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# Molecular epidemiology of *Flavobacterium psychrophilum* from Swiss fish farms

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ABSTRACT: Bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS) caused by *Flavobacterium psychrophilum* are 2 of the major diseases causing high fish mortality in salmonid fish farms. The molecular epidemiology of *F. psychrophilum* is still largely unknown. Multilocus sequence typing (MLST) has been previously used for this pathogen and underscored a correlation between clonal complexes and host fish species. Here we used MLST to study the relationships among 112 *F. psychrophilum* isolates from rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta fario* and *S. t. lacustris* in Swiss fish farms between 1993 and 2012. The isolates belonged to 27 different sequence types (STs). Most of the Swiss outbreaks were associated with strains belonging to clonal complexes CC-ST2 and CC-ST90, found in both rainbow trout and brown trout and represented by several STs. Eight ST singletons could not be connected to any known clonal complex. Already reported from other parts of Europe and North America, CC-ST2 was the most frequent clonal complex observed, and it caused the majority of outbreaks in Switzerland, with CC-ST90 being the second most important type. In the tightly interconnected Swiss fish farms, no association between clonal complex and host fish was detected, but a temporal evolution of the frequency of some STs was observed. The occurrence of sporadic STs suggests high *F. psychrophilum* diversity and may reflect the presence of different sequence types in the environment.

KEY WORDS: *F. psychrophilum* · BCWD · RTFS · Myxobacteria · Pathology · Clonal complex · Sequence complex · Allele type · MLST · Flavobacteriosis



## INTRODUCTION

Bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS) cause high mortality in salmonid fishes infected by the pathogen *Flavobacterium psychrophilum* (*Flavobacteriaceae*, *Bacteroidetes*). Infection of gills, skin, and fins of the fish may lead to high mortality rates, resulting in large economic losses (Cipriano et al. 1995, Kondo et al. 2003, Nematollahi et al. 2003, Hesami et al. 2008). *F. psychrophilum* outbreaks were first described in North America from Coho salmon *Oncorhynchus kisutch* (Borg 1948); the disease was then also identified in France, Germany, the UK, Finland, Denmark, Norway, and Switzerland as well as in Asian countries such as Japan and Korea, reported in ayu *Plecoglossus altivelis* (Nematollahi et al. 2003, Barnes & Brown 2011). Although the role of the commercial fish and egg trade in the dissemination of the pathogen has yet to be clarified, *F. psychrophilum* infections in salmonid fish farms can now be considered cosmopolitan (Bernardet & Kerouault 1989, Cipriano et al. 1995, Ekman et al. 1999).

Only scant information is available on the epidemiology of *Flavobacterium psychrophilum*. Attempts have been made to characterize and type it by differ-

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ent methods such as serotyping, ribotyping, and plasmid profiling (Madetoja et al. 2002, Hesami et al. 2008, Kim et al. 2010). Multilocus sequence typing (MLST) allows the characterization of bacteria based on the nucleotide sequences of several housekeeping genes. Each fragment represents an allele type (AT), and each combination of alleles allows assigning each isolate to a sequence type (ST). This approach is highly reproducible and standardized, as it depends directly on DNA sequences (Maiden et al. 1998). While MLST has also been used to identify species, for instance in the genera *Aeromonas* (Martino et al. 2011) and *Wolbachia* (Baldo et al. 2006), its main use is linked to epidemiological investigations. It has been used to study the epidemiology of selected pathogenic bacterial species such as community and healthcare-associated strains of multidrug resistant *Staphylococcus aureus* (MRSA; Peterson et al. 2012) and animal pathogenic strains of *Brachyspira hydysenteriae* among farmed and wild pigs in Spain and other countries (Osorio et al. 2012). In aquaculture, this technique has been used to type isolates of the fish pathogen *Yersinia ruckeri* from different geographical areas, different hosts, and different environments (Bastardo et al. 2012).

Nicolas et al. (2008) published a detailed protocol for MLST typing of *Flavobacterium psychrophilum* based on the whole genome sequence of strain JIP02-86 (Duchaud et al. 2007). Eleven housekeeping, single-copy genes were used to characterize strains and to classify them into STs. The number of genes was subsequently reduced from 11 to 7 loci, providing higher discrimination power (Nicolas et al. 2008). Data from 50 isolates demonstrated that recombination between *F. psychrophilum* strains occurs frequently and that there is a tight association between some widespread clonal complexes and some host fish species (Nicolas et al. 2008).

