Stress Tolerance-Related Genetic Traits of Fish Pathogen Flavobacterium psychrophilum in a Mature Biofilm

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Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease and rainbow trout fry syndrome, and hence this bacterium is placed among the most important salmonid pathogens in the freshwater aquaculture industry. Since bacteria in biofilms differ substantially from free-living counterparts, this study sought to find the main differences in gene expression between sessile and planktonic states of F. psychrophilum LM-02-Fp and NCMB1947T, with focus on stress-related changes in gene expression occurring during biofilm formation. To this end, biofilm and planktonic samples were analyzed by RNA sequencing to detect differentially expressed candidate genes (DECGs) between the two growth states, and decreasing the effects of interstrain variation by considering only genes with $\log_2$-fold changes $\leq -2$ and $\geq 2$ at $P_{adj}$-values $\leq 0.001$ as DECGs. Overall, 349 genes accounting for $\sim 15\%$ of total number of genes expressed in transcriptomes of F. psychrophilum (n = 2327) were DECGs between biofilm and planktonic states. Approximately 83 and 81% of all up- and down-regulated candidate genes in mature biofilms, respectively, were assigned to at least one gene ontology term; these were primarily associated with the molecular function term “catalytic activity.” We detected a potential stress response in mature biofilms, characterized by a generalized down-regulation of DECGs with roles in the protein synthesis machinery (n = 63, primarily ribosomal proteins) and energy conservation (seven ATP synthase subunit genes), as well as an up-regulation of DECGs involved in DNA repair ($rurC$, $recO$, $phrB1$, $smf$, and $dnaQ$) and oxidative stress response (cytochrome C peroxidase, probable peroxiredoxin, and a probable thioredoxin). These results support the idea of a strategic trade-off between growth-related processes and cell homeostasis to preserve biofilm structure and metabolic functioning. In addition, LDH-based cytotoxicity assays and an intraperitoneal challenge model for rainbow trout fry agreed with the transcriptomic evidence that the ability of F. psychrophilum to form biofilms could contribute to the virulence. Finally, the reported changes in gene expression, as induced by the plankton-to-biofilm transition, represent the first transcriptomic guideline to obtain insights into the F. psychrophilum biofilm lifestyle that could help understand the prevalence of this bacterium in aquaculture settings.

Keywords: Flavobacterium psychrophilum, fish pathogen, gene expression, RNA sequencing, planktonic cells, sessile cells, stress response
INTRODUCTION

*Flavobacterium psychrophilum* is the infectious freshwater bacterium (Nilsen et al., 2011) responsible for bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS) in aquaculture settings (Starliper, 2011). Additionally, this bacterium can infect a variety of non-salmonid freshwater fish (Sudheesh et al., 2012). Before the clinical onset of disease, *F. psychrophilum* can be detected by DNA-based real time-PCR in fish tank water rather than inlet water, being in turn undetectable through classical culture-based methods (Strepparava et al., 2014). Therefore, fish tanks can be an important risk factor (Madetoja et al., 2002) for bacterial replication and persistence in aquaculture settings, for instance, allowing biofilm establishment in different interfaces (e.g., solid-liquid, air-liquid). Consequently, when *F. psychrophilum* moves from a free-living state to a mature biofilm lifestyle, the bacterium may acquire emergent properties (Flemming et al., 2016) that favor persistence in an aquaculture setting, as well as infection recurrence (Sundell and Wiklund, 2011) in salmonids after biofilm-released cells return to the surrounding water. This idea is supported by a recent report on a coherent set of differentially expressed virulence- and biofilm-related genes between sessile and free-living cells, with most of these genes being significantly up-regulated in mature *F. psychrophilum* biofilms (Levipan and Avendaño-Herrera, 2017).

