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Structure of *Flavobacterium psychrophilum* populations infecting farmed rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: *Flavobacterium psychrophilum* isolated from rainbow trout *Oncorhynchus mykiss* suffering from bacterial cold-water disease (BCWD) can dissociate into 2 morphological colony types, rough (R) and smooth (S). However, the presence of the 2 morphotypes in disease outbreaks has not yet been investigated thoroughly. We examined the occurrence of R and S morphotypes in rainbow trout from BCWD outbreaks and in unfertilized eggs from a hatchery. The isolated colony types were characterized by pulsed field gel electrophoresis (PFGE), plasmid analysis, and oxolinic acid susceptibility testing. From most outbreaks, both morphotypes were isolated, although the S type only was isolated from the majority of individual fish. PFGE analysis showed both diverse and indistinguishable genetic patterns among the concurrent morphotypes. While PFGE patterns common to both fish and egg isolates were identified, this was not always the case. Resistant and sensitive isolates of both colony types were isolated from individual disease outbreaks. The plasmid pattern was partly associated with the colony type, showing identical or completely different patterns for the R and S types isolated from the same outbreak. The study showed that within a BCWD outbreak, *F. psychrophilum* cells with different morphology, plasmid content, antibiotic susceptibility, and PFGE pattern can be isolated, suggesting that *F. psychrophilum* populations infecting rainbow trout in farm environments can be diverse and thus complicate the control of the disease.

KEY WORDS: Population structure · Phase variation · Morphotype · Bacterial cold-water disease · Oxolinic acid · Pulsed field gel electrophoresis · Plasmid profiling

INTRODUCTION

Flavobacterium psychrophilum, the causative agent of bacterial cold-water disease (BCWD), is one of the most important bacterial pathogens in freshwater rainbow trout *Oncorhynchus mykiss* aquaculture. Although the bacterium has been extensively studied, much remains unknown regarding the infection process of the pathogen. Phase variation in *F. psychrophilum* and the subsequent dissociation into 2 different morphological colony phenotypes, rough (R) and smooth (S), has previously been reported by Högfors-Rönnholm & Wiklund (2010a). However, the presence of the 2 morphotypes in BCWD outbreaks has not yet been investigated thoroughly. Phase variation is believed to be an adaptive process that involves a change in gene expression resulting in phenotypic differences between bacterial cells of a clonal population (van der Woude 2006). It has been suggested that pathogenic bacteria can switch phenotype in order to survive the host immune responses and to shift between a planktonic and a biofilm-forming growth mode (van der Woude 2006).

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To assess the clonality of bacterial populations involved in disease outbreaks and for epidemiological tracking purposes, it is necessary to investigate the genetic relatedness between infective isolates. The genotypic variability in *Flavobacterium psychrophilum* has previously been analyzed by random amplification of polymorphic DNA (RAPD; Chakroun et al. 1997), restriction fragment length polymorphism of PCR products (PCR-RFLP; Izumi et al. 2003), ribotyping (Cipriano et al. 1996, Chakroun et al. 1998, Dalsgaard & Madsen 2000, Madsen & Dalsgaard 2000, Madetoja et al. 2002), polymorphism in 16s rRNA genes (Soule et al. 2005a), reciprocal suppression subtractive hybridization by microarray analysis (Soule et al. 2005b), and multilocus sequence typing (MLST; Nicolas et al. 2008). Alone or in combination with other genotyping methods, plasmid profiling has also been used as a tool for genetic characterization of *F. psychrophilum* isolates (Lorenzen et al. 1997, Chakroun et al. 1998, Izumi & Aranishi 2004a, Kim et al. 2010). Of the molecular typing methods, pulsed-field gel electrophoresis (PFGE) has been considered the 'gold standard' for bacterial subtyping and epidemiological tracking and has even proven to be a successful method for typing of *F. psychrophilum* (Arai et al. 2007, Chen et al. 2008, Del Cerro et al. 2010) because of its high discriminatory ability and reproducibility.

Despite the significant economic impact of *Flavobacterium psychrophilum* on the global aquaculture industry today, no commercial vaccines are available, and treatment of BCWD mainly relies on oral antimicrobial therapy. The use of antimicrobials in aquaculture settings has resulted in the emergence of resistant *F. psychrophilum* strains, which may hamper the control of recurrent disease outbreaks. Oxytetracycline and oxolinic acid have been widely used in European aquaculture, but the rapid development of resistance towards the antimicrobials has led to therapeutic treatments with florfenicol.