Typing of the pathogenic strains causing high mortality in fish farms is necessary to monitor the rate of recombination/adaptation of bacteria in response to the environment, the dispersion of highly pathogenic strains, and also to monitor strains imported through international trade. In the present study, we applied MLST to examine diversity and relationships among *Flavobacterium psychrophilum* strains isolated from rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta fario* and *S. t. lacustris* in Swiss fish farms from 1993 to 2012. This project is part of the ERA-NET EMIDA PATHOFISH project, which aims to understand the population structure and the epidemiology of *F. psychrophilum* infections provoked in European fish farms.

## MATERIALS AND METHODS

### Isolation of bacterial strains

From 1993 to 2012, 112 *Flavobacterium psychrophilum* strains were isolated from rainbow trout and brown trout from 29 fish farms in Switzerland. The fish showed symptoms of either RTFS, BCWD, or skin and gill infections. Spleen and skin samples were plated on CY-Agar (medium 67 DSMZ for *F. psychrophilum*: 0.3% casitone, 0.136% $CaCl_2 \cdot H_2O$, 0.1% yeast extract, 1.5% agar) and Enriched Cytophaga Agar Medium (medium 1133 DSMZ for *F. columnare*: 0.2% tryptone, 0.05% beef extract, 0.05% yeast extract, 0.02% sodium acetate, 1.5% agar). Yellow colonies were isolated and, when available, several colonies originating from the same fish or from different fish belonging to the same fish farm were isolated and stored at −80°C.

Approval for fish manipulation and collection of bacterial isolates was obtained from the Federal Veterinary Office (FVO, Switzerland) and the Ticino Cantonal Veterinary Office (Authorization 03/2010 and 04/2010) for the experiments carried out after 2009. All isolates obtained before 2009 were received from the Swiss National Reference Center for Fish Diseases (FIWI), where the strains were isolated from diseased fish received for diagnostic purposes.

### DNA extraction and identification of bacterial strains

DNA was extracted from all samples using the InstaGene kit (Bio-Rad). *Flavobacterium psychrophilum* was identified by 16S rRNA gene sequencing. PCR was carried out using the universal primers uniL 26f (5′-ATT CTA GAG TTT GAT CAT GGC TCA-3′) and uniR 1392r (5′-ATG GTA CCG TGT GAC GGG CGG TGT GTA-3′) (Lane 1991). PCR amplifications were performed in a total volume of 50 µl containing 1× *Taq* PCR Master Mix Kit (Qiagen), primers (0.3 µM), and 5 µl of DNA at the following conditions: 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C, with a final elongation of 7 min at 72°C. PCR products were purified using Sephadex® G-100 (Sigma-Aldrich) according to the manufacturer's instructions. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instruction. The PCR primers (0.2 µM) were used for 16S rRNA gene sequencing. Thermal cycling conditions were 1 min at 96°C followed by 25 cycles of 10 s

at 96°C, 5 s at 50°C, and 4 min at 60°C. Amplicons were purified by Sephadex™ G-50 (GE Healthcare-Illustra™) and sequenced with HiDiTM Formamide (Applied Biosystems) on an AB 3500 Genetic Analyzer (Applied Biosystems). The obtained sequences were compared with data included in GenBank (http://blast.ncbi.nlm.nih.gov/).

### MLST conditions and sequencing

MLST PCR was carried out using the primers described by Nicolas et al. (2008) and listed in Table 1. PCR was carried out using 1× *Taq* PCR Master Mix Kit (Qiagen) in a final volume of 25 µl, with primers at 0.3 µM and the addition of 2.5 µl of DNA. Conditions for amplification were 3 min at 94°C followed by 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 2 min, and a final elongation at 72°C for 8 min. Sequencing was performed with the same primers (0.2 µM) as for 16S rRNA gene sequencing, using the BigDye Terminator v3.1 Cycle Sequencing kit.

### MLST analysis

Each sequence was assembled separately for each isolate and locus and screened for potential errors using Phred/Phrap (Ewing & Green 1998, Ewing et al. 1998; single nucleotide polymorphism Phred quality above 40). When the software highlighted potential errors, sequences were validated manually using Consed (Gordon et al. 1998, 2001). As in Siekoula-Nguedia et al. (2012), target sequences were shortened by approximately 70 bp at their extremities compared to the initial MLST scheme (Nicolas et al. 2008). After alignment of the sequences for the whole collection of isolates, arbitrary numbers were used to unambiguously identify each AT and ST.