However, the study of mature bacterial biofilms is technically complex. For example, RNA degradation is a major barrier for gene expression analysis in biofilm cells using certain RNA-based approaches, such as RNA sequencing (RNA-seq). This is due to that mature biofilms are mainly composed of cells over the entire range of physiological states, from highly active to dormant and even dead (Flemming and Wingender, 2010; Flemming et al., 2016). The wide spectrum of metabolic states in biofilm cells is probably due to the influence of diverse stressor agents that mature biofilms often experience, thereby contributing to the development of enhanced stress resistance among sessile cells (Lee et al., 2014). Thus, conclusions derived from experimental designs that ensure optimal RNA-sample quality may not be representative of in-situ properties of some biological systems (Romero et al., 2014) such as bacterial mature biofilms. Nonetheless, because of the high sensitivity in transcript detection and a relatively low cost, RNA-seq technologies have been progressively adopted in biofilm research (Tan et al., 2015; Yan et al., 2017), providing high-quality data for analysis of gene-gene interactions based on noncoding RNAs, protein-coding genes, and even novel transcriptionally active sites (Nobile et al., 2012; Castro et al., 2017).

Interestingly, no stress-related gene expression pattern in *F. psychrophilum* biofilms has been reported hitherto, which in turn may not be easily discernible solely from the study of sessile cells. The present study identified 349 differentially expressed candidate genes (DECGs) between biofilm and planktonic growth states of *F. psychrophilum* LM-02-Fp and NCMB1947\textsuperscript{T} using a RNA-seq approach. A potential stress-resistance pattern in mature *F. psychrophilum* biofilms was found, as characterized by an important down-regulation of a gene repertory encoding for ribosomal proteins, transcriptional factors, different subunits of DNA-directed RNA polymerase, translation factors, elongation factors, electron carriers, and subunits of ATP synthase. In addition, an important up-regulation of genes responsible for DNA repair and some genes associated with the oxidative stress response was detected in sessile cells. Finally, the obtained results represent an initial baseline for future research on the biofilm lifestyle in *F. psychrophilum*.

MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions**

Strains LM-02-Fp and NCMB1947\textsuperscript{T} were confirmed as *F. psychrophilum* through standard phenotyping and 16S rDNA-based PCR as previously described (Urdaci et al., 1998; Bernardet et al., 2002). The two strains were routinely cultured with agitation (150 rpm) at 17 ± 1°C in half-strength TYES medium (Holt et al., 1993), and with not more than two subcultures grown from glycerol-amended stock cultures (15%, final concentration) and stored at −80°C. Briefly, 6-well microtiter plates (flat-bottom TPPR plates, Switzerland) containing a 5-cm\textsuperscript{2} sterile glass slide in every well were inoculated with 1.8 mL of culture to achieve an average density per strain of 1.77 ± 0.46 × 10\textsuperscript{7} CFU cm\textsuperscript{−2} (±SD). Recently, it has been demonstrated experimentally that maximum biofilm formation in these two strains occurs between 96 and 120 h (Levipan and Avendaño-Herrera, 2017) and hence, mature biofilms were allowed to form on glass slides with agitation (40 rpm) at 17 ± 1°C for 96 h. Afterward, 12 glass slides were collected per strain and washed by immersion in chilled sterile milli-Q water (10 s) for subsequent RNA extraction from biofilm cells. These samples were compared with 48-h-old planktonic samples (i.e., late exponential growth stage), since we could not obtain RNA extracts from 96-h-old biofilm supernatants with a RNA integrity number (RIN) adequate for RNA-seq. Planktonic cells from 48-h-old cultures of *F. psychrophilum* LM-02-Fp and NCMB1947\textsuperscript{T} were harvested by centrifugation (5780 RCF) at 4°C for 2.5 min for RNA isolation.

**RNA Extraction and RT-qPCR**

Total RNA was extracted with the TRizol\textsuperscript{®} Max\textsuperscript{TM} Bacterial RNA Isolation Kit (Ambion, Thermo Fisher Scientific, NY, USA) from planktonic and biofilm samples of *F. psychrophilum* LM-02-Fp and NCMB1947\textsuperscript{T}. To do so, planktonic cell pellets and biofilms formed on glass slides were incubated with 1 mL of TRizol reagent for cell lysis, according to the manufacturer’s specifications. Concentrations and preliminary quality (A\textsubscript{260}/A\textsubscript{280} ratio) of extracts were determined using a Nanodrop\textsuperscript{®} ND-1000 spectrophotometer (Thermo Fisher Scientific) and formaldehyde-agarose gel electrophoresis (Rosen and Villa-Komaroff, 1990). Total RNA from four independent experiments was pooled per strain and growth state (biofilm vs. planktonic) at the same final concentration. The RNA integrity numbers of the four resultant RNA pools were determined on an Agilent Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies, CA, USA). Afterward, each pool of total RNA was treated with the TURBO DNA-free\textsuperscript{TM} Kit (Applied Biosystems,
Austin, TX, USA) to be used as a RNA template for subsequent RNA-seq experiments.