The aim of the present work was to investigate the occurrence and coexistence of R and S morphotypes within BCWD outbreaks. Rainbow trout with clinical signs of BCWD and unfertilized eggs were examined, and the genetic relatedness between isolated R and S colony phenotypes was studied by PFGE and plasmid profiling. To provide additional differentiation, susceptibility to oxolinic acid was examined. *In vitro* converted S-to-R (2R) forms of *Flavobacterium psychrophilum* were also included in the study.

MATERIALS AND METHODS

Bacterial sampling and identification

In total, 60 rainbow trout from 3 different Finnish fish farms (Farms 1 to 3) with 2 suspected BCWD outbreaks each, and 10 unfertilized fish egg samples from 1 hatchery in Farm 3 were examined for *Flavobacterium psychrophilum* infections by agar plate culturing. Bacteriological samples from fish were taken from the spleen and kidney on tryptone yeast extract salts (TYES) agar plates (0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2 \times 2H_2O$, 0.05% $MgSO_4 \times 7H_2O$, 1.1% agar, pH 7.2; Holt et al. 1993). The fish egg samples (10 eggs sample^{-1}) were crushed with a sterile glass rod in a 15 ml Falcon tube. Then, 100 µl of the crushed egg suspension was diluted 10× and 100× in 0.5% NaCl, after which 100 µl of each dilution was streaked onto TYES agar plates. All plates were incubated at 15°C for 7 d after inoculation, after which the presence of the 2 different colony types was recorded under a microscope (Fig. 1). A representative number of colonies of the 2 phenotypes derived from infected fish individuals (Samples a to u, Table 1) were sub-cultured and stored at −70°C in TYES broth + 20% glycerol for further examination.

All isolates used in the study (Table 1) were analyzed by a rapid colony PCR for *Flavobacterium psychrophilum* in which the DNA template was prepared by suspending a colony from the TYES agar plate in 50 µl double-distilled water (ddH_2O). One µl of the suspension was used as the template

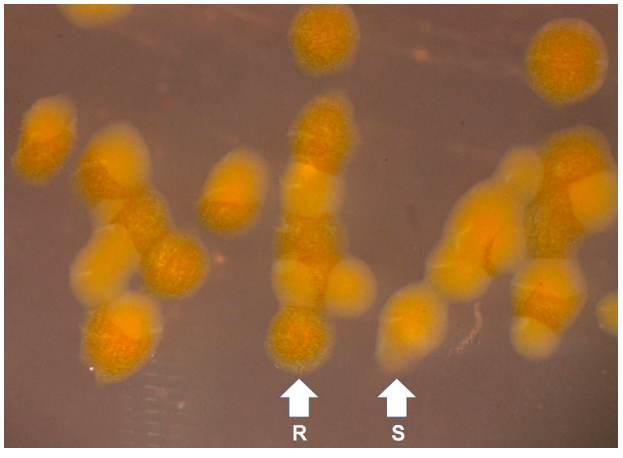


Fig. 1. *Flavobacterium psychrophilum.* Stereomicroscopic image of rough (R) and smooth (S) colony types on a TYES agar plate

Table 1. *Flavobacterium psychrophilum.* Origin (farm, outbreak, sample, year of isolation, and fish organ), pulsed field gel electrophoresis (PFGE) profile, and plasmid content of the rough (R), smooth (S), and smooth-to-rough (2R) converted morphotypes analyzed in this study. Samples sharing the same lowercase letter were isolated from the same fish individual. Asterisk indicates an inhibition zone diameter (IZD) ≤13 mm, indicating resistance to oxolinic acid. ND: not detected; NO: non-outbreak