Isolates harboring the same ST collected from the same fish species in the same farm within a period of less than 2 mo were considered to have originated from the same outbreak, treated as duplicates, and removed from the final analysis. Thus, of 112 *Flavobacterium psychrophilum* isolates studied, only 84 were included in the final analysis (see Table S1 in the supplement at www.int-res.com/articles/suppl/d105p203_supp.pdf). Accession numbers from GenBank are available for each different AT for each gene (JX947346–JX947418).

Clonal complex structures were investigated with eBURST version 3 (Feil et al. 2004, Spratt et al. 2004). A neighbor-joining tree based on Jukes-Cantor distance was built with Phylip (Felsenstein 2004) to depict the nucleotide divergence between isolates. Statistical analyses to investigate association between genotype and host and year were conducted in R (R Development Core Team 2011), including analysis of molecular variance (AMOVA) described by Excoffier et al. (1992). The p-values that could not be computed analytically were estimated with 1 000 000 permutations.

## RESULTS

From 1993 to 2012, we identified 84 isolates responsible for 75 outbreaks in the 29 Swiss fish farms considered in this study. Seven outbreaks were characterized by multiple infections by different STs,

Table 1. Primers used in this study (Nicolas et al. 2008)

| Primer name | Sequence (5′–3′) | Gene target | PCR product (bp) |
|---|---|---|---|
| atpA_F | CTT GAA GAA GAT AAT GTG GG | ATP synthetase, α subunit | 978 |
| atpA_R | TGT TCC AGC TAC TTT TTT CAT | | |
| dnaK_F | AAG GTG GAG AAA TTA AAG TAG G | Chaperone heat shock protein 70 | 897 |
| dnaK_R | CCA CCC ATA GTT TCG ATA CC | | |
| fumC_F | CCA GCA AAC AAA TAC TGG GG | Fumarate hydratase class II | 849 |
| fumC_R | GGT TTA CTT TTC CTG GCA TGA T | | |
| gyrB_F | GTT GTA ATG ACT AAA ATT GGT G | DNA gyrase, β subunit | 1149 |
| gyrB_R | CAA TAT CGG CAT CAC ACA T | | |
| murG_F | TGG CGG TAC AGG AGG ACA TAT | Glycosyltransferase murein G | 825 |
| murG_R | GCA TTC TTG GTT TGA TGG TCT TC | | |
| trpB_F | AAG ATT AAT GGT AGG CCG CCC | Tryptophane synthetase, β subunit | 933 |
| trpB_R | TGA TAG ATT GAT GAC TAC AAT ATC | | |
| tuf_F | GAA GAA AAA GAA AGA AAT ATT AC | Elongation factor Tu | 939 |
| tuf_R | CAC CTT CAC GGA TAG CGA A | | |

while the remaining 68 outbreaks were associated with only 1 ST. The most prevalent ST was ST2 (32 strains; 38%) followed by ST10 (12 strains; 14%), ST90 (6 strains; 7%), ST98 (5 strains; 6%), ST1 (3 strains; 4%), and ST20, ST209, ST79, and ST212 (2 strains each; 2%). The remaining 19 STs caused only 1 outbreak each. The 4 most frequent STs (ST2, ST10, ST90, and ST98) were present in different parts of Switzerland and in different host species. They caused repetitive infections within the same fish farms, the same species of fish, or in different species. On the other hand, some strains seemed to be restricted to 1 outbreak and to 1 fish farm (Table 2).

Clonal complex (CC) relationships are based on all ST profiles separated by a difference of 1 or 2 alleles. Three CCs were detected in our study (Fig. 1a); Fig. 1b shows a tree summarizing the nucleotide divergence between the ST profiles. The largest CC was CC-ST2, taking its name from the most abundant ST (ST2, also probably the founder ST) and includes 13 additional STs. STs belonging to this group can be divided into single locus variants (SLVs) of ST2 that differ from the founder by a single gene (ST10, ST89, ST98, ST114, ST208, ST212) and in double locus variants (DLVs: ST20, ST79, ST214, ST92, ST174, ST209, ST216) that diverge by 1 gene from the closest SLV (e.g. ST20, ST79, and ST214 are