Nine DECGs in RNA-seq data (see below) were randomly selected for designing qPCR primers (Supplementary Table 1) using the software Primer3 (version 4.0.0, Rozen and Skaletsky, 2000) and Beacon Designer™ (free edition). Reverse transcription-qPCR (RT-qPCR) assays were conducted with TURBO-treated RNA samples obtained from three independent experiments and following the procedures described elsewhere (Levipan and Avendaño-Herrera, 2017) with two modifications. (1) The complementary DNA (cDNA) was synthetized using random primers from the ImProm-II™ Reverse Transcription System (Promega, Madison, CA, USA). (2) The qPCR program consisted of an initial denaturation (3 min at 95°C) followed by 40 amplification cycles consisting of denaturation at 95°C for 30 s, 1 min annealing at a temperature chosen based on the primer pair to be used (Supplementary Table 1), and 1 min extension at 72°C. The efficiencies and correlation coefficients of standard curves for DECG quantification were 97.99 ± 2.86% and 0.9975 ± 0.0022 (mean ± SD), respectively. qPCR assays were carried out on a Stratagene Mx3000P real-time PCR device (Agilent Technologies-Stratagene) and data were processed with the software MXPro (version 4.10; Agilent Technologies).

RNA-seq and Data Processing

Next-generation sequencing libraries were constructed in the AUSTRAL-omics Laboratory, Universidad Austral de Chile (Valdivia, Chile). Efficient rRNA depletion from each pool of total RNA was only achieved once the probes of three hybridization-based kits (i.e., RiboMinus™ Transcriptome Isolation Kit; Ribo-Zero™ rRNA Removal Kit; and MICROBExpress™ Bacterial mRNA Enrichment Kit) were mixed and used in two rounds of depletion, as per the MICROBExpress™ KIT protocol (Ambion, Thermo Fisher Scientific, NY, USA). Afterward, 16S- and 23S-rRNA-depleted samples were processed for the removal of ssRNAs < 100 nt and dsRNAs < 200 bp using the MEGAclear™ Kit (Ambion). An Agilent Bioanalyzer profile of rRNA-depleted samples confirmed the efficient removal of 16S- and 23S-rRNAs, as well as of small RNAs. Briefly, libraries were generated with the KAPA Stranded mRNA-seq Kit (Kapa Biosystems Inc., MA, USA) and labeled with a barcode for pooled-library sequencing. Library size determinations were performed by running the libraries on a Fragment Analyzer™ System (Advanced Analytical Technologies, IA, USA) and using the DNF-479 Standard Sensitivity NGS Fragment Analysis Kit. Library concentrations were determined on a LightCycler® Nano Real-Time PCR instrument (Roche Diagnostics, GmbH) and using the KAPA Library Quantification Kit (Kapa Biosystems, Inc.) to adjust concentrations to 10 nM. Libraries were pooled in an equimolar ratio using a protocol for denaturing and diluting libraries from the MiSeq System. The pooled libraries were then loaded (at 12 pM with 1% PhiX, final concentration) on an Illumina MiSeq instrument (Illumina, CA, USA) to create paired-end reads (2 x 150 bp) by using MiSeq Chemistry v2.