Isolate	Farm	Outbreak	Sample	Year	Organ	Morphotype	PFGE profile	IZD (mm)	Plasmid size (kb) I	II	III
P1-4/09	1	A	a	2009	Spleen	S	VIII	41	4.7	3.7	2.15
P1-4/2R/09	1	A	a	2009	Spleen	2R	VIII	41	4.7	3.7	2.15
P1-10/09	1	A	b	2009	Spleen	R	VI	46	9.45	5.1	3.7
P15-2/09	1	B	c	2009	Kidney	R	VI	13*	4.8	3.4	ND
P15-4R/09	1	B	d	2009	Kidney	R	VI	13*	4.8	3.4	ND
P15-4S/09	1	B	d	2009	Kidney	S	VII	38	2.25	ND	ND
P15-4S/2R/09	1	B	d	2009	Kidney	2R	VII	37	2.25	ND	ND
P15-6R/09	1	B	e	2009	Kidney	R	VI	0*	4.8	3.4	ND
P15-6S/09	1	B	e	2009	Kidney	S	VIII	35	2.25	ND	ND
P15-7A/09	1	B	f	2009	Kidney	S	VIII	38	2.25	ND	ND
P15-7B/09	1	B	f	2009	Ulcer	S	VIII	25	2.25	ND	ND
P15-7A/2R/09	1	B	f	2009	Kidney	2R	VIII	33	2.25	ND	ND
P15-7B/2R/09	1	B	f	2009	Ulcer	2R	VIII	36	2.25	ND	ND
P15-9/09	1	B	g	2009	Kidney	R	VI	0*	4.8	3.4	ND
P7-1A/10	2	A	h	2010	Kidney	R	I	9*	4.4	2.1	1.3
P7-1B/10	2	A	h	2010	Kidney	S	I	0*	4.4	2.1	1.3
P7-2A/10	2	A	i	2010	Kidney	R	II	0*	4.4	2.1	1.3
P7-2B/10	2	A	i	2010	Kidney	S	I	0*	4.4	2.1	1.3
P7-7A/10	2	A	j	2010	Kidney	S	I	9*	4.4	2.1	1.3
P7-7B/10	2	A	j	2010	Kidney	R	I	0*	4.4	2.1	1.3
P7-9/10	2	A	k	2010	Kidney	S	I	8*	4.4	2.1	1.3
P7-9/2R/10	2	A	k	2010	Kidney	2R	I	9*	4.4	2.1	1.3
P30-2A/09	2	B	l	2009	Spleen	S	II	0*	3.4	2.2	ND
P6-1/10	3	A	m	2010	Kidney	S	IV	9*	2.25	1.75	1.4
P6-1/2R/10	3	A	m	2010	Kidney	2R	IV	11*	2.25	1.75	1.4
P6-2/10	3	A	n	2010	Kidney	S	IV	9*	2.25	1.75	1.4
P6-2/2R/10	3	A	n	2010	Kidney	2R	IV	8*	2.25	1.75	1.4
P6-6/10	3	A	o	2010	Spleen	S	IV	7*	2.25	1.75	1.4
P6-6/2R/10	3	A	o	2010	Spleen	2R	IV	7*	2.25	1.75	1.4
P12-1/10	3	B	p	2010	Kidney	S	I	0*	4.0	3.4	2.0
P12-3/10	3	B	q	2010	Spleen	S	X	35	ND	ND	ND
P12-8B/10	3	B	r	2010	Kidney	S	III	0*	3.4	2.2	ND
P12-8C/10	3	B	r	2010	Kidney	R	III	0*	3.4	2.1	ND
P12-9A/10	3	B	s	2010	Spleen	S	X	19	ND	ND	ND
P12-9B/10	3	B	s	2010	Kidney	S	III	0*	3.4	2.2	ND
P12-9C/10	3	B	s	2010	Kidney	R	III	0*	3.4	2.1	ND
P1-9/10	3	NO	t	2010	Eggs	S	V	0*	2.15	1.6	1.3
P1-9/2R/10	3	NO	t	2010	Eggs	2R	V	0*	2.15	1.6	1.3
P1-10A/10	3	NO	u	2010	Eggs	R	XI	28	3.9	ND	ND
P1-10B/10	3	NO	u	2010	Eggs	S	V	0*	2.15	1.6	1.3
P1-10B/2R/10	3	NO	u	2010	Eggs	2R	V	0*	2.15	1.6	1.3

in a PCR mastermix containing 1× Phire® reaction buffer (Finnzymes), 0.5 µM of primers PSY1 (5′-GTT GGC ATC AAC ACA CT-3′) and PSY2 (5′-CGA TCC TAC TTG CGT AG-3′; Toyama et al. 1994), 200 µM of each dNTP, 0.4 µl Phire® Hot Start II DNA polymerase (Finnzymes), and ddH_2O to a total volume of 20 µl. The samples were initially denatured for 30 s at 98°C in a thermal cycler (Arktik™, Finnzymes), followed by 34 amplification cycles including denaturation for 5 s at 98°C, annealing of primers for 5 s at 55°C, and extension for 15 s at 72°C. After the last cycle, the PCR mixture was incubated at 72°C for 60 s. Total PCR amplification time was 65 min. The amplified PCR product (1089 bp) was electrophoresed (100 V, 30 min) on a 1% agarose-TEB gel stained with ethidium bromide and detected with ultraviolet transillumination.

