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RESEARCH ARTICLE

Biotyping reveals loss of motility in two distinct *Yersinia ruckeri* lineages exclusive to Norwegian aquaculture

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Abstract

Non-motile strains of *Yersinia ruckeri*, known as *Y. ruckeri* biotype 2, now dominate amongst clinical isolates retrieved from rainbow trout internationally. Due to an acute increase in the number of yersiniosis cases in Norway in recent years, followed by introduction of widespread intraperitoneal vaccination against the disease, an investigation on the prevalence of *Y. ruckeri* biotype 2 in Norwegian aquaculture was conducted. We biotyped 263 *Y. ruckeri* isolates recovered from diseased salmonids in Norway between 1985 and 2020. A total of seven biotype 2 isolates were identified, four of which were collected between 1985 and 1987, and three of which belong to the current epizootic clone, isolated from two different sea-farms in 2017. Whole-genome sequencing revealed single non-synonymous nucleotide polymorphisms in the flagellar genes *flhC* in isolates from the 1980s, and in *fliP* in isolates from 2017. In both variants, motility was restored both by complementation with wild-type alleles *in trans* and via spontaneous mutation-driven reversion following prolonged incubation on motility agar. While biotype 2 strains do not yet seem to have become broadly established in Norwegian aquaculture, the seven isolates described here serve to document a further two independent cases of *Y. ruckeri* biotype 2 emergence in salmonid aquaculture.

KEYWORDS
aquaculture, Atlantic salmon, biotype, flagella, *Yersinia ruckeri*, yersiniosis

1 | INTRODUCTION

The Gram-negative bacterium *Yersinia ruckeri* causes yersiniosis, also known as enteric redmouth disease, predominantly in farmed salmonids (Busch, 1978; Ewing et al., 1978; Ross et al., 1966). Found throughout the world wherever salmonid fish are farmed, yersiniosis is considered primarily a disease of rainbow trout, but also affects farmed Atlantic salmon in Norway, Australia, Chile and the UK (Bastardo et al., 2011; Costa et al., 2011; Gulla et al., 2018; Wheeler et al., 2009). Several studies have demonstrated an epidemic population structure for *Y. ruckeri*, with both host species specific- and geographically limited lineages forming discrete clonal complexes (CC) (Barnes et al., 2016; Bastardo et al., 2011; Calvez et al., 2014). Most recently, multi-locus variable number of tandem repeat analysis (MLVA) revealed a single clonal complex (CC2) as responsible for the majority of yersiniosis outbreaks in rainbow trout globally, while distinct, geographically restricted clones were linked to outbreaks in farmed Atlantic salmon in Norway (CC1), the UK (CC4) and Australia (CC5) (Gulla et al., 2018).

While wild-type *Y. ruckeri* are flagellated and motile, non-flagellated, non-motile strains do occur (Davies & Frerichs, 1989). As lipase secretion is dependent upon the flagellar export apparatus (Evenhuis et al., 2009; Young et al., 1999), motility and lipase phenotypes are genetically linked with non-motile strains also lacking lipase activity (Welch et al., 2011). Non-motile, lipase negative strains are often termed biotype 2 (BT2), while motile, lipase-positive strains are termed biotype 1 (BT1) (Davies & Frerichs, 1989).

In recent years, non-motile BT2 *Y. ruckeri* strains have emerged independently on different continents following detrimental mutations in flagellar genes (Welch et al., 2011; Wheeler et al., 2009), and have become the dominating form associated with yersiniosis in rainbow trout in the USA and Europe (Fouz et al., 2006; Gulla et al., 2018; Welch et al., 2011; Wheeler et al., 2009). Observations of vaccine failure coinciding with BT2 emergence in the UK, Spain, USA and Australia (Austin et al., 2003; Fouz et al., 2006; Arias et al., 2007; Costa et al., 2011) have resulted in speculations around vaccine-escape as the primary selective force responsible (Wheeler et al., 2009; Welch et al., 2011; Tinsley et al., 2011; Barnes et al., 2016). Conversely, reports of BT2 from salmon farming industries worldwide are scarce, with only a single non-motile isolate reported from Norway in 1985 (Sparboe et al., 1986), a few UK isolates from the 1980s and 1990s (Wheeler et al., 2009), and the more recent emergence within a local endemic clonal complex in Australia (Barnes et al., 2016; Gulla et al., 2018).

In Norway, a single *Y. ruckeri* clonal complex (CC1) has almost entirely dominated the yersiniosis situation in salmon farming in both freshwater and seawater since the turn of the millennium (Gulla et al., 2018). In response to an increasing number of outbreaks in recent years, and as a supplement to immersion vaccination, many Norwegian farms now utilize intraperitoneally administered vaccines against yersiniosis, which have thus far proven to be effective in preventing outbreaks in large fish post sea transfer (Gulla & Olsen, 2020). Given the putative association between vaccination and BT2 development, the present study was performed to investigate possible emergence and spread of BT2 *Y. ruckeri* in Norwegian aquaculture.

A non-motile isolate from diseased rainbow trout in Sweden, NVI-11000, was also included in the study as Swedish *Y. ruckeri* BT2 strains have not, to the best of our knowledge, been characterized previously.

2 | MATERIALS AND METHODS

2.1 | Strains and culture

Detailed information on the 263 Norwegian *Y. ruckeri* isolates assessed by biotyping is provided in Table S1, including 46 isolates previously biotyped in our laboratory (Gulla et al., 2018) as well as BT2 isolate NVI-344, which was first described by Sparboe et al. (1986) and also mentioned by Wheeler et al. (2009) and Ormsby (2015) under the designation RD154. Isolates used in complementation and biotype reversion experiments are listed in Table 1. Bacterial cultures were prepared from cryopreserved stocks held at −80°C in Tryptic Soy Broth (TSB) with 20% glycerol. *Y. ruckeri* was cultured on 5% bovine blood agar (BA) or in TSB at 22°C. *E. coli* was cultured at 37°C on Luria agar (LA) or in Luria broth (LB), supplemented with 0.3 mM 2, 6-diaminopimelic acid (DAP) (Alfa Aesar, Ward Hill, Massachusetts, USA) for culture of *E. coli* MFD-pir (Ferrières et al., 2010). The pMJH-46 plasmid was maintained in *E. coli* SM10(λpir), grown with 100 mg/L ampicillin (Merck, Darmstadt, Germany) and 30 mg/L chloramphenicol (Merck) at 30°C. Agar and liquid media for maintaining the pAR3 plasmid vector and derivatives in *Y. ruckeri* and *E. coli* were supplemented with 50 mg/L kanamycin (Merck).

2.2 | Biotyping

Methods for phenotypic biotype assessment were adapted from those described by Evenhuis et al. (2009). Motility was assayed on semi-solid Tryptic Soy Agar (TSA) with 0.3% agar incubated at 22°C. The assay was prepared by surface-inoculating overnight BA cultures of *Y. ruckeri* at the centre of the TSA plate. A visible expanding zone of motile cells within the semi-solid TSA after 24 or 48 h was considered positive for motility. Lipase activity on Tween 80 medium was assayed by incubation at 22°C. Visible calcium precipitation after 48 h incubation was considered positive. Due to inconsistent calcium precipitation reactions for many CC1 isolates that also displayed flagellar motility (Figure S1), biotype assessment of isolates belonging to this lineage did not include lipase activity.