Technical sequence trimming and quality checking were performed with Trimmomatic (Bolger et al., 2014) and PRINSEQ (Schmieder and Edwards, 2011), respectively. Bases (or sequences) with low quality Phred scores (Phred < 30) were discarded. Genes in the reference genome for *F. psychrophilum* strain JIP02/86 (NCBI accession number NC_009613.3) were extracted using the “gffread” option in BedTools (Quinlan and Hall, 2010), thus generating a transcript FASTA file with all gene coordinates in the genome. The “.bam” files were obtained after mapping high-quality reads against extracted genes using the Burrows-Wheeler Alignment Tool (Li and Durbin, 2009). The number of sequence reads per gene for each “.bam” file was computed using the FASTA file of transcripts and the “multiBamCov” option in BedTools. High-quality reads were functionally annotated using the Blast2GO software (Conesa et al., 2005) based on BLAST algorithm run against the non-redundant (NR) database at NCBI and gene ontology (GO) for the hit sequences. The resultant GO terms for each gene were mapped against a total of 124 Go-Slim categories (Hu et al., 2008) in three major biological ontologies: biological processes, molecular functions, and cellular components.

RNA-seq data were deposited in the European Nucleotide Archive (ENA) under the PRJEB14670 accession number for planktonic (ERS1231641, ERS1231642) and biofilm (ERS1231643, ERS1231644) samples. The sequencing data of each sample are shown in Supplementary Table 2.

Virulence Testing

Chinook CHSE-214 cells (ATCC 1681) were grown into 24-well cell culture plates (5 x 10^4 cells per well) using the Leibovitz’s L-15 medium (HyClone Laboratories, Inc., Logan, Utah) supplemented with: 10% fetal bovine serum (Gibco, Invitrogen Laboratories), 6 mM L-glutamine (Gibco), 15 mM HEPES pH 7.3 (Gibco), and 100 µg mL^-1^/100 IU mL^-1^ streptomycin/penicillin (Gibco). Salmon embryo cells were incubated at 18°C and grown to 70–80% confluence. Before bacterial infection assays, chinook cells were washed with phosphate buffered saline (1X PBS, pH 7.0) and 2 mL of fresh antibiotic-free medium supplemented with 2% fetal bovine serum (Gibco) were added per well. The infection was performed with biofilm and planktonic cells of *F. psychrophilum* LM-02-Fp and NCMB1947T grown as previously described. Briefly, planktonic cells were harvested from supernatants containing mature biofilms by centrifugation (5780 RCF) at 4°C for 2.5 min. Planktonic pellets were washed once with chilled sterile milli-Q water and resuspended in the same solvent at 5.80 ± 2.82 and 4.03 ± 2.70 x 10^4 CFU mL^-1^ (mean ± SD) for inoculation with LM-02-Fp and NCMB1947T strains, respectively. Glass slides with 96-h-old biofilms were washed by immersion (10 s) in chilled sterile milli-Q water before cell detachment with sterile cell scrapers. Biofilm-detached cells were concentrated in chilled sterile milli-Q water at 6.56 ± 2.22 and 3.35 ± 2.71 x 10^4 CFU mL^-1^ (mean ± SD) for infection with LM-02-Fp and NCMB1947T strains, respectively. One hundred microliters of planktonic and biofilm inocula were separately added to wells with CHSE-214 monolayers to study cytotoxic effects of *F. psychrophilum* in three independent experiments performed in triplicate at 18°C for 58 h. In addition, to evaluate the background cytotoxicity (BC) due to aging effects of the medium caused by bacterial growth, every experiment included CHSE-214 monolayers inoculated in triplicate with 100 µL (at 1.0 ± 0.72 x 10^4 CFU mL^-1^) of the exponentially growing non-pathogenic *Escherichia coli*.
Cytotoxic effects induced by *F. psychrophilum* and *E. coli* (including CFU counts) were measured at 0, 10, 24, 34, and 58 h post-infection (hpi) using 100 μL- aliquots of cell culture supernatants and the lactate dehydrogenase (LDH) cytotoxicity detection kit (Takara Bio Inc., Otsu, Japan), in accordance with the manufacturer’s instructions. This kit allows for the colorimetric detection of LDH activity-based cytotoxicity in cell-free supernatants by spectrophotometric reading at 500 nm (Tecan Microplate Reader, Infinite 200 PRO, Männedorf, Switzerland). Absorbance measurements were corrected for the background in low controls (i.e., chinkook cells incubated without bacteria) and expressed as a percentage of the LDH activity in high controls (i.e., chinkook cells incubated without bacteria with 1% Triton X-100) using the equation provided by the manufacturer. Cytotoxicity percentages induced by sessile and planktonic cells of *F. psychrophilum* were corrected for the average percentage of the BC determined with the Stbl2 strain, which was about 10%. Thus, we assumed that the growth of *F. psychrophilum* itself was not usually associated with higher cytotoxicities than this threshold percentage. In fact, *E. coli* Stbl2 had a faster growth in the cell culture medium (from 6.0 ± 3.46 × 10^2 to 1.1 ± 0.61 × 10^2 CFU mL^{-1}} within 58 hpi) than *F. psychrophilum*, for example, compared with LM-02-Fp biofilm cells (from 1.4 ± 0.51 × 10^3 to 2.3 ± 0.85 × 10^2 CFU mL^{-1}} within 58 hpi), but did not induce evident degenerative damage in the CHSE-214 cell line after 58 hpi (Supplementary Figure 1).