PFGE

DNA of the isolates was examined using enzyme restriction and PFGE. For reference, the type strain NCIMB1947 of *Flavobacterium psychrophilum* isolated from coho salmon *Oncorhynchus kisutsch* was included in the analysis. For PFGE, DNA plugs were prepared according to the protocol described by Ribot et al. (2006) with minor modifications. Briefly, the bacterial cells were washed in phosphate-buffered saline and suspended to an optical density of 2.0 at 610 nm. Proteinase K was added to the suspensions before mixing with equal volumes of 1% SeaKem Gold agarose (Lonza) and dispensing into plug molds. Bacterial cells were then lysed overnight with Proteinase K, and the plugs were washed twice overnight with TEN buffer (Tris-EDTA-NaCl) at 4°C. DNA from the lysed cells was restricted with 20 units of *Stu*I (Roche) and loaded into 1.2% SeaKem Gold agarose. To separate the DNA fragments, the electrophoresis was run in Bio-Rad CHEF DRIII in HEPES buffer with 5.0 V cm^{-1} for 18 h at 14°C. The pulse times ranged from 0.2 to 5 s for 8 h and from 5 to 15 s for 10 h. The PFGE profiles were analyzed with BioNumerics software (Applied Maths), and a similarity dendrogram (see Fig. 2) was constructed using an unweighted pair-group method with arithmetic mean (UPGMA) and the Dice similarity coefficient.

Plasmid profiling

Plasmid DNA from *Flavobacterium psychrophilum* isolates grown on TYES agar was isolated with the NucleoSpin Plasmid-kit (Macherey-Nagel) according to the manufacturer's instructions. The plasmid DNA was separated by electrophoresis (80 V, 60 min) on a 1% agarose-TEB gel stained with ethidium bromide and visualized under ultraviolet transillumination (Alpha Innotech Multi Image light cabinet). A 1 kb DNA ladder (NEB, N3232) was used for size confirmation.

Antimicrobial susceptibility testing

Susceptibility to oxolinic acid was determined by disc diffusion on TYES agar plates. First, 5 d old colonies of the isolates were suspended in 5 ml sterile 0.5% NaCl, corresponding to a McFarland turbidity standard of 1–2. Then, 40 µl of the bacterial suspension was mixed with 5 ml 0.5% NaCl and spread onto an agar plate. After excess suspension had been removed and the agar plate had been allowed to dry, a disc containing 2 µg oxolinic acid (Oxoid) was placed onto the agar plate. The inhibition zone diameter (IZD) was measured after 5 d of incubation at 15°C. Isolates showing an IZD ≤13 mm were considered resistant, whereas isolates exhibiting an IZD >16 mm were considered sensitive (Casals & Pringler 1991).

S-to-R conversion

Ten *in vitro* converted S-to-R (2R) *Flavobacterium psychrophilum* isolates were prepared from isolated S colony types by repeated passages in TYES broth (Högfors-Rönnholm & Wiklund 2010a) and analyzed in parallel with the parent S isolates.