2.3 | General molecular techniques

DNA extraction for MLVA and qualitative PCRs was performed by boiling a bacterial colony for 7 min in nuclease-free water as described previously (Gulla et al., 2018). Genomic DNA templates for whole-genome sequencing, Sanger sequencing and PCR amplification for cloning was extracted with the Gentra Puregene Yeast/Bact. Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations for Gram-negative bacteria. Plasmid templates for PCR amplification for cloning were purified with the Qiagen Plasmid Mini Kit (Qiagen).

Oligonucleotides (Table 2) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HotStarTaq Master Mix (Qiagen) was used for PCR amplification prior to Sanger sequencing and for qualitative PCR assays, with standard cycling conditions according to the manufacturer's recommendations including 30 s annealing at 60°C and 60 s extension time. All PCR products were verified by agarose gel electrophoresis. Sanger sequencing was performed on an Avant 3500xl Genetic Analyzer (Applied Biosystems Waltham, MA, USA) using BigDye version 3.1 reagents according to manufacturer's description.

The MLVA genotyping was performed according to Gulla et al. (2019). Briefly, DNA extracted from each isolate was used as

TABLE 1 Bacterial strains and plasmids used for biotype reversion and complementation experiments

Bacterial strain or plasmid	Description	Source or references
Yersinia ruckeri strains		
NVI–344 (RD154)	CC10, BT2	This study; Sparboe et al. (1986); Wheeler et al. (2009)
NVI–492	CC10, BT1, source of wild-type *flhDC*	This study
NVI–10990	CC1, BT2	This study
NVI–10705	CC1, BT1, source of wild-type *fliP*	This study
Escherichia coli strains		
MFD-pir	MG1655 RP4–2-Tc::[ΔMu1::*aac(3)IV*-Δ*aphA*-Δ*nic*35-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA*	Ferrières et al. (2010)
Plasmids		
pMJH–46	Source of the *bla* promoter	Addgene plasmid #67,272; Hossain et al. (2015)
pAR3	pBBR1 *oriT KanR amilCP*	This study (Materials S1)
pAR3-fliP	pAR3 containing the *bla* promoter and wild-type *fliP*	This study
pAR3-flhDC	pAR3 containing wild-type *flhDC*	This study

Note: NVI-344 is identical to the isolate described by Sparboe et al. (1986) and synonymous to RD154 mentioned in Wheeler et al. (2009) and Ormsby (2015).

MFD-pir was acquired from Biological Resource Center of the Institut Pasteur (CRBIP).

pMJH-46 was acquired from Addgene, deposited by Mark Liles.

A complete description of pAR3 is provided in Materials S1.

template in two five-plex PCR assays featuring fluorescently labelled primers, with subsequent capillary electrophoresis for size calling of PCR products and in silico calculation of 10-loci MLVA profiles. A minimum spanning tree based on MLVA results was generated in BioNumerics v7.6.3 (Applied Maths NV, Sint-Martens-Latem, Belgium).

2.4 | Whole-genome analyses

Genome assemblies for a total of 26 *Y. ruckeri* isolates (Table 3) of diverse origin, covering various genetic lineages and both biotypes, were utilized in the present study. Of these, seven were downloaded from the National Center for Biotechnology Information (NCBI), while the remaining 19 were generated as described below.

Sequencing libraries were prepared by use of either NEBNext Ultra DNA Library Prep- (New England Biolabs, Ipswich, MA, USA), TruSeq DNA PCR-Free- (Illumina, San Diego, CA, USA) or NexteraFlex- (Illumina, San Diego, CA, USA) kits. Subsequent Illumina sequencing was performed on either a HiSeq or MiSeq platform, with paired end read lengths of 125, 150 or 300 base pairs. See Table S2 for per-strain sequencing details. Adapter sequences in raw reads were removed and low-quality nucleotides trimmed with Trimmomatic v0.38 (Bolger et al., 2014), prior to de novo assembly using SPAdes v3.13.0 (Bankevich et al., 2012) with the -careful option.

All 26 *Y. ruckeri* genome assemblies were annotated with Prokka v1.13 (Seemann, 2014) utilizing default settings, prior to generation of a core gene alignment using Roary v3.12.0 (Page et al., 2015) with the MAFFT aligner. The alignment was concatenated with snp-sites v2.4.1 (Page et al., 2016) and a maximum likelihood tree was generated using MEGA v10.2 (Kumar et al., 2018) with 1000 bootstrap replicates and visualized in R using ggtree (Yu et al., 2017).

The flagellar sequence region (position 2355800–2427400) was extracted from the genome of *Y. ruckeri* NHV_3758 (accession no. CP023184.1) and used as a reference for alignment of flagellar genes (*flh*, *fli* and *flg*) in MEGA.

2.5 | Complementation experiments

Complementation experiments for restoration of motility in BT2 strains were performed by supplying wild-type variants of flagellar genes *in trans* on the plasmid vector pAR3 (Materials S1). This plasmid contains the pBBR1 plasmid origin, kanamycin resistance, amilCP chromoprotein and an origin-of-transfer which allows for mobilization by the chromosomally encoded conjugation machinery in the *E. coli* MFD-pir donor strain.

Linearized vector (by restriction with XbaI, New England Biolabs, Catalog # R0145) and target inserts were amplified by PCR with sequence-overlapping primers (Table 2), using Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) as specified by the manufacturer, with annealing temperatures calculated with the NEB Tm Calculator tool. PCRs included 22 cycles of amplification, with extension at 72°C for 30 s or 60 s, respectively for amplification of inserts or the vector backbone. PCR products were verified by agarose gel electrophoresis.

TABLE 2 Primer sequences used during complementation experiments

Oligo name	Sequence (5′–3′)	Usage
fliH_f	GAGAGTGGCGAAATCGTRATTGG	Sanger sequencing, *fliH*
fliH_r	CAATTTGTCCAATGTAGACAACCAACG	
flhC_f	GCCACTTACTGCATGAGTTATCGTTG	Sanger sequencing, *flhC*
flhC_r	GCCAGACAGATAAGACATCCATATCG	
fliP_f	ATGATGTCCCTGCACTGTGAATCCAAAG	Sanger sequencing, *fliP*
fliP_r	GGGAGATTAACTGTAGAAACTTTGCG	
pAR3_f	TAATGACTAGTCAAGTGGCTCCTCGCTC	pAR3 vector backbone amplification
pAR3_r	TCTAGAGCTTGCCCTCATCTGTTACG	
fliP_OL_f	**GAAAAAGGAAGAGTATCTAGACTAGT**ATGATGTCCCTGCACTGTGAATCC	Complementation, *fliP* expression
fliP_OL_r	CGAGGAGCCACTTGACTAGTCATTAGGGAGATTAACTGTAGAAACTTTGCG	
bla_P_OL_f	CAGATGAGGGCAAGCTCTAGATTTCAGGTGGCACTTTTCGGGGAAATGTG	Complementation, *fliP* expression
bla_P_OL_r	**CATACTAGTCTAGATACTCTTCCTTTTTC**AATATTATTGAAGC	
flhDC_OL_f	CGTAACAGATGAGGGCAAGCTCTAGACCACATTCAGTTATGTCTTCCTTGC	Complementation, *flhDC*
flhDC_OL_r	GCGAGGAGCCACTTGACTAGTCATTACCAGACAGATAAGACATCCATATCG	
oriT_f	GCTTGCCCTCATCTGTTACG	PCR verification of vector
oriT_r	GTTCGTGTAGACTTTCCTTGGTG	

Note: Underlined nucleotides indicate overlapping sequence to the pAR3 vector backbone.
Bold nucleotides indicate overlap sequence between the *bla* promoter and *fliP*.