Also, planktonic and biofilm cells of *F. psychrophilum* LM-02-Fp and NCMB1947^T^ were evaluated for their virulence capacity on rainbow trout fry (*Oncorhynchus mykiss*) using an intraperitoneal challenge model for 26 days. Before bacterial challenge, fish were anesthetized by immersion in a benzocaine solution (30 mg L^{-1}) at 18°C. In addition, the absence of *F. psychrophilum* or other bacterial pathogens was previously confirmed by bacteriological and PCR analyses of samples (gills, mucus, skin, spleen, and kidney) from randomly collected fish (Urdaci et al., 1998; Bernardet et al., 2002). Fish (5–8 g) were deposited and acclimatized (for 1 week) into 10 L tanks with aerated and dechlorinated water (7 L) to get an average density of 10.2 kg m^{-3} in chilled sterile milli-Q water (pH 7.6–7.8) was changed bi-daily and fish were fed daily for each tested growth state and strain, including control tanks. *F. psychrophilum* and NCMB1947^T^ strains were deposited and acclimatized (for 1 week) into 10 L tanks with aerated and dechlorinated water (7 L) to get an average density of 10.2 kg m^{-3} using a 12 h: 12 h light: dark regime. Tank itself was not usually associated with higher mortality than control tanks, and the absence of other bacterial pathogens was previously confirmed by bacteriological and PCR analyses of samples (gills, mucus, skin, spleen, and kidney) from randomly collected fish (Urdaci et al., 1998; Bernardet et al., 2002). Fish (5–8 g) were deposited and acclimatized (for 1 week) into 10 L tanks with aerated and dechlorinated water (7 L) to get an average density of 10.2 kg m^{-3} in chilled sterile milli-Q water (pH 7.6–7.8) was changed bi-daily and fish were fed daily for each tested growth state and strain, including control tanks.

The LDH-based cytotoxicity of *F. psychrophilum* NCMB1947^T^ and LM-02-Fp on CHSE-214 cells was modeled in response to changes in predictor variables such as the growth state (biofilm vs. plankton), temporality, strain, and the interaction among the first two variables. This was done using generalized additive models for location, scale and shape from R “gamlss” package (Rigby and Stasinopoulos, 2005). Before running the analysis, the temporality was scaled (Z-transformed) in order to determine its effect on the rest of variables (Schielzeth, 2010). The two strains were modeled together and separately using the beta inflated distribution (BEINF) with the time as a continuous variable. The BEINF is similar to the beta distribution but allows zeros and ones as values for the response variable (Ospina and Ferrari, 2010). Gehan-Breslow-Wilcoxon and Log-rank (Mantel-Cox) tests were performed with GraphPad Prism 7 (GraphPad Software, Inc., CA, USA) to determine statistically significant differences (P < 0.05) among mortality curves constructed from fish challenge data.

