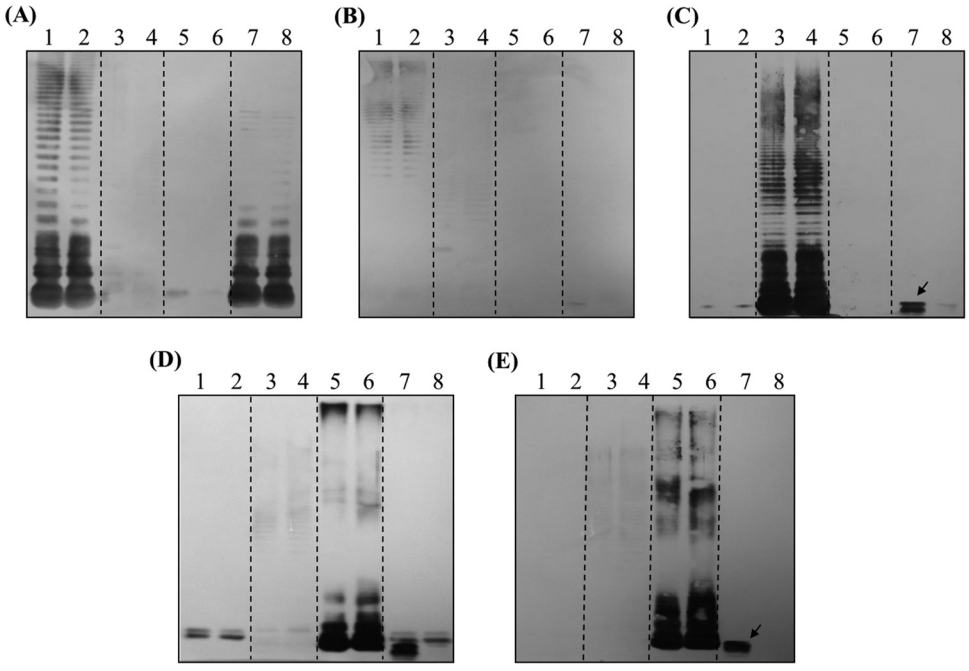

FIG 3 Western blotting of *Y. ruckeri* LPSs. (A to E) Reactions with anti-O1 antiserum, anti-O1 antiserum cross absorbed with an O8 isolate, anti-O2 antiserum, anti-O5 antiserum, and O5 antiserum cross absorbed with an O8 isolate, respectively. Lanes 1 to 8 represent isolates RD332, RD358 (serotype O1), RD464, RD486 (serotype O2), RD362, RD372 (serotype O5), RD428, and RD474 (serotype O8), respectively.