Strain NVI-10990 was complemented with pAR3-fliP, containing *fliP* (corresponding to nucleotides 2,381,401–2,382,198 in acc.no. CP023184) amplified with primers fliP_OL_f and fliP_OL_f from the motile CC1 isolate NVI-10705, coupled with the *E. coli* wild-type *bla* promoter for weak constitutive expression, amplified from pMJH-46 (acc.no. JQ070344, nucleotides 9530–9662) with primers bla_P_OL_f and bla_P_OL_r.

Strain NVI-344 was complemented with pAR3-flhDC, containing the *flhDC* operon (corresponding to nucleotides 2,428,041–2,426,303 in acc.no. CP023184) from a motile CC10 isolate, NVI-492, amplified using primers flhDC_OL_f and flhDC_OL_r.

The PCR-amplified backbone and respective inserts were assembled with NEBuilder HiFi (New England Biolabs) and cloned into chemically competent *E. coli* MFD-pir by heat shock according to the manufacturer's instructions. Kanamycin-resistant colonies were isolated followed by PCR-verification of the plasmid backbone with primers oriT_f and oriT_r, and of the respective inserts with primers fliP_f and fliP_r, and primers flhC_f and flhC_r. Assembly of the *bla* promoter and *fliP* was confirmed by PCR amplification using the bla_P_OL_f and fliP_OL_r primers, with an expected amplicon size of 986 bp.

Complementing plasmids were transferred to *Y. ruckeri* by conjugation, performed by combining two 25 µl PBS suspensions containing approximately equal densities of *E. coli* MFD-pir donor and *Y. ruckeri* recipients respectively. The 50 µl suspension was plated and incubated overnight at 28°C on non-selective LB-agar supplemented with DAP. The resulting bacterial lawn was collected and washed twice in 1 ml PBS by centrifugation (5 min at 6000G), followed by suspension of the bacterial pellet in 0.1ml PBS and plating on 50 mg/L kanamycin LA without DAP. Kanamycin-resistant transconjugants were isolated with subsequent species confirmation by MALDI-TOF (Biotyper Microflex LT; Bruker Daltonics, Bremen, Germany) and PCR-verification of the expression vector backbone with primers oriT_f and oriT_r. Complemented strains were cured of the vector by repeated subculture on non-selective agar media until kanamycin-sensitive colonies were recovered and confirmed negative for the vector by PCR with the oriT primer-set.

2.6 | Biotype reversion experiments

To facilitate reversion to BT1, BT2 strains NVI-344 and NVI-10990 were initially prepared in 10 parallels each consisting of 5 ml TSB-culture grown to stationary phase by overnight shaking at room temperature. From each replicate culture, a 10 µl droplet was deposited at the centre of a semi-solid TSA plate, which was then sealed with parafilm and incubated for up to 6 weeks at 22°C in a sealed box with a water reservoir to maintain humidity. Motile cells from the leading edge of expanding growth zones were isolated and flagellar genes *fliP* and *flhC* amplified by PCR and Sanger sequenced.

2.7 | Microscopy

Staining of flagella was performed using a tannic acid and alum mordant with a crystal violet stain as described by Heimbrook et al. (1989). Cells were grown overnight in TSB at 22°C without agitation, before being stained and observed by phase-contrast microscopy at 1000× magnification.

TABLE 3 26 *Yersinia ruckeri* genome assemblies downloaded from NCBI or produced in the current study for phylogenetic evaluation and alignment of flagellar genes

Strain	Biological source	Country	Year	Sero-type	Bio-type	MLVA-CC[a]	Accession no.	Genome reference
NVI–10990	*S. salar*	Norway	2017	O1	2	1	JAJIBN000000000	This study
NVI–10974	*S. salar*	Norway	2017	O1	2	1	JAJIBM000000000	This study
NVI–10705	*S. salar*	Norway	2016	O1	1	1	JAJJIH000000000	This study
NVI–9967	*S. salar*	Norway	2015	O1	1	1	JAJIBK000000000	This study
NVI–3629	*S. salar*	Norway	1996	O1	1	1	JAJIBH000000000	This study
NHV_3758	*S. salar*	Norway	1987	O1	1	1	CP023184	Wrobel et al. (2018)
NVI–494	*S. salar*	Norway	1987	O1	2	10	JAJIBF000000000	This study
NVI–344	*S. salar*	Norway	1985	O1	2	10	JAJIBV000000000	This study
NVI–492	*S. salar*	Norway	1987	O1	1	10	JAJIBE000000000	This study
NVI–9681	*S. salar*	Norway	2014	O2	1	3	JAJIBJ000000000	This study
NVI–6225	*S. salar*	Norway	2008	O2	1	3	JAJIBI000000000	This study
NVI–1347	*S. salar*	Norway	1988	O2	1	3	JAJIBG000000000	This study
NCTC12268	*O. mykiss*	Canada	1985	O5	1	s	JAJIBS000000000	This study
QMA0431	*S. salar*	Australia	2007	O1	2	5	GCA_001882895	Barnes et al. (2016)
QMA0427	*S. salar*	Australia	2004	O1	2	5	GCA_001883575	Barnes et al. (2016)
QMA0435	*S. salar*	Australia	2009	O1	1	5	GCA_001882945	Barnes et al. (2016)
NVI–11076	Salmon farm (b.f.)	Norway	2017	O1	1	7	JAJIBQ000000000	This study
NVI–11050	*S. salar* (e.f.)	Norway	2017	O1	1	9	JAJIBP000000000	This study
NVI–11000	*O. mykiss*	Sweden	2017	O1	2	2	JAJIBO000000000	This study
NCTC12266	*O. mykiss*	USA	pre 1990	O1	1	2	JAJIBR000000000	This study
CSF007–82	*O. mykiss*	USA	1982	O1	1	2	LN681231	Nelson et al. (2015)
ATCC29473	*O. mykiss*	USA	1961	O1	1	2	KN150747 & KN150748	Daligault et al. (2014)
NCTC12269	*O. mykiss*	Canada	pre 1990	O6	1	s	JAJIBT000000000	This study
BigCreek74	*O. tshawytscha*	USA	1974	O2	1	s	CP011078	Unpublished
NVI–10587	*S. salar* (e.f.)	Norway	2015	O1	1	8	JAJIBL000000000	This study
NCTC12270	*A. anguilla*	Denmark	pre 1990	O7	1	s	JAJIBU000000000	This study

Note: Abbreviations used: *O.* (*Oncorhynchus*), *S.* (*Salar*), *A* (*anguilla*), e.f. (egg-fluid), b.f. (biofilm).

Public nucleotide sequences identified as plasmids were not included for the phylogenetic analysis.