### Results

#### Differentially Expressed Candidate Genes (DECGs) between Biofilm and Planktonic States of *F. psychrophilum* LM-02-Fp and NCMB1947^T^

We identified 349 DECGs between biofilm and planktonic states, which accounted for ~15% of total number of genes expressed in transcriptomes of the two strains (i.e., 2327 expressed genes).
Out of 349 DECGs, 151 and 198 were significantly up- and down-regulated in the mature biofilm state of both strains, respectively (Figure 1). Some 83% of all up-regulated candidate genes were associated with at least one GO term, resulting in a total of 392 term occurrences (Table 1 and Supplementary Table 3). Among these, 182 unique GO terms were grouped in different GO_Slim categories into biological processes (36.81%), molecular functions (59.34%), and cellular components (3.85%) (Figure 2). Similarly, 81% of all down-regulated candidate genes were associated with at least one GO term, resulting in a total of 591 term occurrences (Table 1 and Supplementary Table 3). The identified 218 unique GO terms were grouped in different GO_Slim categories into biological processes (38.99%), molecular functions (53.21%), and cellular components (7.8%) (Figure 2).

**Potential Adaptive Response to Stress of** *F. psychrophilum* **Biofilms**

Most DECGs linked to protein synthesis were significantly down-regulated in mature biofilms of *F. psychrophilum* LM-02-Fp and NCMB1947T. Specifically, those encoding ribosomal proteins (*n* = 47), initiation factors IF1 and IF3 (FP1318 and FP0894, respectively), and elongation factors EF-Ts, EF-P, EF-Tu, and EF-G (FP0453, FP0975, FP1184, and FP1341, respectively) (Table 1). The same pattern was found for some DECGs encoding proteins involved in cell survival induced by growth arrest (universal stress protein UspA, FP0467); the prevention of functional ribosome formation (FP0602); the maturation of tRNAs (FP1794); and RNA synthesis using DNA as a template (FP1175, FP1176, and FP1313) (Table 1). Furthermore, transcriptional factors DksA (RNA polymerase-binding transcription factor, FP0218) and NusG (transcriptional elongation factor, FP1181), as well as the sigma factor RpoD (primary sigma factor during exponential growth of bacteria, FP2048), were significantly down-regulated in mature biofilms (Table 1). In contrast, DECGs encoding transcriptional factors from the families AraC (FP0253, FP0984, and FP0994), TetR (FP0423), LysR (FP0763), and ArsR (FP1595) were significantly up-regulated (Table 1). The same was true for DECGs encoding...
### TABLE 1 | Changes in gene expression as part of the potential adaptive response of *F. psychrophilum* biofilms (NCMB1947\(^T\) and LM-02-Fp) to face stressful conditions.

| Category | Locus tag* | DECG description | Hit name** | GO number | Log\(_2\) FC*** |
|----------|------------|------------------|------------|-----------|----------------|