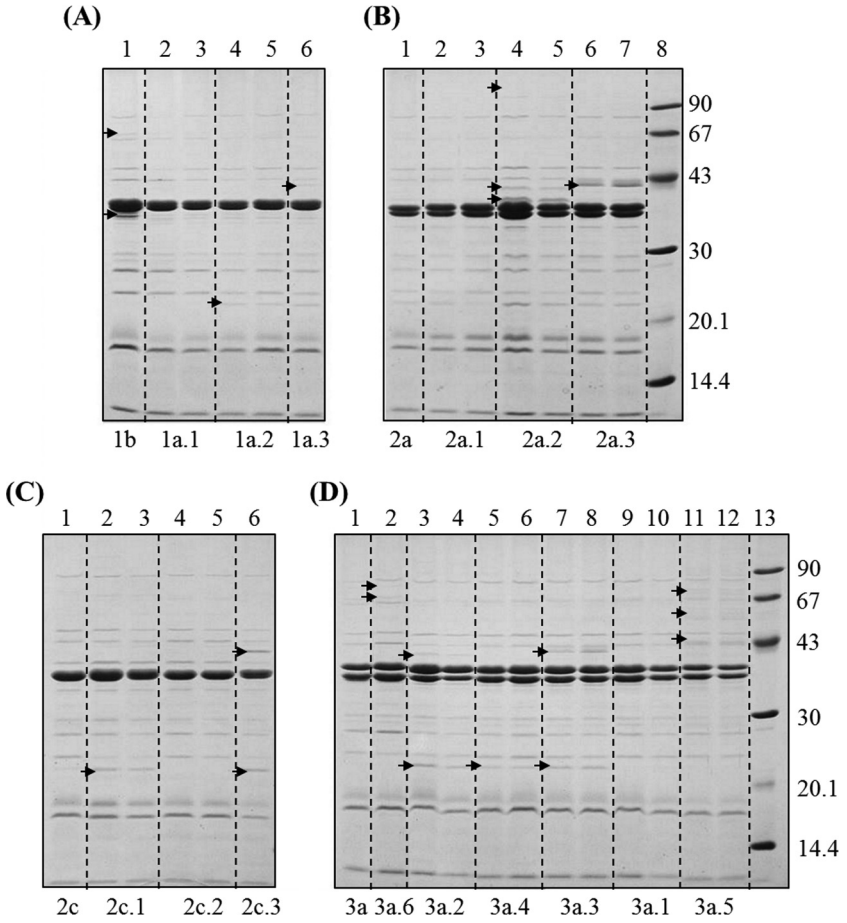

FIG 4 OMP profiles and OMP subtypes of selected paired isolates of *Y. ruckeri*. (A to D) Lane 1 represents a reference isolate from the original typing scheme (31). (A) OMP type 1a: lanes 1 to 6 represent isolates RD6 (reference isolate representing OMP type 1b with an additional 36-kDa protein [lower arrow]), RD400 and RD402 (OMP type 1a.1), RD396 and RD522 (OMP type 1a.2), and RD394 (OMP type 1a.3), respectively. (B) OMP type 2a: lanes 1 to 7 represent isolates RD158 (reference isolate), RD336 and RD342 (OMP type 2a.1), RD436 and RD480 (OMP type 2a.2), and RD418 and RD464 (OMP type 2a.3), respectively; lane 8 represents molecular mass standards. (C) OMP type 2c: lanes 1 to 6 represent isolates RD154 (reference isolate), RD370 and RD412 (OMP type 2c.1), RD362 and RD380 (OMP type 2c.2), and RD374 (OMP type 2c.3), respectively. (D) OMP type 3a: lanes 1 to 12 represent isolates RD124 (reference isolate), RD386 (OMP type 3a.6), RD422 and RD440 (OMP type 3a.2), RD532 and RD534 (OMP type 3a.4), RD458 and RD468 (OMP type 3a.3), RD426 and RD496 (OMP type 3a.1), and RD382 and RD492 (OMP type 3a.5), respectively; lane 13 represents molecular mass standards. Arrows indicate differences in protein expression with respect to the reference isolates.

some of our isolates under standard aerobic conditions for preparation of OMPs, we observed that the expression of the three major proteins (the heat-modifiable protein, OmpA, and the porins, OmpC and OmpF) differed from that previously described (28). However, bacteria were previously grown statically under conditions of low aeration (28). To confirm that these subtle differences in aeration accounted for the observed differences in protein expression, we compared the OMP profiles of eight reference isolates from the previous study (28) that were grown under aerobic and anaerobic growth conditions (see Fig. S3 in the supplemental material). Two major proteins (OmpA and OmpF) were clearly expressed under aerobic conditions (see Fig. S3A), whereas a third major protein (OmpC) was expressed under anaerobic growth conditions (see Fig. S3B, arrow). To confirm the identities of the three major proteins as OmpA, OmpC, or OmpF and allow OMP types to be assigned, OMP profiles of four reference isolates (RD6, RD124, RD154, and RD158) that were grown under aerobic and anaerobic growth conditions were compared after heating the samples at 50, 60, 70, 80, 90, and 100°C prior to SDS-PAGE (see Fig. S4 in the supplemental material). This allowed OmpA to be differentiated from OmpC and OmpF based on its heat-modifiable properties and OmpC to be distinguished from OmpF based on its behavior under different conditions of aeration. In this way, OmpC was confirmed to represent the higher-molecular-mass protein that is upregulated under low-oxygen growth conditions.