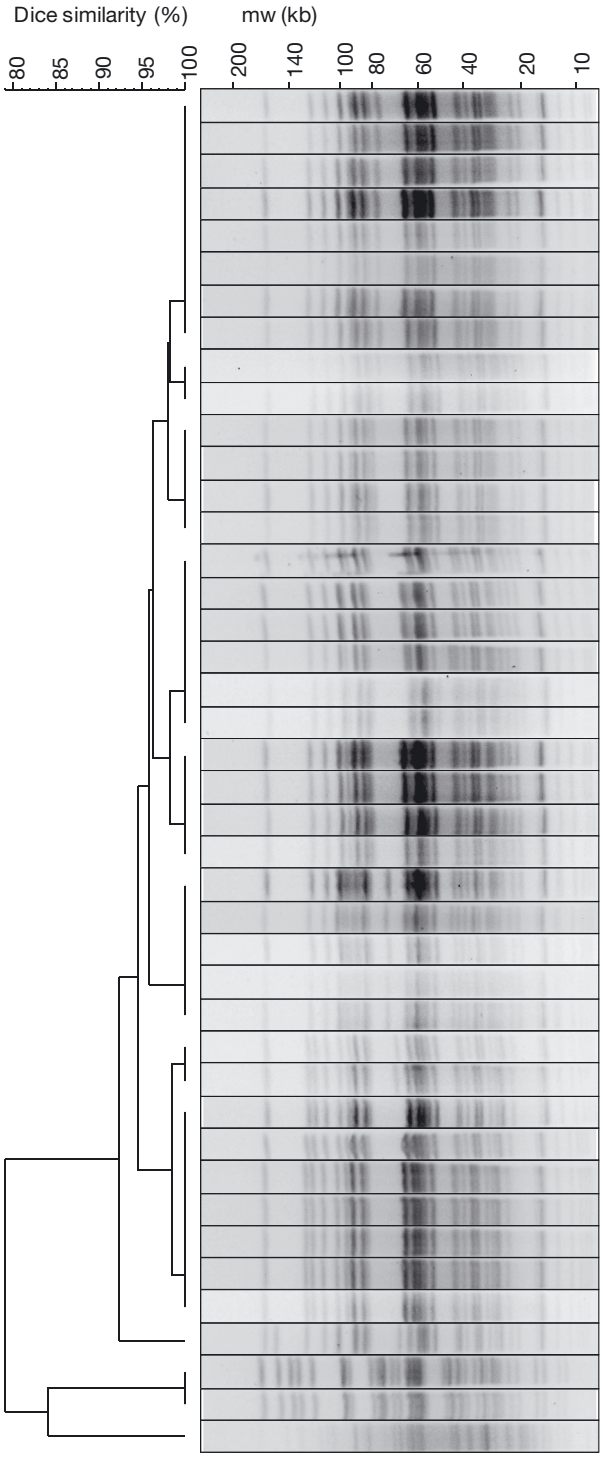
RESULTS

Bacterial sampling and identification

Yellow pigmented *Flavobacterium psychrophilum* colonies were isolated from 37 out of a total of 60 fish examined. From 5 out of 6 BCWD outbreaks, both R and S colony types were isolated, although the sole isolation of the S form dominated in individual fish. Concurrent R and S types were isolated from 10 out of a total of 37 infected fish, whereas the R and the S type alone were sampled from 6 and 21 individuals, respectively (data not shown). A total of 28 isolates from infected fish (10, 8, and 10 from Farms 1, 2, and 3, respectively) were subcultured for further examination. In addition, *F. psychrophilum* was isolated from 2 unfertilized egg samples from Farm 3. From these 2 samples, only 3 colonies were isolated, 1 R and 2 S colony phenotypes. The concentration of *F. psychrophilum* in the unfertilized eggs corresponded to about 20 to 100 colony forming units (CFU) ml^{-1}, equal to a concentration of 1 to 5 CFU egg^{-1}. All isolates used in this study (Table 1) were identified as *F. psychrophilum* by PCR.

PFGE analysis

All 42 *Flavobacterium psychrophilum* isolates included in the PFGE analysis were typeable, and a total of 11 PFGE profiles (I–XI) were identified (Table 1). The isolates from the 3 different fish farms showed a high genetic homogeneity indicated by the band similarity between 38 of the 42 isolates exceeding 94% (Fig. 2). Apart from this main cluster, 3 farm