Table 2. *Flavobacterium psychrophilum*. Prevalence of sequence types (STs) and clonal complexes (CCs) found in Swiss isolates. Samples were collected between 1993 and 2012; the number of fish farms providing samples was 29. Multiple samplings per year and per farm were possible, as different fish species may have been present in each fish farm. 'Years' indicates first and last sampling years. RT: rainbow trout *Oncorhynchus mykiss*; BTF: brown trout *Salmo trutta fario*; BTL: brown trout *S. t. lacustris*; C: char (*Salvelinus* sp.). AT: allele type for each locus shown

| | No. of isolates | % of population | No. of farms infected | Years | Fish species | AT | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *trpB* | *gyrB* | *dnaK* | *fumC* | *murG* | *tuf* | *atpA* |
| **CC-ST2** | **64** | **76.1** | **27** | **14[a]** | | | | | | | | |
| ST2 | 32 | 38.0 | 18 | 1993–2012 | RT, BTF, BTL | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| ST10 | 12 | 14.3 | 8 | 1993–2009 | RT, C | 2 | 8 | 2 | 2 | 2 | 2 | 2 |
| ST98 | 5 | 5.9 | 3 | 2011 | RT, BTF, BTL | 2 | 2 | 2 | 2 | 2 | 48 | 2 |
| ST20 | 2 | 2.4 | 2 | 1994 | RT | 8 | 8 | 2 | 2 | 2 | 2 | 2 |
| ST79 | 2 | 2.4 | 2 | 1993–1994 | RT | 2 | 8 | 8 | 2 | 2 | 2 | 2 |
| ST209 | 2 | 2.4 | 1 | 2003 | RT | 2 | 20 | 27 | 2 | 2 | 2 | 2 |
| ST212 | 2 | 2.4 | 1 | 1993–1996 | | 2 | 2 | 2 | 2 | 41 | 2 | 2 |
| ST89 | 1 | 1.2 | 1 | 2011 | RT | 2 | 2 | 2 | 2 | 2 | 47 | 2 |
| ST92 | 1 | 1.2 | 1 | 2009 | RT | 3 | 2 | 2 | 2 | 2 | 41 | 2 |
| ST114 | 1 | 1.2 | 1 | 2009 | RT | 2 | 2 | 2 | 2 | 2 | 49 | 2 |
| ST174 | 1 | 1.2 | 1 | 2011 | RT | 1 | 2 | 2 | 2 | 2 | 48 | 2 |
| ST208 | 1 | 1.2 | 1 | 2003 | RT | 2 | 2 | 22 | 2 | 2 | 2 | 2 |
| ST216 | 1 | 1.2 | 1 | 2011 | RT | 2 | 2 | 2 | 2 | 2 | 47 | 41 |
| ST214 | 1 | 1.2 | 1 | 1998 | RT | 2 | 8 | 2 | 2 | 2 | 2 | 50 |
| **CC-ST90** | **10** | **11.9** | **7** | **6[a]** | | | | | | | | |
| ST90 | 6 | 7.1 | 6 | 2009–2011 | RT, BTF, BTL | 1 | 1 | 1 | 1 | 1 | 2 | 1 |
| ST1 | 3 | 3.6 | 2 | 1993–2003 | RT | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| ST210 | 1 | 1.2 | 1 | 2011 | RT | 1 | 1 | 1 | 1 | 1 | 2 | 2 |
| **CC-ST116** | **2** | **2.4** | **2** | **2[a]** | | | | | | | | |
| ST116 | 1 | 1.2 | 1 | 2009 | RT | 27 | 45 | 24 | 10 | 16 | 48 | 41 |
| ST118 | 1 | 1.2 | 1 | 2010 | RT | 27 | 8 | 24 | 10 | 16 | 48 | 41 |
| **Other STs** | **8** | **9.6** | **7** | **4[a]** | | | | | | | | |
| ST31 | 1 | 1.2 | 1 | 1993 | RT | 3 | 19 | 13 | 9 | 12 | 16 | 15 |
| ST58 | 1 | 1.2 | 1 | 2011 | BTF | 21 | 32 | 10 | 7 | 24 | 31 | 29 |
| ST115 | 1 | 1.2 | 1 | 2009 | BTF | 30 | 28 | 8 | 15 | 50 | 5 | 3 |
| ST117 | 1 | 1.2 | 1 | 2009 | BTF | 31 | 46 | 25 | 10 | 33 | 50 | 14 |
| ST119 | 1 | 1.2 | 1 | 2009 | BTF | 18 | 47 | 8 | 3 | 34 | 3 | 11 |
| ST211 | 1 | 1.2 | 1 | 2011 | RT | 27 | 58 | 28 | 10 | 33 | 48 | 49 |
| ST213 | 1 | 1.2 | 1 | 1993 | RT | 15 | 14 | 9 | 10 | 6 | 47 | 49 |
| ST215 | 1 | 1.2 | 1 | 1999 | RT | 21 | 3 | 22 | 3 | 3 | 3 | 2 |