Based on the variation of the three major OMPs, each of the 135 isolates was assigned to one of four distinct OMP types, 1a, 2a, 2c, or 3a (Fig. 4), using the previously described OMP typing scheme (28). The Atlantic salmon isolates were relatively diverse since 32 isolates belonged to OMP type 2a, 17 belonged to OMP type 2c, and 60 belonged to OMP type 3a. In contrast, the rainbow trout isolates were much more homogeneous since the majority (24 of 26) belonged to OMP type 1a; two isolates belonged to OMP type 3a, and these were representative of the classical biotype 1 Hagerman type strain. However, the isolates can be further subtyped based on the variation of minor proteins (Fig. 4), and the association of isolates representing the various OMP types and

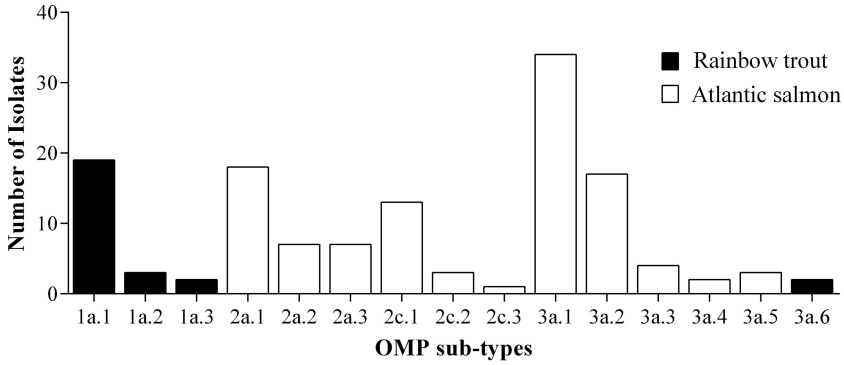

FIG 5 OMP subtypes of *Y. ruckeri* isolates recovered from Atlantic salmon and rainbow trout.

subtypes in Atlantic salmon and rainbow trout is summarized in Fig. 5. From these data, it is clear that the association of OMP types and subtypes in Atlantic salmon and rainbow trout isolates is not random. All 109 isolates, representing OMP types 2a, 2c, and 3a, and associated subtypes (with the exception of 3a.6), were recovered exclusively from Atlantic salmon; none of these were obtained from rainbow trout. Similarly, all 24 OMP type 1a isolates and two OMP type 3a.6 isolates were recovered only from rainbow trout; none of these were obtained from Atlantic salmon.

Association of biotypes, serotypes, and OMP types. Each of the four O serotypes was strongly associated with a specific OMP type (Fig. 6). Thus, OMP type 1a isolates were predominantly (92.3%) members of serotype O1, OMP type 2a isolates were almost exclusively (93.8%) members of serotype O2, OMP type 2c isolates were mostly (58.8%) members of serotype O5 (serotypes O1 and O2 were also represented), and the majority (88.5%) of OMP type 3a isolates belonged to serotype O8. However, multiple serotypes were also associated with specific OMP types and subtypes, although this was more apparent within Atlantic salmon isolates than within rainbow trout isolates (Fig. 6). Thus, serotypes O1, O2, and O5 were associated with OMP subtypes 2a.1 and 2c.1, serotypes O2, O5, and O8 were associated with OMP subtype 3a.1, serotypes O1, O5, and O8 were associated with OMP subtype 3a.2, and serotypes O1, O2, and O8 were associated with OMP subtype 3a.5 (Fig. 6). When all three phenotypic characters (biotype, serotype, and OMP type) were considered together, the clonal groups associated with each species became apparent (Fig. 6). Thus, Atlantic salmon isolates were represented by 19 clonal groups, whereas rainbow trout isolates were represented by five different clonal groups; significantly, no clonal group was associated with both species. It was also apparent that a small number of clonal groups were associated with the majority of disease in Atlantic salmon and rainbow trout. Thus, 65.0% of rainbow trout disease was caused by biotype 2, serotype O1, OMP type 1a.1 strains, and 57.8% of Atlantic salmon infections were due to biotype 1, serotype O8, OMP type 3a.1/3a.2 isolates and biotype 1, serotype O2, OMP type 2a.1 isolates (Fig. 6).