Isolate	Morphotype	Farm	Organ	Susceptibility	PFGE profile
P7-7A/10	S	2	Kidney	Resistant	I
P7-7B/10	R	2	Kidney	Resistant	I
P7-9/10	S	2	Kidney	Resistant	I
P7-9/2R/10	2R	2	Spleen	Resistant	I
P12-1/10	S	3	Kidney	Resistant	I
P7-1A/10	R	2	Kidney	Resistant	I
P7-1B/10	S	2	Kidney	Resistant	I
P7-2B/10	S	2	Kidney	Resistant	I
P7-2A/10	R	2	Kidney	Resistant	II
P30-2A/09	S	2	Spleen	Resistant	II
P12-8B/10	S	3	Kidney	Resistant	III
P12-8C/10	R	3	Kidney	Resistant	III
P12-9B/10	R	3	Kidney	Resistant	III
P12-9C/10	R	3	Kidney	Resistant	III
P6-2/2R/10	2R	3	Kidney	Resistant	IV
P6-6/10	S	3	Spleen	Resistant	IV
P6-6/2R/10	2R	3	Spleen	Resistant	IV
P6-1/2R/10	2R	3	Kidney	Resistant	IV
P6-1/10	S	3	Kidney	Resistant	IV
P6-2/10	S	3	Kidney	Resistant	IV
P1-10B/10	S	3	Egg	Resistant	V
P1-10B/2R/10	2R	3	Egg	Resistant	V
P1-9/2R/10	2R	3	Egg	Resistant	V
P1-9/10	S	3	Egg	Resistant	V
P1-10/09	R	1	Spleen	Sensitive	VI
P15-9/09	R	1	Kidney	Resistant	VI
P15-2/09	R	1	Kidney	Resistant	VI
P15-4R/09	R	1	Kidney	Resistant	VI
P15-6R/09	R	1	Kidney	Resistant	VI
P15-4S/09	S	1	Kidney	Sensitive	VII
P15-4S/2R/09	2R	1	Kidney	Sensitive	VII
P1-4/09	S	1	Spleen	Sensitive	VIII
P1-4/2R/09	2R	1	Spleen	Sensitive	VIII
P15-7A/09	S	1	Kidney	Sensitive	VIII
P15-7A/2R/09	2R	1	Kidney	Sensitive	VIII
P15-7B/09	S	1	Ulcer	Sensitive	VIII
P15-7B/2R/09	2R	1	Ulcer	Sensitive	VIII
P15-6S/09	S	1	Kidney	Sensitive	VIII
NCIMB1947	R		Kidney		IX
P12-3/10	S	3	Spleen	Sensitive	X
P12-9A/10	S	3	Spleen	Sensitive	X
P1-10A/10	R	3	Egg	Sensitive	XI

Fig. 2. *Flavobacterium psychrophilum.* Similarity dendrogram of pulsed field gel electrophoresis (PFGE) results for the isolates analyzed in this study. The type strain NCIMB1947 was included as a reference. R: rough morphotype, S: smooth morphoype, 2R: smooth-to-rough converted morphotype, mw: molecular weight; 'susceptibility' refers to the level of resistance to oxolinic acid

isolates had clearly different PFGE profiles (Profiles X–XI). Of the total 11 PFGE profiles, Farms 1, 2, and 3 contained *F. psychrophilum* isolates with 3, 2, and 6 different profiles, respectively. The number of PFGE profiles found in each farm correlated with the number of isolates (10, 8, and 13) derived from the respec-

tive farms (1, 2, and 3). The most predominant profile (I) contained 7 isolates and included 6 isolates from Farm 2 and 1 isolate from Farm 3. More than 1 PFGE profile was obtained from 4 out of the total 6 outbreaks. The PFGE analysis also showed that the R and S colony types of *F. psychrophilum* isolated from the same BCWD outbreak can be genetically indistinguishable, but they can also have different genetic profiles. *F. psychrophilum* isolated from eggs were genetically similar or different from those infecting fish in the same farm. None of the examined isolates clustered together with the type strain NCIMB1947 (Profile IX; Fig. 2).

Plasmid profiling

Most *Flavobacterium psychrophilum* isolates contained from 1 to 3 plasmids each, with sizes ranging from 1.3 to 4.8 kb, and only 2 isolates without plasmids were observed (Table 1). The plasmid profile was partly associated with the colony type, showing identical or completely different patterns for the R and S colony types isolated from the same disease outbreak. In Farms 1 and 3, the R types showed a different profile compared to the S isolates, while the 2 colony phenotypes from the same outbreak in Farm 2 shared the same plasmid content. Isolates with identical plasmid profiles often shared indistinguishable PFGE profiles.

Antimicrobial susceptibility testing

A high level of oxolinic acid-resistant isolates was found in this study (Fig. 2). All 8 isolates from Farm 2 were resistant, and only 26% of all tested isolates were susceptible to the antimicrobial. Both resistant and sensitive cells of both colony types were isolated, indicating that there was no correlation between colony phenotype and oxolinic acid susceptibility. However, resistant R types and sensitive S types with a different genotypic profile were isolated from inner organs of the same fish individual. Genetically indistinguishable oxolinic acid-resistant R and S colony types of *Flavobacterium psychrophilum* were also isolated simultaneously.