[a]Total number of years when infection by a given CC was detected

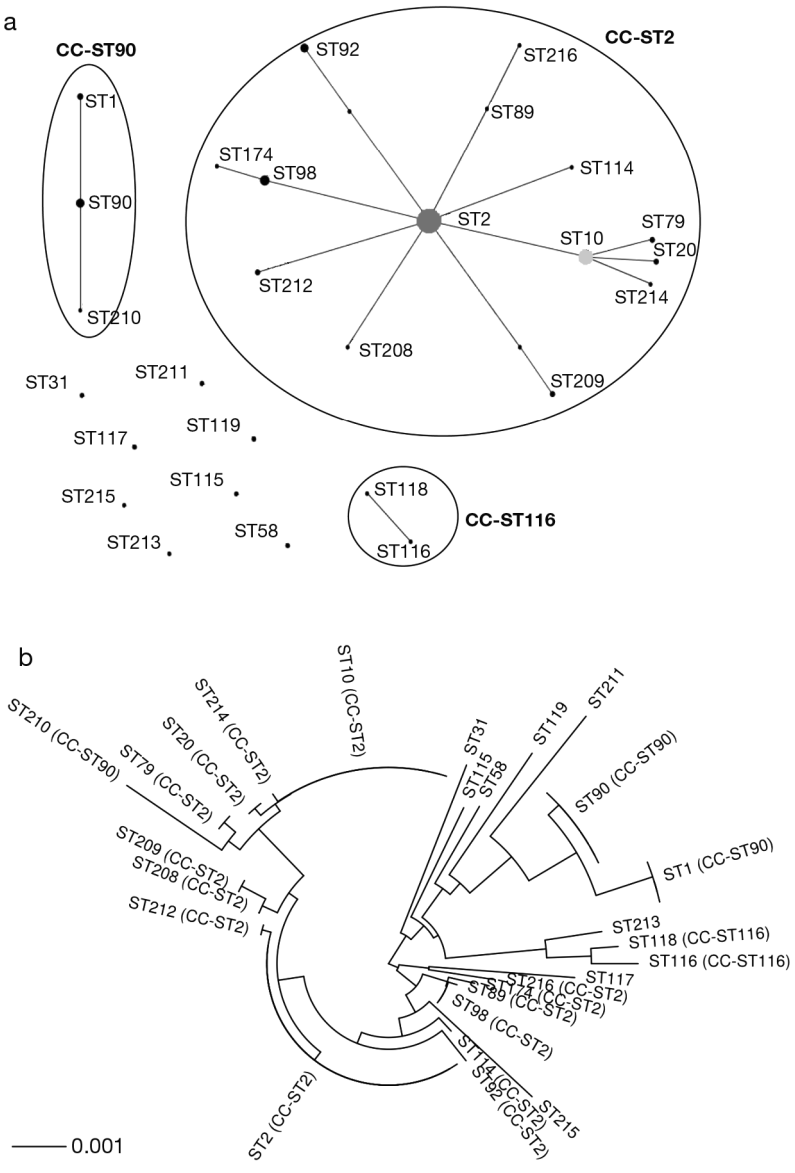


Fig. 1. *Flavobacterium psychrophilum*. Relationships between isolates. (a) eBurst diagram showing links between the sequence types (STs) and indicating the presence of 3 clonal complexes (CCs). Each step represents 1 allele type (AT) difference from the linked ST. The intermediate isolates between ST2 and ST92 or between ST2 and ST209 have not been identified but were nevertheless introduced in the diagram as a potential link to the founder ST2. (b) Neighbor-joining tree summarizing the nucleotide divergence between the genotypes. The scale bar corresponds to an average divergence of 0.001 nucleotide changes per site