The two contigs of ATCC29473 were combined into a single file prior to analysis.

[a]MLVA clonal complex according to Gulla et al. (2018) or present study, with 's' indicating singleton or minor/undefined clonal complex.

3 | RESULTS

3.1 | Genotypic background

Ongoing efforts to MLVA genotype the collection of more than 800 Norwegian *Y. ruckeri* isolates cryopreserved at the Norwegian Veterinary Institute continue to verify the long-standing dominance of the CC1 lineage amongst domestic yersiniosis outbreaks (Gulla et al., 2018). Conversely, Norwegian isolates recovered in other contexts, e.g. from disease in individual wild fish, ovarian fluid of clinically healthy broodstock, or environmental biofilms, are, as previously described, much more diverse. Such isolates represent a wide range of putatively non- or low-virulent lineages, minor clonal complexes and singletons. Inclusion of a large number of older isolates in the present study further allowed identification of a previously unrecognized Norwegian clonal complex proposed here as CC10. CC10 currently harbours six isolates recovered between 1985 and 1988 from two geographically distant Norwegian salmon farms, and includes the single BT2 isolate previously reported from Norway (Sparboe et al., 1986), NVI-344 (also referred to as RD154). An up-to-date minimum spanning tree visualizing results from MLVA genotyping of 601 Norwegian *Y. ruckeri* isolates, and associated biotyping results (see below), is shown in Figure 1. MLVA further revealed that NVI-11000, isolated from rainbow trout in Sweden, belongs to CC2 (not shown in Figure 1), the dominant clonal complex amongst international rainbow trout isolates. CC2 has not yet been identified from Norway.

3.2 | Phenotypical analyses

Of 263 Norwegian *Y. ruckeri* isolates biotyped in the present study (see Table S1 and Figure S1), seven isolates, originating from four different locations and recovered over a period of 33 years, were identified as BT2 by being non-motile and lacking lipase activity. Four of these isolates originate from two neighbouring farms in northern Norway in the 1980s and belong to CC10. The remaining two CC10 isolates studied, from a different location in mid-Norway, are both motile BT1. The remaining three BT2 isolates, which were recovered in 2017 from two Atlantic salmon sea farms located in neighbouring fjords in mid-Norway, belong to CC1 and display identical MLVA profiles.

While lipase activity and motility were linked in all non-CC1 isolates, some motile CC1 isolates displayed varying lipase activity ranging from weak to absent (Figure S1). Such isolates were recorded here as BT1. Non-motile CC1 isolates (BT2) displayed no lipase activity.

3.3 | Whole-genome analyses

A core genome of 2838 genes (protein identity ≥97%) was identified across the 26 *Y. ruckeri* genomes assayed, resulting in a 14,670 bp SNP-alignment as the basis for phylogenetic reconstruction. Clustering in the resulting tree demonstrates deep branching

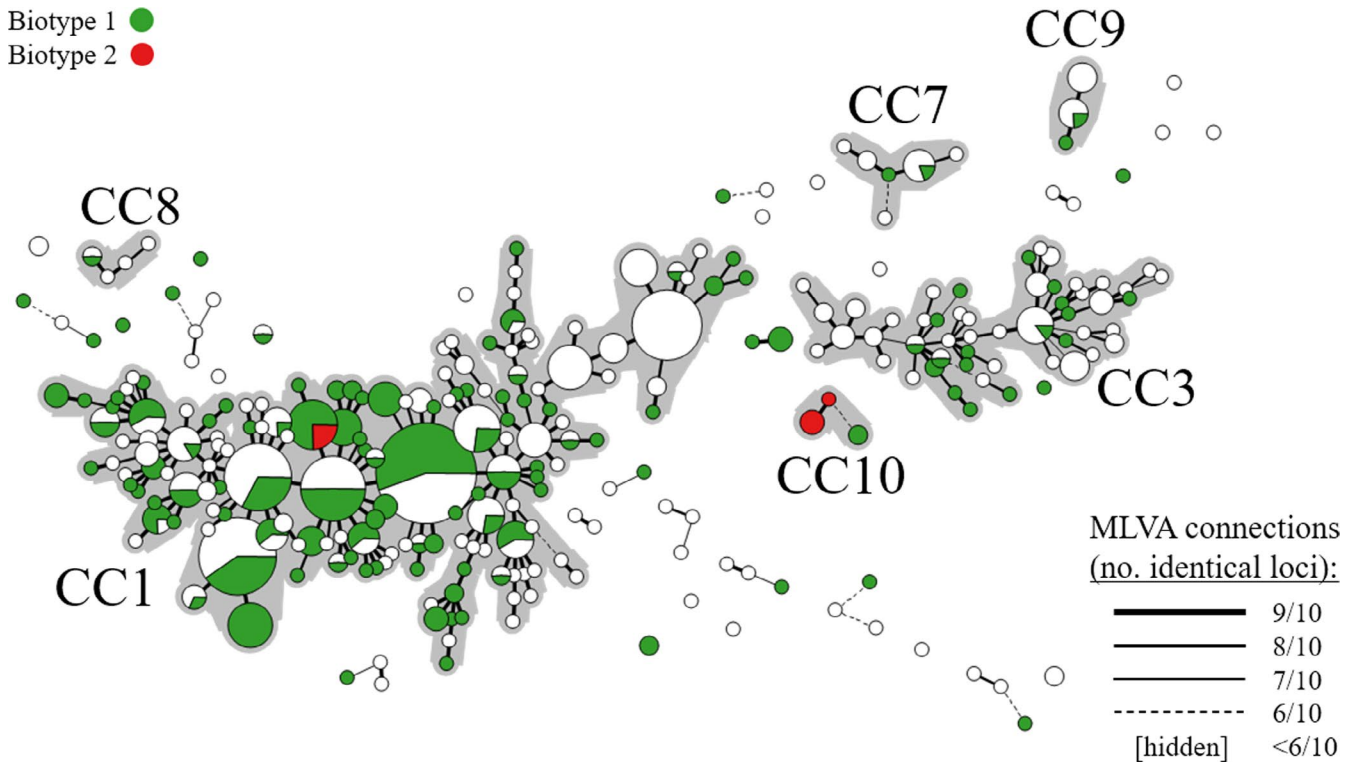

FIGURE 1 Minimum spanning tree based upon multi-locus variable number of tandem repeat analysis (MLVA) genotyping (Gulla et al., 2018) of 601 Norwegian *Y. ruckeri* isolates, collected from 1985 to 2020. Declining MLVA similarity correlates with the declining thickness of branch connections (see bottom right legend). Defined clonal complexes, interconnected throughout via ≥6/10 identical loci, are bound by a grey border. Biotyping results for assayed isolates are shown in green (biotype 1) and red (biotype 2)

between the MLVA-defined clonal complexes and highlights the independent origins of BT2 within CC's 1, 2, 5 and 10 (Figure 2).

Alignment of flagellar gene sequences extracted from the NVI-11000 genome assembly revealed the same 10 bp deletion in *fliR* as previously observed in BT2 isolates from rainbow trout in Denmark and Finland, *fliR*Δ2, which results in a frameshift from amino acid 147 onwards with the protein terminated early by a stop codon in position 169 (Welch et al., 2011).