S-to-R conversion

The *in vitro* converted S-to-R (2R) isolates always showed identical oxolinic acid susceptibility patterns, plasmid profiles, and PFGE patterns compared to the wild S type isolates (Table 1, Fig. 2).

DISCUSSION

Isolation of concurrent R and S colony phenotypes of *Flavobacterium psychrophilum* from diseased rainbow trout has previously been reported by Högfors-Rönnholm & Wiklund (2010a), but genotypic homogeneity between the 2 morphotypes has not been confirmed. The fact that S colony types can be converted to R types *in vitro* shows that phase variation in *F. psychrophilum* exists, at least under laboratory conditions, even though in our experience, the conditions for R-to-S conversion are still not fully optimized. Here we report the coincidental isolation of R and S *F. psychrophilum* morphotypes from diseased rainbow trout and eggs, which, based on PFGE analysis and plasmid profiling, share indistinguishable genetic characteristics, suggesting the occurrence of phase variation *in vivo*. Our results also indicated that both phase variants can simultaneously be present in the same infected fish individual.

The role of phase variation in the pathogenesis of *Flavobacterium psychrophilum* and the mechanisms by which it occurs are still unknown. A switch between phenotypes in pathogenic bacteria can occur through multiple mechanisms involving DNA rearrangements or epigenetic regulation (van der Woude & Bäumler 2004). Since the 2R isolates included in this study maintained their plasmids after *in vitro* conversion from S to R, it is unlikely that a change in colony morphology in *F. psychrophilum* is connected to the acquisition or loss of a plasmid. It has recently been discovered that phase variation in the human pathogenic bacterium *Staphylococcus aureus* is a self-organized stochastic event, involving a large-scale inversion of the chromosome, switching phenotypic characteristics either on or off (Cui et al. 2012). This genetic event has been shown to influence the colony morphology, antimicrobial susceptibility, and hemolytic activity of the bacterium (Cui et al. 2012). It is not yet known under which circumstances phase variation in *F. psychrophilum* occurs in natural environments and whether it takes place inside or outside the host. R and S colony types of *F. psychrophilum* have been shown to be nearly equal in virulence when injected into rainbow trout, but to possess different biochemical characteristics in terms of agglutination, hydrophobicity, and adhesion, and to express different hemolytic and antiphagocytic activity (Högfors-Rönnholm & Wiklund 2010a,b,

2012). Thus, the sporadic generation of phenotypically different subpopulations could enhance the chance of pathogen survival and infection. Phase variation could therefore be an important feature in the pathogenesis of *F. psychrophilum*, enabling the bacterium to colonize different anatomical niches in the host and thereby escape its defense mechanisms. It is also possible that both variants are needed for efficient colonization of the host or modulation of the host immune response.

Although there is evidence of horizontal (Madetoja et al. 2002) and vertical transmission of *Flavobacterium psychrophilum* (Brown et al. 1997, Taylor 2004), it is not known how these 2 infection routes contribute to the recurrent outbreaks of BCWD in fish farm environments. In our study, *F. psychrophilum* was found in unfertilized egg samples, albeit in low concentrations. Nevertheless, some of these *F. psychrophilum* isolates were genetically similar to those isolates causing disease in the same farm, indicating that outbreaks in rainbow trout fry may originate from infected eggs. Because of the possible presence of viable non-culturable cells (Michel et al. 1999), the isolated number of CFU might not necessarily have corresponded to the actual number of live *F. psychrophilum* cells present in the egg samples. The contribution of vertical transmission to the infection pressure in fish farms might therefore be greater than estimations based on the number of viable *F. psychrophilum* cells isolated from unfertilized egg samples.