SLVs of ST10 and DLVs of ST2). It was not possible to link ST209 and ST92 directly with other STs, but both STs are DLVs of ST2 so they are also connected to CC-ST2 (Fig. 1). In the other 2 CCs, it was not possible to determine the founder STs due to low abundances: CC-ST90 (ST1, ST90, ST210) and CC-ST116 (St116 and ST118) were represented only by 10 and 2 isolates, respectively. Eight other STs (ST31, ST58, ST115, ST117, ST119, ST211, ST213, ST 215) were isolated only once each and did not cluster with any other ST or CC (Fig. 1).

The 64 strains belonging to CC-ST2 represent 76.1% of all strains causing outbreaks in 25 of 29 fish farms (89.3%; Table 2) over 14 yr. CC-ST90 caused fewer outbreaks (10 outbreaks [11.9%] in 8 fish farms over 6 yr) than CC-ST2, but only 3 different STs are members of this complex. CC-ST116 caused 2 outbreaks, each in a different year and also in different fish farms.

Most of the outbreaks we studied were apparently caused by isolates that cluster within CC-ST2, CC-ST90, and CC-ST116. These clones are present all over the country with the exception of 2 fish farms where no evidence of infection by these types was detected (Fig. 2) and the outbreaks (1 in each fish farm) were caused by singletons (ST58 and ST115 during 2011 and 2009) not related to any other strain.

Our data set includes 67 *Oncorhynchus mykiss*, 16 *Salmo trutta* (13 *S. t. fario* and 3 *S. t. lacustris*) and 1 char *Salvelinus* sp. Fisher exact test (based on STs) and AMOVA (based on nucleotide divergence) did not allow identifying a statistically significant association between genotype and host fish species. In contrast, the frequency of the STs evolved over time. The association between genotype and sampling date was highly significant (Fisher exact test, $p = 5.3 \times 10^{-6}$) when the isolates were pooled in 2 groups according to their sampling year (46 isolates collected in the period 1993–2003 versus 38 isolates collected in the period 2009–2012; none of the isolates was collected between 2004 and 2008). Although the fraction of variance linked to sampling date as computed by the AMOVA was weak (8.8% when isolates were grouped by years, 8.3% when isolates were grouped in 2 sampling periods), permutation analyses indicated that the associ-

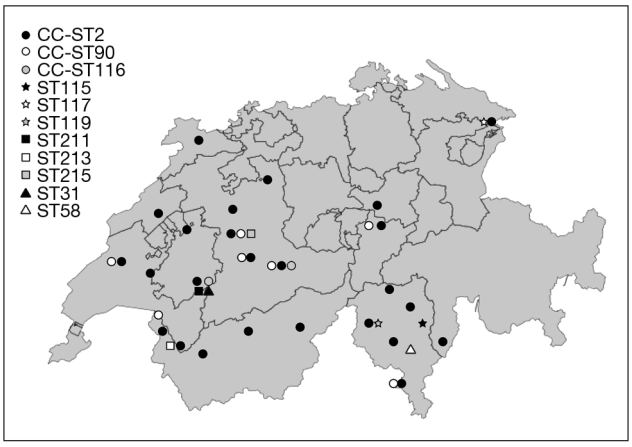


Fig. 2. *Flavobacterium psychrophilum*. Geographical distribution of clonal complexes (CCs) and sequence types (STs) in the 29 fish farms sampled in Switzerland. Adjacent spots designate different sequence types collected from the same fish farm

ation was statistically significant. Furthermore, the association between genotype and sampling period as measured by Fisher exact test and AMOVA remained higher than expected ($p < 0.005$) even when only genotypes from a same host fish and from a same fish farm were permuted, indicating that the observed evolution of genotype frequencies cannot be explained by simple differences in the origin of the isolates. Closer examination revealed that evolution was marked for 3 STs: ST90 was sampled 6 times after 2009 but never before 2003; ST10 was recorded 11 times before 2009 but only once after 2009; ST98 was sampled 5 times in 2012 but never before.