DISCUSSION

Yersinia ruckeri is primarily a pathogen of rainbow trout, and very few studies have focused on the comparison of isolates recovered primarily or exclusively from Atlantic salmon (8, 9). The present analysis of 109 *Y. ruckeri* isolates recovered from diseased Atlantic salmon in Scotland over a 14-year period represents the most detailed study of isolates from this species performed to date. In contrast to the majority of the isolates recovered from rainbow trout, the Atlantic salmon isolates were exclusively members of biotype 1. This observation suggests that the biotype 2 phenotype has not yet emerged in Scottish Atlantic salmon isolates as it has in European and North American rainbow trout isolates (16, 17, 22–27); however, it should be noted that the biotype 2 phenotype was previously described in a small number of older Atlantic salmon isolates originating from Norway (18) and the United

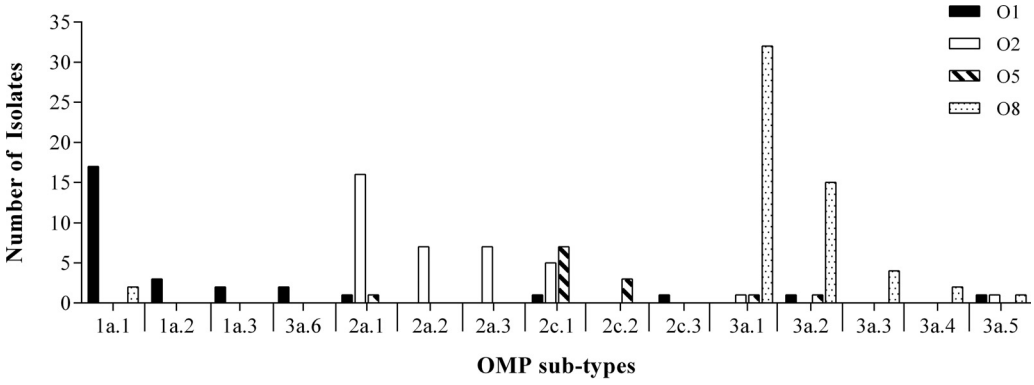

FIG 6 OMP subtypes and O serotypes (O1, O2, O5, and O8) of *Y. ruckeri* isolates recovered from Atlantic salmon and rainbow trout. Strains of OMP types 1a.1, 1a.2, 1a.3, and 3a.6 were associated exclusively with rainbow trout; strains of the other OMP types were associated only with Atlantic salmon.

Kingdom (24). The biotype 2 phenotype has arisen independently on at least four occasions within serotype O1 rainbow trout isolates in Europe and the United States by specific mutations in the genes (*fliR*, *flhA*, and *flhB*) involved in flagellar secretion; its distribution in Europe is not due simply to the expansion and distribution of a single clone (24, 42). Possible explanations for the loss of flagellar motility in *Y. ruckeri* isolates from rainbow trout have been discussed by Welch et al. (42), but it is intriguing that, in the present study, the biotype 2 phenotype was not identified in a single Atlantic salmon isolate. The complete absence of biotype 2, serotype O1 isolates in Atlantic salmon over the 14-year period is striking because these strains continue to be the predominant cause of disease in rainbow trout in Scotland since they were first identified in the 1980s (22). Clearly, the biotype 2 phenotype has neither emerged independently in Atlantic salmon isolates by mutation of one or more of the flagellar secretion genes, nor has it been acquired from rainbow trout by strain transmission. However, it is also notable that *Y. ruckeri* strains isolated from Atlantic salmon in Australia and Chile are also members of biotype 1 and not biotype 2 (7, 9). It has been postulated that vaccination may be providing the selective pressure that is driving the loss of flagellar motility in *Y. ruckeri* (20, 42). If this is the case, it is surprising that biotype 2 has emerged in rainbow trout but not in Atlantic salmon because Atlantic salmon are generally vaccinated with the same serotype O1-based vaccines as those used for rainbow trout (9).

