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Introduction, expansion and coexistence of epidemic *Flavobacterium psychrophilum* lineages in Chilean fish farms

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ABSTRACT

Chile is one of the countries where the development of salmonid farming has been the most successful. The first importation of salmonids in Chile from the northern hemisphere dates back to the late 19th century and the country now ranks as the world second largest producer of farmed salmon. However, the fast increase of infections caused by the bacterium *Flavobacterium psychrophilum* is a growing concern for this local industry. This pathogen, also recognized as an important problem worldwide, has been first reported in Chile in 1993 and is currently affecting all three cultivated salmonid species: Atlantic salmon (*Salmo salar*), Coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*O. mykiss*). Here we conducted a MLST (multi-locus sequence typing) analysis of the local genetic diversity of *F. psychrophilum* to better understand its origin and propagation in the country, and to suggest practices that could contribute to its control in the future. A total of 94 bacterial isolates, collected from the main production zones, were analyzed and compared to those of other origins already available. The data reveal the country-wide distribution of several genotypes closely related to those that are the most prevalent in European and North American fish farms, and overlapping host fish species of the different lineages. This population structure is probably the direct consequence of local fish farming practices that relied until recently on massive import of fish eggs (e.g., 78 million of eggs in 2012) and where mixed-species farms and fish transportation across the country are common.

1. Introduction

Chile is currently the second largest producer of farmed salmon in the world (Ibieta et al., 2011) despite the absence of native population of salmonids in the Southern hemisphere. All salmon and trout species found in different water bodies (rivers, lakes, fjords and sea) of the country have been imported for aquaculture purpose.

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It has been estimated that Chile has bought over the years about two billion of salmonid eggs from various countries and continents of the Northern hemisphere, especially USA, Norway, Scotland, Denmark and Finland (Ibieta et al., 2011), since the first import of eggs in 1885 (Bluth et al., 2003). Besides the absence of native salmonid species and the over 80% importation of eggs from foreign sources through the 1990s, several other aspects also contribute to distinguish salmonid farming in Chile from the rest of the world. In particular, whereas in other salmonid farming countries facilities are generally dedicated to the production of a unique species, many Chilean fish farms grow different salmonid species (i.e., rainbow trout, Atlantic salmon and Coho salmon). Moreover, specialized production zones have emerged (i.e., for fry and juveniles; for smolts and adults; for broodstock and hatching) to optimize the use of the different water bodies. As a consequence, eggs and live fish need to be moved across the country to complete their full life cycle (Rosefield and Manley, 2010).

Flavobacterium psychrophilum (Bernardet et al., 1996), a Gram-negative, filamentous, psychrotrophic bacterium belonging to the phylum *Bacteroidetes*, is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS) in freshwater salmonid fish worldwide (see review Nematollahi et al., 2003; Barnes and Brown, 2011). In Chile, this condition is observed in freshwater aquaculture facilities since 1993 and the incidence of *F. psychrophilum* has dramatically increased since then (Bustos et al., 1995; Avendaño-Herrera et al., 2009). The bacterium is now routinely isolated from rainbow trout and Atlantic salmon raised in open and closed flow systems as well as from lake fish cultures; a total of 1937 cases were reported by the diagnostic laboratories between 2005 and 2010 (Godoy and Avendaño-Herrera, 2012). According to the Chilean National Fishing Service and Aquaculture (SERNAPESCA), the bacterium is responsible for significant to high mortality rates (5–70%) in fingerlings, resulting in economic losses which rank second after those caused by *Piscirickettsia salmonis* (Valdebenito and Avendaño-Herrera, 2009). Importantly, *F. psychrophilum* infections in Chile are usually chronic because the water temperature in fish freshwater farms rarely exceed 14 °C and is therefore particularly appropriate for the development of the pathogen. To date, antimicrobial therapies represent the only recourse to control the condition in farmed fish: it has been estimated that 55 tons of florfenicol and 55 tons of oxytetracycline were applied at the Chilean farms to control outbreaks between 2006 and 2009 (Henríquez-Núñez et al., 2012).

Despite the impact of *F. psychrophilum* infections in Chile, relatively little is known about its genetic diversity in the country. This knowledge would be useful for understanding epidemiological factors associated with the bacterium (i.e., host species, geographical distribution, virulence), in order to define appropriate management strategies to minimize the risks of pathogen introduction or transmission. Valdebenito and Avendaño-Herrera (2009) using RAPD, 16S rRNA alleles and repetitive extragenic palindromic PCR (REP-PCR) observed a relative genetic homogeneity using 20 Chilean *F. psychrophilum* strains isolated from farmed Atlantic salmon and rainbow trout. Other molecular techniques have been used to study the genetic variability of this species but only a few Chilean isolates have been included (Chakroun et al., 1998; Soule et al., 2005; Ramsrud et al., 2007; Nicolás et al., 2008). Among these approaches, multi-locus sequence typing (MLST) is currently regarded as the gold standard for molecular typing of many bacterial species, including *F. psychrophilum* (Nicolás et al., 2008; Siekoula-Nguedia et al., 2012; Fujiwara-Nagata et al., 2013; Strepparava et al., 2013). Recently, Apablaza et al. (2013) reported MLST sequence data for 25 Chilean *F. psychrophilum* isolates obtained from 2006 to 2010, showing some genotypical links with isolates from Europe and North America. However, the MLST scheme used in this latter study did not fit the standards of the other *F. psychrophilum* MLST studies, hampering comparisons with previously published and future data.

Here, we subjected a large collection of *F. psychrophilum* isolates retrieved from farms located in the main Chilean areas of salmonid production to the MLST scheme of the previous studies (Nicolás et al., 2008; Siekoula-Nguedia et al., 2012; Fujiwara-Nagata et al., 2013; Strepparava et al., 2013). This allowed to analyze genetic diversity and the comparison of the genotypes to those found in other countries.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Only 3 *F. psychrophilum* isolates from Chilean origin were included in Nicolás et al. (2008). In this study, we genotyped a total of 91 additional isolates. This collection consists of isolates that were retrieved by the authors either from fish (rainbow trout, Atlantic salmon and Coho salmon) directly sampled in the field or from fish sent to diagnostic laboratories; most fish presented typical clinical signs of BCWD or RTFS. The bacterium was obtained from internal organs (kidney or spleen), or less frequently, from external organs (gill, fin and skin). The identity of each isolate was confirmed as *F. psychrophilum* by standard phenotyping procedures (Bernardet et al., 2002): colony morphology and pigmentation, cell morphology, gliding motility, Gram-staining, cytochrome oxidase and catalase activities, oxidation/fermentation reactions, presence of cell wall-associated flexirubin type pigments and absorption of Congo red.

The *F. psychrophilum* type strain ATCC 49418^T was included as a positive control in all analyses. The bacteria were grown on TYES agar plates (tryptone yeast extract salts medium, consisting of: 0.4% tryptone, 0.05% yeast extract, 0.02% anhydrous calcium chloride, 0.05% magnesium sulphate heptahydrate and 1.2% agar, pH 7.2) and incubated aerobically at 15 °C for 3–5 days. Stock cultures were maintained frozen at −80 °C in Cryobille tubes (AES Laboratory) or in TYES with 15% glycerol.

2.2. DNA extraction and confirmation of the bacterium species

Chromosomal DNA was extracted using InstaGene Matrix (Bio-Rad) following the manufacturer's recommendations.