Sequence alignment of flagellar genes from the NVI-344 genome with those of motile CC10 isolates revealed two non-synonymous SNPs not previously identified in *Y. ruckeri*, that is, *fliH (A205V)* and *flhC (G143V)*. Sanger sequencing of these two genes in all currently known CC10 isolates subsequently verified that while the *fliH*-variant is omnipresent within the CC10 lineage irrespective of biotype, the *flhC*-variant was found exclusively in BT2 isolates (Figure 3). The *flhC* mutation is situated in a sharp turn in the tertiary structure where FlhC binds a zinc ion and interacts with FlhD in the $FlhD_4C_2$ complex (Wang et al., 2006). This complex functions as a master transcriptional activator for flagellar and chemotaxis genes (Liu & Matsumura, 1994), and is also involved in the regulation of other genes (Bleves et al., 2002; Jozwick et al., 2017). Deletions or detrimental mutations in *flhDC* are known to silence expression of flagellar genes (Al Mamun et al., 1996; Chain et al., 2004; Jozwick et al., 2017; Monday et al., 2004).

Alignment of flagellar genes further revealed a single non-synonymous SNP, *fliP(A224D)* in NVI-10990, not present in any publicly available *Yersinia fliP* sequences. This SNP was also confirmed in the remaining non-motile CC1 isolates (NVI-10990 -10974 and -10975) by Sanger sequencing, while being absent in motile CC1 isolates. The flagellar export protein FliP functions with FliQ and FliR in a $P_5Q_4R_1$ complex, which constitutes the export gate of the flagellar secretion channel, a key component of the flagellar export apparatus (Kuhlen et al., 2018; Minamino & Macnab, 1999). Mutations in the export gate complex proteins have been shown to cause loss of motility in *Y. ruckeri* previously, specifically two variants of frameshift-inducing deletions in *fliR* (Welch et al., 2011). The *fliP(A224D)* mutation results in a shift in amino acid class from non-polar to polar close to the critical Asp197–Lys222 intramolecular bridge, which has been shown previously to result in motility defects when mutated (Kuhlen et al., 2018; Ward et al., 2018).

3.4 | Biotype reversion

From 10 replicate cultures each of isolates NVI-344 and NVI-10990, incubated on semi-solid agar for detection of spontaneous BT reversion, motile mutants were successfully recovered from a single culture of NVI-344, and from five cultures of NVI-10990.

Sanger sequencing of *flhC* in the reversal mutant of NVI-344 revealed a non-synonymous alanine-valine mutation at residue 143 of *flhC* (Figure 3). Although different from the wild-type glycine found in motile strains, alanine is more similar to glycine than to the branched amino acid valine found in the non-motile NVI-344.

Sanger sequencing of *fliP* in reverted mutants of NVI-10990 revealed non-synonymous SNPs in residue 224 in all five mutants, resulting in reversion to non-polar amino acid residues in this position (Figure 4). Reversion to the original wild-type allele of *fliP* was observed in one case.

All of the spontaneously reverted BT2 strains were phenotypically indistinguishable from their wild-type motile counterparts in motility and lipase assays, and by microscopic observation of flagella.

3.5 | Complementation experiments

Plasmid-mediated complementation of NVI-344 and NVI-10990 with wild-type variants of the *flhDC* operon and constitutively expressed *fliP*, respectively, restored motility and lipase activity for both strains (Table 4), with observable flagella in flagella-stained phase-contrast microscopy (Figure 5). Motility and lipase activity was lost when the strains were cured of the complementing plasmids.

4 | DISCUSSION

While non-motile BT2 *Y. ruckeri* mutants have emerged and established independently on multiple occasions in international farming of rainbow trout, often shortly after introduction of yersiniosis vaccines, these variants remain rarely reported from farmed Atlantic salmon. This study presents the first characterization of BT2 *Y. ruckeri* recovered from farmed salmon in Norway, where an increase in the use of yersiniosis vaccines has occurred in recent years. Two novel BT2 variants, belonging to distant phylogenetic lineages and isolated three decades apart, were detected amongst the 263 *Y. ruckeri* isolates biotyped. Neither of the two mutants seem to have established widespread dominance, however, and are now possibly extinct *in natura*, although at least one of the progenitor lineages still thrives. The mutations responsible for loss of motility were identified, and motility could in both cases be readily restored experimentally via culture-based reversion and plasmid-mediated complementation.

As for all previously characterized BT2 *Y. ruckeri*, the non-motile phenotypes of the two Norwegian variants described here are linked to mutations in essential flagellar genes. Both phenotype-altering mutations differed, however, from most previously documented cases of BT2 development in that they represented non-synonymous shifts rather than frameshifting deletions (Barnes et al., 2016; Welch et al., 2011). As detrimental non-synonymous SNPs are arguably less conspicuous than frameshift-inducing indels when exploring sequence data, they may be more difficult to identify as a likely cause for altered phenotype. This was certainly the case with BT2 isolate NVI-344, which displayed two unique non-synonymous SNPs in flagellar genes. The discovery of closely related motile isolates by MLVA genotyping was of key importance to enable identification of the responsible mutation by comparing the suspected genes between CC10 isolates of both biotypes. In

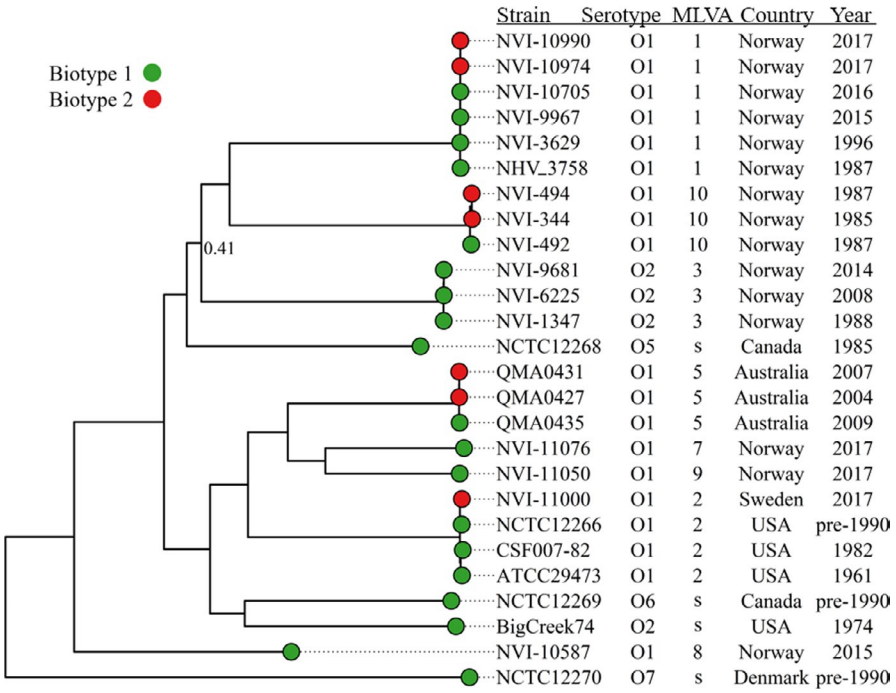

FIGURE 2 Maximum likelihood phylogenetic tree based on a core-gene alignment of 26 *Y. ruckeri* isolates produced with Roary using the MAFFT aligner. Assemblies were downloaded from NCBI or produced in current study (details in Table 3). Bootstrap values are from 1000 replicates with values >0.8 hidden. Multi-locus variable number of tandem repeat analysis (MLVA) 's' indicates the isolate does not belong to any of the MLVA clonal complexes defined here or by Gulla et al. (2018). Biotypes are shown in green (biotype 1) and red (biotype 2)