| Protein synthesis machinery | FP0094 | 23S rRNA-intervening sequence protein | FPSM_00097 | GO:0005840 | −2.38 |
| FP0146 | SOS ribosomal protein L25/general stress protein Ctc | rpsY | GO:0005840, GO:0003735, GO:0008097, GO:0006412 | −3.13 |
| FP0218 | Molecular chaperone | FPSM_00244, dksA | GO:0008270 | −2.63 |
| FP0253 | Probable transcriptional regulator, AraC family | FP0253, FPSM_00280 | GO:0003700, GO:0043565, GO:0006351, GO:0006355 | 2.82 |
| FP0344 | 30S ribosomal protein S16 | rpsP | GO:0005840, GO:0003735, GO:0006412 | −4.52 |
| FP0423 | Probable transcriptional regulator, TetR family | FPSM_00466, FP0423 | GO:0003677, GO:0006351, GO:0006355 | 2.64 |
| FP0453 | Elongation factor Ts (EF-Ts) | tsf | GO:0005737, GO:0003748, GO:0006414 | −3.45 |
| FP0454 | 30S ribosomal protein S2 | rpsB | GO:0005840, GO:0003735, GO:0006412 | −3.62 |
| FP0455 | 30S ribosomal protein S9 | rpsI | GO:0005840, GO:0003735, GO:0006412 | −4.15 |
| FP0456 | 50S ribosomal protein L13 | rplM | GO:0005840, GO:0003735, GO:0006412 | −4.51 |
| FP0467 | Universal stress protein UspA | uspA, FPSM_00512, FP0467 | GO:0006950 | −2.98 |
| FP0602 | RsfS-ijap-like ribosome-associated protein (ribosomal silencing factor) | FP0602, rtsS | GO:0005737, GO:0017148, GO:0042256, GO:0090071 | −2.26 |
| FP0763 | Probable transcriptional regulator, LysR family | FPSM_01554, FP0763 | GO:0003677, GO:0003700, GO:0006351, GO:0006355 | 4.12 |
| FP0892 | 50S ribosomal protein L20 | rplT | GO:0005840, GO:0003735, GO:0006412 | −3.87 |
| FP0894 | Translation initiation factor IF3 | inrC, FPSM_01478 | GO:0003743, GO:0006413 | −3.22 |
| FP0975 | Elongation factor P (EF-P) | FPSM_01375, efp | GO:0005737, GO:0003748, GO:0006414 | −3.20 |
| FP0975 | Probable transcriptional regulator, AraC family | FPSM_01384, FP0984 | GO:00016021, GO:0003700, GO:0043565, GO:0006351, GO:0006355 | 4.06 |
| FP0994 | Probable transcriptional regulator, AraC family | FPSM_01394, FP0994 | GO:0003700, GO:0043565, GO:0006351, GO:0006355 | 3.38 |
| FP1135 | SOS ribosomal protein L31 | rRNA_05395, rpmE | GO:0005840, GO:0003735, GO:0006412 | −3.69 |
| FP1175 | DNA-directed RNA polymerase beta’ subunit RpoC | rpoC | GO:0003677, GO:0003899, GO:0006351 | −2.65 |
| FP1176 | DNA-directed RNA polymerase beta subunit RpoB | rpoB | GO:0003677, GO:0003899, GO:00032549, GO:0006351 | −2.65 |
| FP1177 | SOS ribosomal protein L7/L12 | rplL | GO:0005840, GO:0003735, GO:0006412 | −3.53 |
| FP1178 | SOS ribosomal protein L10 | rplU | GO:0005840, GO:0070180, GO:0006412, GO:0042254 | −3.98 |
| FP1179 | SOS ribosomal protein L1 | rplA | GO:00015934, GO:00000049, GO:0003735, GO:0019843, GO:0006412, GO:0006417 | −3.64 |
| FP1181 | Transcription antitermination protein | nusG | GO:0006353, GO:0006354, GO:0031564, GO:00032784 | −2.57 |
| FP1184 | Elongation factor Tu (EF-Tu) | tuf | GO:0005737, GO:0003748, GO:0003924, GO:0005525, GO:0006414 | −4.14 |
| FP1312 | SOS ribosomal protein L17 | rplQ | GO:0005840, GO:0003735, GO:0006412 | −3.40 |
| FP1313 | DNA-directed RNA polymerase subunit alpha | rpoA | GO:0003677, GO:0003899, GO:0046983, GO:0006351 | −4.11 |