Rainbow trout isolates were represented almost exclusively by serotype O1, whereas more extensive serotypic variation occurred within isolates recovered from Atlantic salmon. Isolates belonging to serotypes O8 (56 isolates) and O2 (34 isolates) were responsible for the majority (82.6%) of infections of Atlantic salmon, although isolates belonging to serotypes O5 (14 isolates) and O1 (5 isolates) were also recovered. The discovery of the newly emerged serotype O8 in Atlantic salmon isolates (and also in two rainbow trout isolates) was a highly significant outcome of the present study. However, it is possible that this O serotype may represent the same O1b serotype that has previously been described in Australia (7) and Chile (9). It is clear from the silver-stained LPS profiles (Fig. 2) and from Western blotting (Fig. 3) that serotype O1 and O8 LPSs have common, if not identical, core polysaccharide regions. Although the O-antigen regions of each LPS type have very similar silver-stained ladder patterns, they are, nevertheless, quite distinct by Western blotting. Thus, the evidence suggests that the O8 LPS type has emerged by modification of the O-antigen component of the O1 LPS. O-antigen modification is a well-recognized phenomenon in Gram-negative bacteria and may be generated by horizontal gene transfer (43, 44) or bacteriophage-mediated mechanisms (45, 46). Indeed, horizontal gene transfer involving O-antigen biosynthesis gene clusters has contributed to LPS diversity in the *Aeromonas hydrophila* strains responsible for septicemia in catfish (47). However, mutations within a key set of genes have been responsible for the vaccine-driven evolution of the capsular operon of the fish pathogen *Streptococcus iniae* (48), and this process cannot be discounted to explain the emergence of the O8 O antigen in *Y. ruckeri*.

The OMP profiles of our aerobically grown isolates were, with the exception of the reduced expression of OmpC, consistent with those obtained previously that were representative of bacteria grown under conditions of low aeration (see Fig. S3 and S4 in the supplemental material). As with the biotype and O-serotype data, marked differences were observed in the OMP profiles of Atlantic salmon and rainbow trout isolates. Atlantic salmon isolates were associated with OMP types 2a (three subtypes), 2c (three subtypes), and 3a (five subtypes), whereas rainbow trout isolates were associated almost exclusively with OMP type 1a (three subtypes); two rainbow trout isolates were represented by OMP type 3a (one subtype) (Fig. 4 and 5). These findings demonstrated further that the Atlantic salmon isolates were more diverse than the rainbow trout isolates. Indeed, the combined use of all three phenotypic characters (biotype, serotype, and OMP type) (21) identified 19 clones associated with Atlantic salmon and five clones with rainbow trout; remarkably, none of the Atlantic salmon-specific clones were identified in rainbow trout and *vice versa*. However, the use of other approaches, such as multilocus sequence typing (MLST) or genome sequencing, will almost certainly identify further differences between strains from each species. Taken together, our data strongly suggest that *Y. ruckeri* isolates from Atlantic salmon and rainbow trout represent distinct subpopulations. Wheeler et al. (24) also demonstrated, using pulsed-field gel electrophoresis, that United Kingdom Atlantic salmon isolates were more diverse than rainbow trout isolates (albeit with substantially smaller numbers of salmon strains); the Atlantic salmon isolates clustered into three groups that also correlated with serotypes O1, O2, and O5.