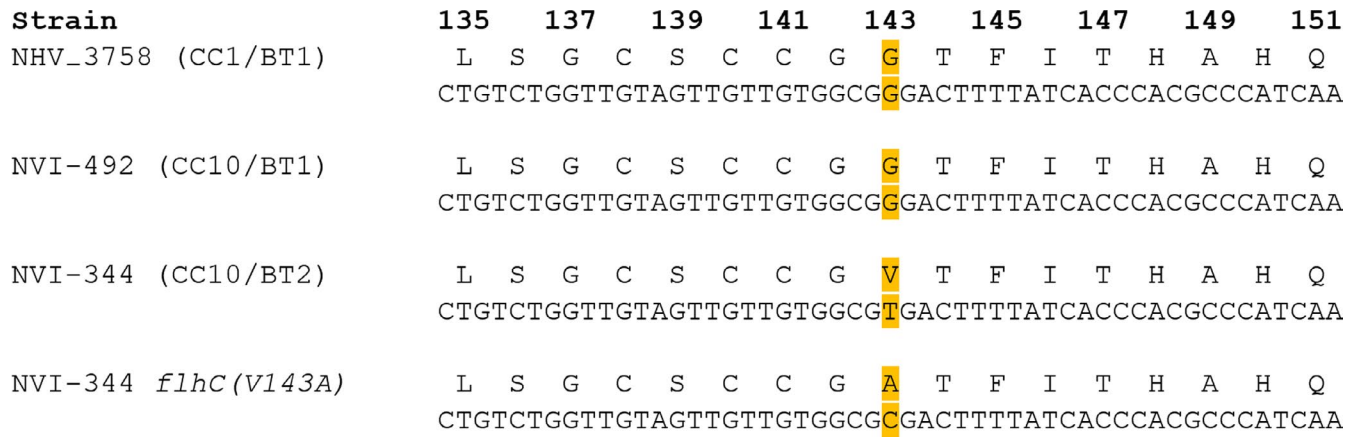

FIGURE 3 Protein- and DNA-sequence data for *flhC* amino acid positions 135–151 in the genomes of isolates NHV_3758 (CC1/BT1; accession no. CP023184), NVI-492 (CC10/BT1), NVI-344 (CC10/BT2), as well as the NVI-344 biotype reversal mutant

contrast, the causative SNP in BT2 isolate NVI-10990 was readily identifiable as several whole-genome sequences from very closely related motile isolates were already at hand. There is notably at least one previous record of BT2 where the causative mutation(s) could not be identified (Calvez et al., 2014; S. Calvez, pers. comm.), which together with the results presented here could suggest that, although more difficult to discover, BT2 development may arise as commonly by non-synonymous SNPs as by indels.

In addition to our findings in *Y. ruckeri* from Norwegian Atlantic salmon, we also investigated a single non-motile CC2 isolate (NVI-11000) recovered in 2017 from rainbow trout farmed in Sweden, revealing the same 10 bp *fliR* deletion as previously described in BT2 isolates from rainbow trout in Denmark and Finland (Welch et al., 2011). This finding contributes to further document the wide distribution of the various BT2 sub-lineages within the internationally dominant rainbow trout-associated CC2 lineage (Gulla et al., 2018). Notably, despite being a prominent producer of rainbow trout, CC2 has not yet been identified in Norway.

NVI-344, confirmed here as a member of the hitherto undescribed CC10 lineage, represents the first recorded instance of *Y. ruckeri* BT2, and indeed yersiniosis, in Norway (Sparboe et al., 1986). This relatively minor clonal complex is restricted to five clinical cases diagnosed in two Norwegian salmon farms between 1985 and 1988. Available information on the severity of these CC10 cases is limited, but the two affected farms being separated by more than 700 km indicates a wider historical distribution than that documented here. Moreover, while BT2 isolates were only detected in one of the farms, these isolations predate BT1 detections from the other farm, indicating that *Y. ruckeri* CC10 (with the BT1 phenotype) was likely also present in Norway at some point prior to 1985.

While identification of *Y. ruckeri* BT2 in CC10 is interesting in the context of independent BT2 emergences in salmonid aquaculture,

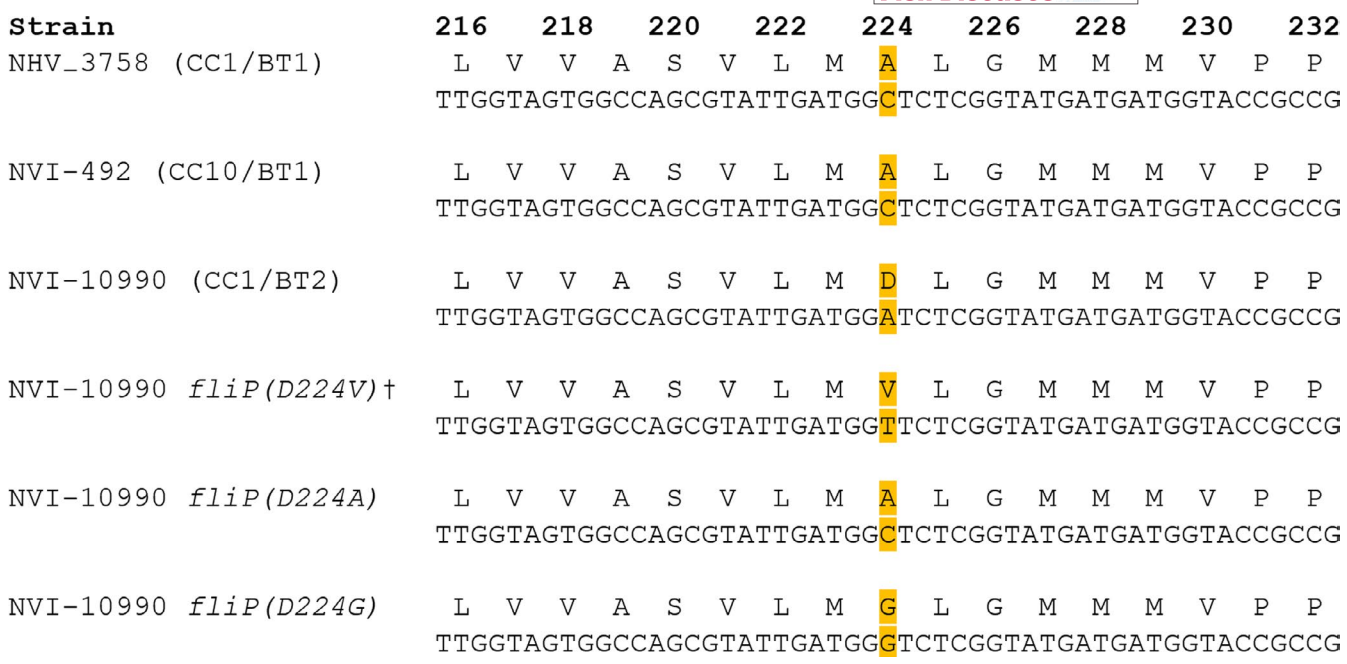

FIGURE 4 Protein- and DNA-sequence data for *fliP* amino acid positions 216–232 in the genomes of isolates NHV_3758 (CC1/BT1; accession no. CP023184), NVI-10990 (CC1/BT2), as well as the five NVI-10990 biotype reversal mutants. (†) NVI-10990 *fliP(D224V)* was observed in three cases