## DISCUSSION

Fish from Swiss fish farms appear to be colonized mainly by 2 large CCs, with CC-ST2 being the most frequent. Most strains within CC-ST2 belong to ST2 (n = 32) and ST10 (n = 12) and are widely distributed throughout Switzerland. These 2 dominating STs are apparently also common in other countries: ST2 strains have already been reported in Europe (Nicolas et al. 2008, Siekoula-Nguedia et al. 2012), and ST10 strains were identified both in Europe and in Oregon, USA (Nicolas et al. 2008). Here we refer to the CC of ST2 and ST10 as a part of CC-ST2, whereas Nicolas et al. (2008) called it CC1. These 2 sequence types (ST2 and ST10) have probably given rise to other pathogenic STs found in Switzerland and, together with ST90 (n = 6), have caused repeated outbreaks in different years. ST2 is probably the founder ST for the large CC-ST2 present in Switzerland; it is possible, however, that ST2 is only a subfounder due to the sampling bias deriving from different sampling strategies used in the 2 periods under study (1993–2008 and 2009–2012).

Eighteen different STs were present as singletons in 18 different fish farms (Table 2). One would thus tend to conclude that different STs may cause isolated outbreaks: when CCs are considered, however, 7 of the 18 ST singletons (39%) belonged to CC-ST2 (Fig. 1) and could easily be found where ST2 or another member of this complex was present. Two of them, ST116 and ST118, formed CC-ST116, and ST210 was linked to CC-ST90. The remaining 8 STs were not linked to any other CC present in our country and could be considered sporadic.

Siekoula-Nguedia et al. (2012) obtained results similar to ours studying *Flavobacterium psychrophilum* isolated from rainbow trout in France near the Atlantic coast. Most of the French STs belonged to CC-ST2 and were represented by several STs, with ST2 being the most prevalent. ST2 and other STs in the CC-ST2 (e.g. ST20, ST89, ST92, ST98) detected in France are also present in Switzerland. ST90 was also detected but not its SLVs ST1 and ST210, whereas a DLV (ST108) was reported from France but not Switzerland. Sporadic STs that do not belong to major CCs and were isolated only once were also found and differed from those reported in our study.

Most of the flavobacteriosis outbreaks in Switzerland were caused by strains belonging to CC-ST2 (76.1%) and represented by 14 STs. The widely distributed CC-ST2 as well as CC-ST90, the second most frequent CC in Switzerland, could correspond to highly pathogenic isolates that are able either to survive locally in the fish farms and cause outbreaks when conditions become favorable to the pathogen or may be repeatedly imported through the fish or egg trade. Our sporadic STs appeared only once in rainbow trout or brown trout. We hypothesize that these isolates reflect the local diversity present in the environment; these strains may not be able to survive for a long time in the fish farm or to spread between fish farms. However, they could act as genetic pools for new STs generated by homologous recombination (Nicolas et al. 2008, Siekoula-Nguedia et al. 2012).

Swiss fish farms are tightly interconnected, as some of them exchange eggs, small fry, and fish each year. These connections could play an important role in spreading the disease, as *Flavobacterium psychrophilum* distribution within the country is not related

to barriers such as mountain ridges or river basins. In contrast to a previously published investigation (Nicolas et al. 2008) describing a relationship between STs and host fish species in a worldwide study, we observed infection by the same ST (and different STs within the same CC) in different fish species (e.g. ST2, ST10, ST98, and ST90 in rainbow trout, brown trout, and char). Our results therefore do not suggest any strong association between STs or CCs and host fish species in Swiss farms and indicate that, at least in certain circumstances, CC-ST2 and CC-ST90 can have a broader host range than previously reported, encompassing members of the genera *Oncorhynchus* and *Salmo*. Additional studies are needed to understand the contributions of host specificity and transmission factors to relationships between CCs and fish species observed elsewhere.

Of note, temporal analysis revealed that the frequency of the genotypes evolved over years, and this observation is apparently robust with respect to the evolution of the origin of our isolates in terms of fish farm and host fish species. It would be interesting to see whether similar temporal trends can be observed in other geographical areas. In the future, more data might shed light on the mechanisms that could underlie these changes, such as random drift and antibiotic selection.

