



Characteristics of epidemic and sporadic *Flavobacterium psychrophilum* sequence types



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ABSTRACT

Population genetic analysis of *Flavobacterium psychrophilum* using multilocus sequence typing (MLST) data has identified a genetic lineage consisting of sequence types (STs) associated with epidemic spread and high mortality in farmed rainbow trout (*Oncorhynchus mykiss*). To identify phenotypic characteristics that discriminate epidemic *F. psychrophilum* STs from sporadically occurring STs, a set of traits of isolates/STs ($n = 32$) with distinct genetic background and clinical impact was studied in vitro. The results showed that epidemic and sporadic STs could not be discriminated by their resistance to povidone-iodine or iron starvation or by their ability to hydrolyze elastin and gelatin. Antimicrobial resistance against flumequine, oxolinic acid and oxytetracycline was significantly more prevalent ($p < 0.05$), although not a prerequisite, in epidemic STs. The two groups showed equal biofilm forming abilities, while the epidemic STs were associated with higher adherence ($p < 0.05$) indicating that adhesion and antimicrobial resistance could be factors contributing to the epidemic potential of *F. psychrophilum*. All isolates/STs of the largest clonal complex CC-ST2 were distinguished from others by the bacterial insertion sequence IS256 in the collagenase encoding gene. IS256 might thus constitute a novel molecular marker for rapid differentiation of epidemiologically important CC-ST2 genotypes.

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1. Introduction

Since a standardized multilocus sequence typing (MLST) scheme based on the sequences of seven loci was released for the fish pathogen *Flavobacterium psychrophilum* (Nicolas et al., 2008), several studies have implemented the method for genetic typing of isolates from diverse origins (Apablaza et al., 2013; Avendaño-Herrera et al., 2014; Fujiwara-Nagata et al., 2013; Nilsen et al., 2014; Siekoula-Nguedia et al., 2012; Strepparava et al., 2013). A web-accessible MLST database (<http://pubmlst.org/fpsychrophilum/>) has also been established which allows comparable typing between laboratories. Population genetic studies of *F. psychrophilum* using MLST data have unambiguously supported the hypothesis of an epidemic population structure where successful clonal complexes (CCs) have expanded within a generally recombinant bacterial population (Nicolas et al., 2008; Nilsen et al., 2014; Siekoula-Nguedia et al., 2012).

Common to previously published MLST studies of *F. psychrophilum* is the identification of a large globally spread CC, hereafter referred to as CC-ST2 according to its currently predicted ancestral sequence type (ST), with an apparent affinity to the rainbow trout (*Oncorhynchus mykiss*) host. STs within CC-ST2 have been responsible for severe bacterial cold water disease (BCWD) outbreaks in many European countries for several consecutive years (Nilsen et al., 2014; Siekoula-Nguedia

et al., 2012; Strepparava et al., 2013). CCs genetically distinct from CC-ST2 have been associated with repeated disease outbreaks in Atlantic salmon (*Salmo salar*) in Norway (Nilsen et al., 2014) and in ayu (*Plecoglossus altivelis altivelis*) in Japan (Fujiwara-Nagata et al., 2013), suggesting either geographical restriction or host adaptation within certain clonal groups. More sporadically occurring *F. psychrophilum* STs appearing as singletons or minor CCs are usually isolated from a broader host range and are considered to be of a lesser clinical impact (Nilsen et al., 2014; Siekoula-Nguedia et al., 2012). The reason for the epidemiological success of the putatively virulent CC-ST2 is not known, but its worldwide distribution has been associated with the international trade of fish and associated products (Avendaño-Herrera et al., 2014; Fujiwara-Nagata et al., 2013; Nicolas et al., 2008; Nilsen et al., 2014). Evidence has linked the rapid increase in *F. psychrophilum* infections in Chile and Norway to a recent introduction of epidemic CC-ST2 genotypes, particularly to ST2 (Avendaño-Herrera et al., 2014; Nilsen et al., 2014). Once established, these epidemiologically important genotypes can become a long term endemic problem.

The global distribution and dominance of CC-ST2 might be due to changes in properties determining the survival of *F. psychrophilum* in the aquatic environment or in the rainbow trout host. In aquaculture environments, where antimicrobials are commonly used, inherent resistance or an enhanced ability to form biofilms (Álvarez et al., 2006; Sundell and Wiklund, 2011) could provide a selective advantage over susceptible *F. psychrophilum* populations. In host-pathogen interactions, the ability to obtain iron (Álvarez et al., 2008) and to

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adhere to (Nematollahi et al., 2003) and degrade host tissue (Duchaud et al., 2007; Madsen and Dalsgaard, 1998) could be crucial factors for the outcome of BCWD.

The main objective of this study was to identify discriminative phenotypic characteristics for *F. psychrophilum* STs of the geographically most widely spread genetic lineage. For this purpose, we compared the ability of *F. psychrophilum* STs with distinct genetic background and clinical impact to resist antimicrobial agents and iron starvation, to adhere and to form biofilms and to hydrolyze elastin and gelatin. In addition, we studied the potential of the bacterial insertion sequence IS256 as a molecular marker for rapid differentiation of epidemiologically important *F. psychrophilum* genotypes.

2. Materials and methods

2.1. eBURST analysis of the global *F. psychrophilum* MLST database

The complete allelic profile list from the *F. psychrophilum* MLST database (<http://pubmlst.org/fpsychrophilum/>) was downloaded (accessed on 28 March 2014) and a population snapshot of the 995 isolates and 166 STs was created using the eBURSTv3 algorithm (Fig. 1). CCs were identified based on sharing 5 (relaxed) or 6 (default) out of 7 alleles using standard eBURST methodology (<http://eburst.mlst.net/>).