TABLE 4 Motility- (assessed on semi-solid TSA), lipase secretion- (assessed on Tween80 agar) and presence of flagella- (assessed by phase-contrast microscopy with flagella-stain) phenotypes of the two BT2 isolates NVI-344 (CC10) and NVI-10990 (CC1), respectively before and after reversion and complementation

Isolate	Motility	Lipase	Flagella
NVI–344	–	–	–
NVI–344 pAR3-flhDC	+	+	+
NVI–344 *flhC(V143A)*	+	+	+
NVI–10990	–	–	–
NVI–10990 pAR3-fliP	+	+	+
NVI–10990 *fliP(D224V)*[a]	+	+	+
NVI–10990 *fliP(D224A)*	+	+	+
NVI–10990 *fliP(D224G)*	+	+	+

[a]Three independent mutants of NVI-10990 displaying the same *fliP(D224V)* mutation and phenotype.

this lineage has not been registered since the late 1980s despite hundreds of later Norwegian isolates being genotyped, and is now possibly extinct. However, the three BT2 isolates belonging to the contemporary and only clinically relevant *Y. ruckeri* lineage in Norway in recent years, CC1, are of considerable current interest. While confirming that BT2 mutants have the potential to arise within this clonal lineage, the fact that of 204 biotyped CC1 isolates, collected over four consecutive decades, only three isolates from early 2017 displayed the BT2 phenotype, indicates a low historical and current prevalence in Norwegian aquaculture. The three isolates in question, NVI-10974, -10975 and -10990, share identical MLVA-profiles and the same causative BT-shifting SNP, indicating an unidentified common origin. Indeed, they were all recovered over a single month from two sea-farms in neighbouring fjords where a de-lousing vessel had visited both facilities shortly prior to the outbreaks, constituting perhaps the most likely route of transmission. Being that this phenotype within CC1 has not been detected since however, this might have represented a dead-end emergence.

While it has been hypothesized that BT2 development may be driven by lower levels of vaccine protection towards strains lacking the strongly immunogenic flagella, any causal correlation has yet to be verified. It is nevertheless interesting to note that the BT2 isolates detected within the exclusively Norwegian CC1 lineage were recovered shortly after introduction of widespread intraperitoneal vaccination within the Norwegian salmon farming industry, although it remains uncertain whether the affected fish had received such a vaccine.

The various *Y. ruckeri* lineages now known to harbour BT2 strains, that is, CC1, CC2, CC5 and CC10, all represent deep-branching phylogenetic lineages within the global *Y. ruckeri* population (Figure 2), demonstrating that BT2 mutations may likely arise within any lineage. As previously mentioned however, while BT2 *Y. ruckeri* have become remarkably successful in farming of rainbow trout, emerging on different continents following multiple independent mutation events within the clonal lineage CC2, this situation is not mirrored in international Atlantic salmon aquaculture. Early BT2 isolates from salmon in the UK in the 1980s and 1990s were then described as 'rainbow trout-like' (Wheeler et al., 2009), a supposition later confirmed by MLVA placing them within the globally dominating rainbow trout lineage CC2 (Gulla et al., 2018). A different *Y. ruckeri* lineage, CC4, has been associated with yersiniosis in farmed salmon in the UK in recent

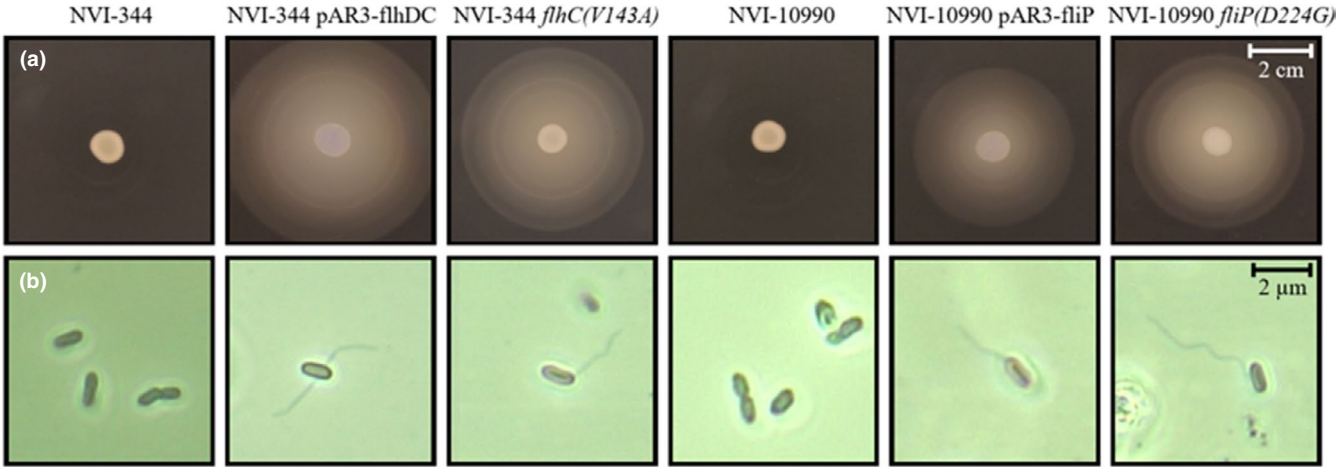

FIGURE 5 Motility assayed on semi-solid Tryptic Soy Agar (a) photographed after 24 h for motile strains and 48 h for non-motile strains, and phase-contrast microscopy with flagella stain (b). Microscopy images are representative except for NVI-10990 complemented with pAR3-fliP which displayed a lower proportion of flagellated cells (approximately 1 in 50). No flagellated cells were observed in the non-motile isolates NVI-344 and NVI-10990

years with no reports of BT2 development (Ormsby et al., 2016), while studies on isolates from Chilean salmon farming found exclusively BT1 (Bastardo et al., 2011). To our knowledge, the well-documented BT2 development within the salmon-associated CC5 from Australia (Barnes et al., 2016) represents the only known post-millennial BT2 development in 'salmon specific' lineages prior to the present study. One conceivable explanation why BT2 emergences have not established a similar dominance in salmon-associated lineages as seen within the rainbow trout-associated CC2, might relate to differing production forms for the two fish species. As such, both of the novel BT2 variants from Norwegian salmon characterized here were found over short time periods and from marine farms only. A mutant of this essentially freshwater pathogen arising in the marine environment, with harvested fish not returning to freshwater, will likely face a dead-end in terms of onwards transmission. As rainbow trout are generally farmed internationally in freshwater, and as *Y. ruckeri* appear well adapted for long-term survival in freshwater environments (Thorsen et al., 1992; Romalde et al., 1994; Coquet et al., 2002), these conditions will likely provide greater opportunities for persistence of eventual BT2 mutants and spread to subsequent fish stocks.