Our finding that biotype 2, serotype O1 isolates remain the principle cause of ERM in rainbow trout in the United Kingdom was in agreement with the results of previous studies (20, 22, 27). These isolates can be distinguished from other strains by having a unique OMP type (OMP type 1a) and represent the same discrete clone previously identified in the United Kingdom (21, 28). Although the number of rainbow trout isolates examined in the present study was smaller than that of Atlantic salmon isolates, they were isolated from widespread geographic regions over a 13-year period and are representative of the rainbow trout *Y. ruckeri* population in the United Kingdom. The observed homogeneity of rainbow trout isolates is also in agreement with various other recent studies (16, 17, 25, 49). Greater sampling from rainbow trout may have revealed increased diversity, but the samples analyzed represented all isolates submitted for serotyping over the 13-year time period (probably reflecting the success of vaccination in rainbow trout). However, the recovery of two serotype O8 isolates from rainbow trout is significant because they represent the emergence of a new biotype 2 clone, which expresses the novel O8 O antigen. Crucially, these isolates belonged to the same biotype (biotype 2) and OMP type (1a.1) as the majority of serotype O1 rainbow trout isolates, suggesting that serotype O1 and O8 rainbow trout isolates are very closely related and possibly have a common ancestral origin. It is reasonable to speculate that the serotype O8 rainbow trout isolates have perhaps acquired O8-specific O-antigen genes from Atlantic salmon isolates by horizontal gene transfer. This hypothesis is supported by the fact that serotype O8 rainbow trout isolates were recovered in 2010 and 2011, a number of years after this serotype was first identified in Atlantic salmon, and also fits with the finding that recombination appears to be more important than mutation for the generation and maintenance of diversity in *Y. ruckeri* (34).

Vaccination has played an important role in controlling ERM/yersiniosis in Atlantic salmon in Australia (50), Chile (9, 51), Norway (12), and Scotland. The vaccines used for Atlantic salmon are generally the same serotype O1-based vaccines as those used for

rainbow trout (9, 20), although, in Scotland, there has been a shift toward the use of multivalent autogenous vaccines in recent years (T. Wallis, Ridgeway Biologicals Ltd., personal communication). Certainly, the use of serotype O1-based vaccines in Scotland most likely accounts for the very low number (five) of serotype O1 isolates recovered from this species. In Chile, serotype O1 vaccines against yersiniosis were introduced in 1995 (51), and Bastardo et al. (9) suggested that vaccination of Atlantic salmon provided the selective pressure that led to the emergence of the O1b serotype identified in 2008. Serotype O1-based vaccines have been used in Scotland for a similar period of time, and it is reasonable to speculate that the widespread use of these vaccines may similarly account for the emergence of the O8 serotype in Atlantic salmon here. This is further supported by the time frame for the emergence of the O1b and O8 serotypes in Chile and Scotland, respectively; in both cases, these serotypes began to become problematic in 2008. Shifts in the predominant serotypes circulating within bacterial populations due to the selective pressures exerted by vaccination are well documented and may occur by serotype replacement (52, 53) or switching (54, 55). It is unclear whether the emergence of the O8 serotype in Atlantic salmon is due to replacement or switching, but, whatever the reason, it is intriguing that similar serotype replacement/switching has not occurred in rainbow trout isolates, either in the United Kingdom or elsewhere, as a consequence of vaccination in this species. In a comparison of two serotype O1-based vaccines in rainbow trout, Tinsley et al. (20) concluded that LPS is the dominant protective antigen of *Y. ruckeri*. Similarly, Welch and LaPatra (56) demonstrated that LPS has exceptionally high potency as a component of whole-cell vaccines, and they concluded that LPS is the only cellular component contributing to protection. Therefore, the emergence of the O8 serotype in both Atlantic salmon and rainbow trout is a potentially significant development because current serotype O1-based vaccines may not provide protection against this new serotype.