The high observed prevalence of oxolinic acid resistance in *Flavobacterium psychrophilum* underpins the importance of antimicrobial susceptibility testing of clinical isolates in order to predict the *in vivo* success or failure of antibiotic therapy and to avoid unnecessary administration of drugs exerting a selective pressure on the target bacterial population. The percentage of oxolinic acid-resistant isolates (74%) found in our investigated BCWD outbreaks was surprisingly high, because the drug has been banned from use in Finnish aquaculture since 2001. Since quinolone resistance in *F. psychrophilum* has been associated with chromosomal mutations (Izumi & Aranishi 2004b, Shah et al. 2012), it is possible that earlier heavy use of oxolinic acid led to a selection of resistant isolates in the studied farm environments. The study showed that R and S morphotypes of *F. psychrophilum* isolated from BCWD outbreaks may differ in their genotype and thus in their antimicrobial sensitivity. Since concurrently isolated R and S colony types can easily be distinguished under a stereomicroscope (Fig. 1), it would be advisable to test both types for antimicrobial susceptibility when considering the proper treatment strategy. The inability to control resistant *F. psychrophilum* cells could lead to repetitive disease outbreaks in farm environments.

Flavobacterium psychrophilum is considered genetically homogeneous as a species (Nicolas et al. 2008), and apparent associations between closely related isolates and the rainbow trout host have been made (Chen et al. 2008, Siekoula-Nguedia et al. 2012). In our study, the *F. psychrophilum* isolates recovered from infected rainbow trout from 3 different fish farms were characterized by limited genetic variability. The results correlate with previous findings of Avendaño-Herrera et al. (2009), who reported a high genetic relatedness between *F. psychrophilum* isolates from BCWD outbreaks in Chile. In another study, Chen et al. (2008) observed that *F. psychrophilum* isolated from rainbow trout showed a more uniform PFGE pattern compared to isolates collected from other salmonids. Our results also showed a high similarity between the PFGE profiles of the apparently virulent isolates collected from diseased rainbow trout and the type strain NCIMB1947.

Despite the documented homogeneity of *Flavobacterium psychrophilum* isolated from rainbow trout, the molecular mechanisms applied in previous studies might not have revealed the true diversity of the pathogen, and the functional impact of heterogeneity might be underestimated. In-depth analyses of the human pathogen *Mycobacterium tuberculosis* have shown that even in highly homogeneous bacterial populations, heterogeneity can have significant functional consequences (Homolka et al. 2010). It is therefore possible that in contrast to clonal outbreaks, BCWD in farmed rainbow trout is caused by a functionally heterogeneous group of *F. psychrophilum*. Since no reproducible immersion challenge models using a single strain or clone of *F. psychrophilum* have yet been reported, it can be speculated based on our findings that *F. psychrophilum* cells with different traits and roles in the pathogenesis might be required to overcome the host defense systems and to establish a systemic infection.

Due to the expansion of genetically closely related isolates, it has been suggested that *Flavobacterium psychrophilum* exhibits an epidemic population structure (Siekoula-Nguedia et al. 2012), in which a particularly successful clone or group of closely related genotypes may become prevalent, prevail for a period of time and then disappear as a result of recombination. Madetoja et al. (2002) observed that a single clone of *F. psychrophilum* can dominate in a

farm over a period of time, but also noticed that strains showing characteristics different from the dominant type were always found concurrently. These results are consistent with our findings, where *F. psychrophilum* isolates with different PFGE profiles were found from each farm.

Although the presence of more than 1 genotype of *Flavobacterium psychrophilum* in a fish farm environment (Madetoja et al. 2002, Madsen et al. 2005, Del Cerro et al. 2010) and in the same fish individual (Chen et al. 2008) has been reported previously, the occurrence of R and S morphotypes within a BCWD outbreak has not been investigated before. Our study showed that *F. psychrophilum* cells with different morphology, plasmid content, antimicrobial susceptibility, and PFGE pattern can be isolated from a disease outbreak, corroborating both genotypic and phenotypic heterogeneity within the bacterial population infecting farmed rainbow trout. In addition to improving the possibility of pathogen survival under stressful environmental conditions, diversity within an infective bacterial population might have a significant impact on its global gene expression, on host interactions, and thus, on the severity of a disease outbreak.

Our study showed that most of the examined BCWD outbreaks in farmed rainbow trout were caused by more than 1 genotype of *Flavobacterium psychrophilum* and often included both R and S morphotypes. The concurrent isolation of both morphotypes sharing indistinguishable genetic patterns suggested that phase variation in *F. psychrophilum* also occurs *in vivo*. In addition, both oxolinic acid resistant and sensitive cells were isolated together from eggs and infected fish. Such phenotypic and genotypic diversity simultaneously present within *F. psychrophilum* populations in fish farm environments might complicate the control of BCWD.

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