2.2. Selection of bacterial isolates and culture conditions

Thirty-two *F. psychrophilum* isolates representing 16 epidemic and 16 sporadic STs (Table 1) were selected from the MLST database based on eBURST grouping (Fig. 1). Epidemic STs were genetically associated with the globally spread CC-ST2 (Fig. 1) and were considered part of the same lineage whereas sporadic STs consisted of singletons and minor CCs genetically distinct from CC-ST2. The colony morphology of *F. psychrophilum* is either rough or smooth (Högfors-Rönholm and Wiklund, 2010) when grown on tryptone yeast extract salts (TYES) (Holt et al., 1993) agar. The rough and smooth *F. psychrophilum* morphotypes have been shown to express different phenotypic characteristics in vitro (Högfors-Rönholm, 2014). Hence, only the smooth morphotype was selected because of its higher prevalence within BCWD outbreaks (Sundell et al., 2013). Although none of several

screened ST2 isolates showed typical smooth morphological characteristics, this genotype was included in the study because of its significance and predominance in the global MLST database. All isolates were grown on TYES agar for 5 days at 15 °C prior to testing. An approximate 10^9 CFU mL⁻¹ stock culture of each *F. psychrophilum* isolate was prepared by inoculating 5-day-old colonies from TYES agar into TYES broth to an optical density of 1.0 at 520 nm (Unicam, HeXios β) and maintained at -70 °C. Dilutions from the stock cultures were used in all analyses unless otherwise stated.

2.3. Minimum inhibitory concentration (MIC) testing

Antimicrobial resistance of the isolates was tested by measuring the MIC of the aquaculture drugs flumequine (FLU), oxolinic acid (OXA) and oxytetracycline (OTC) by broth microdilution in round bottomed 96-well microtiter plates (Nunc). FLU and OTC were obtained from Sigma-Aldrich and OXA from Santa Cruz Biotechnology Inc. In addition we tested the MIC of Betadine® (Leiras Takeda), a povidone-iodine based chemical used for fish egg surface disinfection. The MIC of the iron chelator 2,2'-Dipyridyl (DPD), obtained from Sigma-Aldrich, was used for studying the capability of epidemic and sporadic *F. psychrophilum* STs to resist iron starvation.

Antimicrobial stock solutions (1000 µg mL⁻¹) of FLU, OXA and OTC were prepared by dissolving in 0.1 M NaOH, 1 M NaOH and methanol respectively and making up to volume with TYES broth. Approximately 10^5 CFU of each isolate was exposed to triplicate two-fold serial dilutions of FLU, OTC and OXA ranging from 64 to 0.125 µg mL⁻¹. The concentrations of Betadine® and DPD diluted in TYES broth were 1000, 800, 600, 400, 200, 100 µg mL⁻¹ and 31.2, 27.3, 23.4, 19.5, 15.6, 11.7, 7.8, 3.9 µg mL⁻¹, respectively. TYES broth was used as negative control for each compound. After static incubation for 96 h at 15 °C, the MIC of the antimicrobial was interpreted as the lowest concentration that prevented visible growth. Because of the slow bacterial growth in iron restricted media the MIC of DPD was determined after 10 days of incubation. To separate fully susceptible wild-type isolates from those with a decreased susceptibility, microbiological criteria were used to define resistance (EUCAST, 2000; Kahlmeter et al., 2003). A tentative wild-type cut-off value (CO_{WT}) was visually assessed from the MIC distribution as the modal MIC ± 1 twofold dilution (the inherent

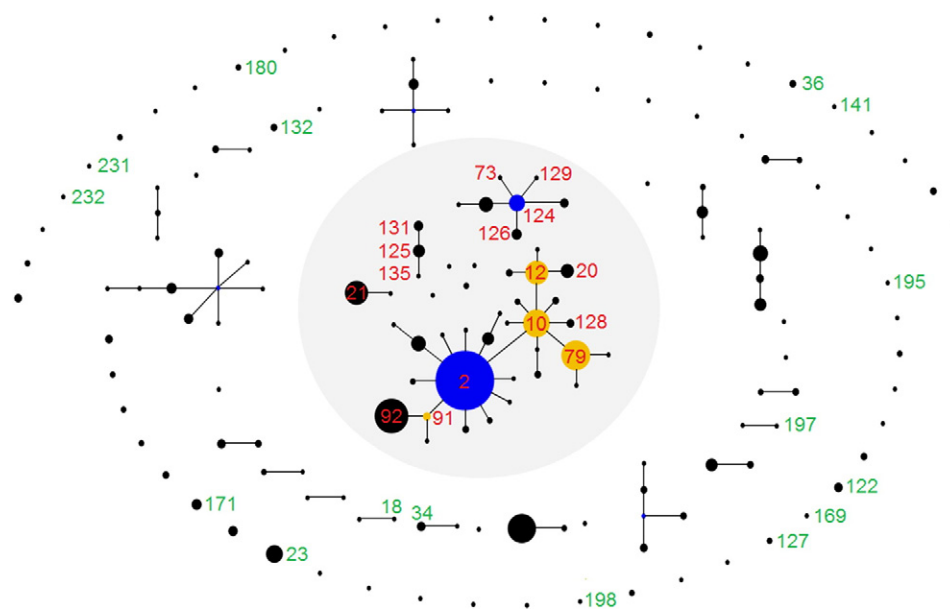


Fig. 1. Population snapshot of the *F. psychrophilum* MLST database with default eBURSTv3 settings (accessed on 28 March 2014). Each dot represents an individual ST and dot size reflects its abundance. Linked clusters represent CCs, singleton STs are unlinked. Clonal founders are shown in blue, subgroup founders in yellow. CCs and STs within the shaded area are linked with the use of a relaxed group definition. Epidemic STs selected for this study are indicated with red numbers, sporadic with green respectively.

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