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## LITERATURE CITED

Baldo L, Dunning Hotopp JC, Jolley KA, Bordenstein SR and others (2006) Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Appl Environ Microbiol 72:7098–7110

Barnes ME, Brown ML (2011) A review of *Flavobacterium psychrophilum* biology, clinical signs, and bacterial cold water disease prevention and treatment. Open Fish Sci J 4:1–9

Bastardo A, Ravelo C, Romalde JL (2012) Multilocus sequence typing reveals high genetic diversity and epidemic population structure for the fish pathogen *Yersinia ruckeri*. Environ Microbiol 14:1888–1897

Bernardet JF, Kerouault B (1989) Phenotypic and genomic studies of '*Cytophaga psychrophila*' isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. Appl Environ Microbiol 55:1796–1800

Borg AF (1948) Studies on myxobacteria associated with diseases in salmonid fishes. PhD dissertation, University of Washington, Seattle, WA

Cipriano RC, Ford LA, Teska JD (1995) Association of *Cytophaga psychrophila* with mortality among eyed eggs of Atlantic salmon (*Salmo salar*). J Wildl Dis 31: 166–171

Duchaud E, Boussaha M, Loux V, Bernardet JF and others (2007) Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. Nat Biotechnol 25: 763–769

Ekman E, Börjeson H, Johansson N (1999) *Flavobacterium psychrophilum* in Baltic salmon *Salmo salar* brood fish and their offspring. Dis Aquat Org 37:159–163

Ewing B, Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res 8:186–194

Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 8:175–185

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 186: 1518–1530

Felsenstein J (2004) PHYLIP (Phylogeny Inference Package) version 3.6. Department of Genome Sciences, University of Washington, Seattle, WA. Available at http://evolution.gs.washington.edu/phylip.html

Gordon D, Abajian C, Green P (1998) Consed: a graphical tool for sequence finishing. Genome Res 8:195–202

Gordon D, Desmarais C, Green P (2001) Automated finishing with autofinish. Genome Res 11:614–625

Hesami S, Allen KJ, Metcalf D, Ostland VE, MacInnes JI, Lumsden JS (2008) Phenotypic and genotypic analysis of *Flavobacterium psychrophilum* isolates from Ontario salmonids with bacterial coldwater disease. Can J Microbiol 54:619–629

Kim JH, Gomez DK, Nakai T, Park SC (2010) Plasmid profiling of *Flavobacterium psychrophilum* isolates from ayu (*Plecoglossus altivelis altivelis*) and other fish species in Japan. J Vet Sci 11:85–87

Kondo M, Kawai K, Okabe M, Nakano N, Oshima S (2003) Efficacy of oral vaccine against bacterial coldwater disease in ayu *Plecoglossus altivelis*. Dis Aquat Org 55:261–264

Lane DJ (1991) 16S/23S rRNA sequencing. Wiley & Sons, Chichester

Madetoja J, Dalsgaard I, Wiklund T (2002) Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. Dis Aquat Org 52:109–118

Maiden MC, Bygraves JA, Feil E, Morelli G and others (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95: 3140–3145

Martino ME, Fasolato L, Montemurro F, Rosteghin M and others (2011) Determination of microbial diversity of *Aeromonas* strains on the basis of multilocus sequence typing, phenotype, and presence of putative virulence genes. Appl Environ Microbiol 77:4986–5000

Nematollahi A, Decostere A, Pasmans F, Haesebrouck F (2003) *Flavobacterium psychrophilum* infections in salmonid fish. J Fish Dis 26:563–574

Nicolas P, Mondot S, Achaz G, Bouchenot C, Bernardet JF, Duchaud E (2008) Population structure of the fish-pathogenic bacterium *Flavobacterium psychrophilum.* Appl Environ Microbiol 74:3702–3709

Osorio J, Carvajal A, Naharro G, La T, Phillips ND, Rubio P, Hampson DJ (2012) Dissemination of clonal groups of *Brachyspira hyodysenteriae* amongst pig farms in Spain, and their relationships to isolates from other countries. PLoS ONE 7:e39082

Peterson AE, Davis MF, Julian KG, Awantang G and others (2012) Molecular and phenotypic characteristics of healthcare- and community-associated methicillin-resistant *Staphylococcus aureus* at a rural hospital. PLoS ONE 7:e38354

R Development Core Team (2011) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. Available at www.r-project.org

Siekoula-Nguedia C, Blanc G, Duchaud E, Calvez S (2012) Genetic diversity of *Flavobacterium psychrophilum* isolated from rainbow trout in France: predominance of a clonal complex. Vet Microbiol

Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ (2004) Displaying the relatedness among isolates of bacterial species — the eBURST approach. FEMS Microbiol Lett 241:129–134

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