Although the success of *Y. ruckeri* CC1 in Norway over the past three decades cannot be attributed to biotype shift, CC1 does display a seemingly intrinsic slow-swimming phenotype in comparison to motile isolates within other examined lineages (Riborg, personal observation). Lipase activity appears linked to this low-motility trait as slow-swimming CC1 isolates display a weak or even negative lipase reaction while remaining motile (Figure S1). This situation therefore contradicts the conventional BT2 definition as described by Davies and Frerichs (1989), which regards lipase-activity and motility as positively correlated traits. This feature appears unique to CC1 as we find all other motile strains to be lipase positive. We further found that lipase activity may be increased to detectable levels in motile CC1 isolates by leaving a liquid culture static for several weeks prior to plating, while at the same time swimming-speed also increases to be on par with motile non-CC1 strains. Due to the phenotypic ambiguity thus observed, the lipase assay was discarded in the present study and biotyping of remaining CC1 isolates was based on motility assessment alone. Reduced motility has notably also been observed previously in the Australian salmon-pathogenic lineage CC5, where a successive reduction in motility was observed over time, in addition to some completely non-motile (BT2) strains also arising (Barnes et al., 2016).

Mechanisms for conditional expression of flagellar motility in *Y. ruckeri* are known, in particular from the temperature-dependant regulation of motility documented in other *Yersinia* species (Cornelis, 1992). Although *Y. ruckeri* is non-motile at lower temperatures, flagella are still expressed (O'Leary et al., 1979) and thus exposed to the immune system in a cold-blooded host. As such, Jozwick et al. (2019) recently demonstrated that flagellin expression was repressed in a motile CC2 strain during infection in rainbow trout. This was, however, a remarkably slow process requiring several days to achieve complete repression of *fliC* transcription, while transcription rates rapidly increased in the host *post mortem* (Jozwick et al., 2019). During infection, tighter regulation of motility could presumably provide some of the same advantages as potentially awarded by the BT2 phenotype, while at the same time retaining access to the flagella on demand. This might be a favoured strategy in some of the salmon-associated lineages, or perhaps a step along an evolutionary path towards ultimately dispensing of motility permanently.

During characterization of the two *Y. ruckeri* BT2 mutants NVI-344 and NVI-10990 it was further discovered that prolonged incubation on motility agar eventually produced spontaneous biotype reversions mutants, which could be easily observed and recovered (Figure 6). This highlights the risk of misidentifying BT2 isolates as slow-swimming BT1, if the agar plate is not frequently monitored throughout the incubation period. Interestingly, while all reverse mutants regained motility by single base conversion at the exact

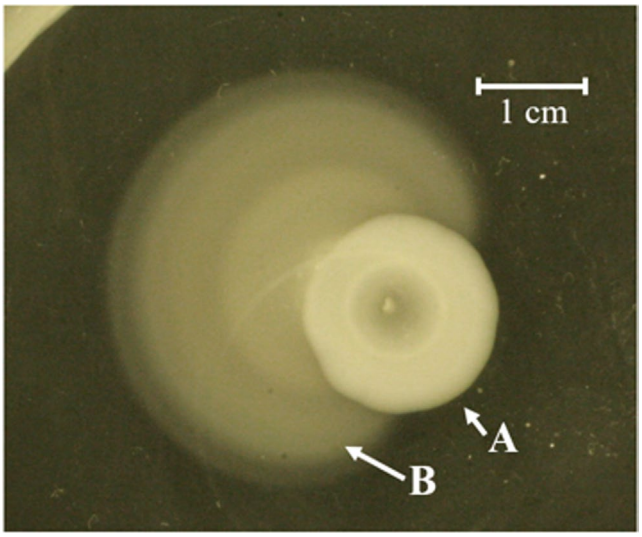

FIGURE 6 Spontaneous biotype reversion after prolonged incubation of the non-motile strain NVI-10990 (a) on semi-solid Tryptic Soy Agar. The motile mutant (b) appeared after 8 days of incubation

site suspected of causing a BT2-shift in the first place, most did not revert to the original amino acid residue found in the motile wild-type, but rather acquired alternative amino acids with similar characteristics. While motility on 0.3% agar appeared fully restored for these variants, it may be that protein function is affected negatively in other ways. Nonetheless, the possibility for spontaneous BT-reversion in *Y. ruckeri* documented here could imply that BT2 variants arising from point mutations in the wild are more flexible than those caused by frameshift-inducing deletions, where spontaneous reversion mutants are probably less likely to arise.

Aside from prolonged culture, motility in both NVI-10990 and NVI-344 could also be restored via plasmid complementation with wild-type functional copies of, respectively, *fliP* and *flhDC*. Complemented NVI-10990 displayed slower swimming than equivalent (CC1) motile isolates, correlating with the proportion of microscopically observable flagellated cells. Similar effects have been observed previously with plasmid-mediated complementation of *flhA(D256G)* and are likely due to competition from the chromosomally situated mutated gene (Welch et al., 2011), and in our specific case also affected by the expression levels of *fliP* from the *bla*-promoter. In contrast, motility in complemented NVI-344 was greatly enhanced, likely due to increased copy number of the plasmid-borne *flhDC* operon.

Identification and characterization of spontaneous biotype reversions, in combination with complementation experiments, verified that the identified mutations in *fliP* and *flhC* are responsible for the BT2 phenotype observed within *Y. ruckeri* lineages CC1 and CC10, respectively. Considering all previously characterized *Y. ruckeri* BT2 mutants have possessed mutations in genes encoding the flagellar export apparatus specifically (Barnes et al., 2016; Welch et al., 2011), the BT2-inducing mutation in *fliP* was unsurprising. The *flhC* mutation was, on the other hand, somewhat unexpected, as mutations in the *flhDC* operon have never before been linked to loss of motility in *Y. ruckeri*, and it has been proposed as a protected locus in this species (Jozwick et al., 2017). It may be the case that CC10 have other adaptations that allow for the loss of *flhDC* function in this lineage, or that some functionality is preserved with this missense mutation in *flhC* even though motility is lost. Mutations in the *flhDC* operon do, however, represent a common cause of motility-loss in other pathogens (Al Mamun et al., 1996; Chain et al., 2004; Monday et al., 2004) and it would represent a seemingly optimal mutation site in terms of preserving resources via silencing the transcription of all flagella-related operons. Interestingly, Jozwick et al. (2017) found their constructed *flhDC*-deletion mutant to be more virulent in challenge trials, in contrast to naturally occurring BT2 mutations known at the time. As such, it could be relevant to compare the virulence of the BT2 isolates in CC10 with their BT1 counterparts. However, given the current state of the yersiniosis situation in Norwegian aquaculture, with CC1 completely dominating and CC10 remaining absent for over 30 years, further examination of the effects of BT2 in *Y. ruckeri* CC1 should be prioritized.

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DATA AVAILABILITY STATEMENT

The genome assemblies have been deposited at DDBJ/ENA/GenBank under accession numbers JAJIBE000000000–JAJIBV000000000 and JAJJIH000000000, as described in Table 3.

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