The association of distinct subpopulations of *Y. ruckeri* with Atlantic salmon and rainbow trout and the increased strain diversity observed among Atlantic salmon strains raise important questions that impact our understanding of the epidemiology and pathogenesis of this pathogen as well as disease prevention strategies. Historically, the majority of disease in rainbow trout in Europe and North America has been caused by serotype O1 strains of biotype 1 (the Hagerman strain) or, more recently, by biotype 2 variants (42). The association of a limited number of clones with a large proportion of disease in rainbow trout fits with the epidemic population structure model proposed for *Y. ruckeri* (34, 57) and is consistent with the belief that *Y. ruckeri* is a recently emerged pathogen that was originally geographically isolated but quickly became widely disseminated (3, 42). These strains presumably represent hypervirulent clones (58) that have adapted to a new niche (intensively cultured rainbow trout) (42) and consequently "exploded" in number and become widely distributed by extensive fish movements (34, 57). In contrast, the evidence presented here and by others (24) clearly shows that *Y. ruckeri* strains that are associated with diseased Atlantic salmon are more diverse than those recovered from rainbow trout. A high degree of strain diversity is a characteristic of natural populations of commensal bacteria and is often associated with opportunistic pathogens. Thus, a possible explanation for the greater diversity of the Atlantic salmon strains is that they may simply represent opportunistic pathogens originating from the commensal flora. Yersiniosis is often associated with stressful conditions, such as overcrowding, elevated water temperature, and changes in salinity. Atlantic salmon may simply be more sensitive to such environmental stressors and more susceptible to opportunistic infection than rainbow trout.

It is also clear that bacterial strains associated with Atlantic salmon and rainbow trout represent distinct subpopulations, and two potential explanations may account for this. First, salmon and trout farms in Scotland are generally located in different geographic regions, and this provides an element of hydrographic separation of the two industries; salmon farms are located mainly in the north and west and trout farms in the south and east (see Fig. S5 in the supplemental material) (59, 60). In addition, a compartmentalized management program has been established in Scotland to help control bacterial kidney disease (BKD), especially in salmon (61). For these reasons, there is very limited transfer of fish between the salmon and trout networks as evidenced by the infrequent transmission of BKD between the two species (59, 60). For the same reasons, there will clearly be limited scope for the transmission of *Y. ruckeri* strains between Atlantic salmon and rainbow trout, and the two species may conceivably harbor distinct, effectively isolated, and separately evolving populations. Nevertheless, the two sectors are not completely independent, and disease transmission between the two species may occasionally occur (59). Indeed, genetic analyses of *Renibacterium salmoninarum* isolates from Atlantic salmon and rainbow trout provided evidence for the transmission of this pathogen between the two species because, in many cases, the strains were indistinguishable (62). Clearly, this is not the case for *Y. ruckeri*. A second explanation to account for the presence of distinct subpopulations of *Y. ruckeri* in Atlantic salmon and rainbow trout is that different strains are specifically adapted to one host species or the other. It has long been recognized that serotype O1 isolates of *Y. ruckeri* have far greater prevalence in, and are more pathogenic toward, rainbow trout than are other serotypes; as pointed out above, it has been proposed that the epidemic expansion and dissemination of serotype O1 *Y. ruckeri* occurred when the pathogen found an alternative niche in intensively cultured rainbow trout (42). Conversely, non-O1 serotypes are usually associated with nonsalmonid species or with the environment, leading to the suggestion that different *Y. ruckeri* isolates exhibit various degrees of host specificity and virulence or adaptation for survival in the environment (16, 31, 63). Different pulsotypes and multilocus sequence types of *Y. ruckeri* are also associated with specific host species, strengthening the proposal for adaptive niche specialization (34, 57). Indeed, experimental challenge studies have also highlighted the differences in virulence between different serotypes and clonal groups of *Y. ruckeri* (33, 58); in particular, serotype O1 strains recovered from species other than rainbow trout were all avirulent in this species (33, 58). Ultimately, it is likely that various selection pressures determined by factors such as ecological niche (e.g., host specificity), geography, the environment, and farm management practices all play a role in influencing the diversity and evolution of *Y. ruckeri* in Atlantic salmon and rainbow trout.

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