(Continued)
| Table 1 (Continued) |
|----------------------|
| **Category** | **Locus tag** | **DECG description** | **Hit name** | **GO number** | **Log2 FC** |
| 30S ribosomal protein S4 | FP1314 | rpsD | GO:0015935, GO:00003735, GO:0019843, GO:00006412 | −4.55 |
| 30S ribosomal protein S11 | FP1315 | rpsK | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −4.00 |
| 30S ribosomal protein S13 | FP1316 | rpsM | GO:00005840, GO:00000049, GO:00003735, GO:0019843, GO:00006412 | −3.67 |
| Translation initiation factor IF1 | FP1318 | iniA | GO:00005737, GO:00003734, GO:0019843, GO:00006412 | −3.53 |
| 50S ribosomal protein L15 | FP1320 | rplO | GO:0015934, GO:000003735, GO:0019843, GO:00006412 | −3.20 |
| 50S ribosomal protein L30 | FP1321 | rpmD | GO:00005840, GO:000003735, GO:00006412 | −4.92 |
| 30S ribosomal protein S5 | FP1322 | rpsE | GO:0015935, GO:00003735, GO:0019843, GO:00006412 | −3.12 |
| 50S ribosomal protein L18 | FP1323 | rplR | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.56 |
| 50S ribosomal protein L6 | FP1324 | rplF | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −4.01 |
| 30S ribosomal protein S8 | FP1325 | rpsH | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.60 |
| 30S ribosomal protein S14 | FP1326 | rpsN | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.72 |
| 50S ribosomal protein L5 | FP1327 | rplE | GO:00005840, GO:00000049, GO:00003735, GO:0019843, GO:00006412 | −4.56 |
| 50S ribosomal protein L24 | FP1328 | rplX | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.32 |
| 50S ribosomal protein L14 | FP1329 | rplN | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.11 |
| 30S ribosomal protein S17 | FP1330 | rpsQ | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −4.81 |
| 50S ribosomal protein L29 | FP1331 | rpmC | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −5.06 |
| 50S ribosomal protein L16 | FP1332 | rplP | GO:00005840, GO:00000049, GO:00003735, GO:0019843, GO:00006412 | −3.34 |
| 30S ribosomal protein S3 | FP1333 | rpsC | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.91 |
| 50S ribosomal protein L22 | FP1334 | rplV | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.47 |
| 30S ribosomal protein S19 | FP1335 | rpsS | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.80 |
| 50S ribosomal protein L2, partial | FP1336 | rplB | GO:00016740, GO:00019843, GO:00006412 | −3.72 |
| 50S ribosomal protein L4 | FP1338 | rplD | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −3.12 |
| 50S ribosomal protein L3 | FP1339 | rplC | GO:00005840, GO:00003735, GO:0019843, GO:00006412 | −2.83 |
| 30S ribosomal protein S10 | FP1340 | rpsJ | GO:00005840, GO:00000049, GO:00003735, GO:0019843, GO:00006412 | −2.97 |
| Elongation factor G (EF-G) | FP1341 | fusA | GO:00005737, GO:00003746, GO:00003924, GO:00005525, GO:00006414 | −3.89 |
| Category | Locus tag* | DECG description | Hit name** | GO number | Log2 FC*** |
|----------|------------|------------------|------------|-----------|-----------|
|          | FP1342     | 30S ribosomal protein S7 | rpsG       | GO:0015935, GO:0000049, GO:0003735, GO:0019843, GO:0006412 | -4.05     |
|          | FP1343     | 30S ribosomal protein S12 | rpsL       | GO:0015935, GO:0000049, GO:0003735, GO:0019843, GO:0006412 | -3.68     |
|          | FP1397     | 30S ribosomal protein S20 | rpsT       | GO:0005840, GO:0003735, GO:0019843, GO:0006412 | -3.51     |
|          | FP1432     | 50S ribosomal protein L33 | rpmG       | GO:0005840, GO:0003735, GO:0019843, GO:0006412 | -3.52     |
|          | FP1466     | Extracytoplasmic function (ECF)-type sigma factor | FP1466, FPSM_01934 GO:0003677, GO:0003700, GO:0016987, GO:0006352, GO:0006355 | 2.79      |
|          | FP1595     | Probable transcriptional regulator, ArsR family | FP 1595, FPSM_01218 GO:0003677, GO:0003700, GO:0006351, GO:0006355 | 2.82      |
|          | FP1793     | 30S ribosomal protein S1 | IA01_08730, rpsA | GO:0005840, GO:0003723, GO:0003735, GO:0006412 | -3.97     |
|          | FP1794     | Ribonuclease Z | FP1794 | GO:0005840, GO:0003735, GO:0019843, GO:0006412 | -2.06     |
|          | FP1849     | 50S ribosomal protein L9 | rplF       | GO:0005840, GO:0003735, GO:0019843, GO:0006412 | -3.37     |
|          | FP1850     | 30S ribosomal protein S18 | rpsR       | GO:0005840, GO:0003735, GO:0019843, GO:0006412 | -3.22     |
|          | FP1851     | 30S ribosomal protein S6 | rpsF       | GO:0005840, GO:0003735, GO:0019843, GO:0006412 | -4.21     |
|          | FP1867     | rRNA methyltransferase | FP1867, FPSM_00933 | GO:0003723, GO:0003735, GO:0006412 | 4.12      |
|          | FP2045     | 30S ribosomal protein S15 | rpsO       | GO:0005840, GO:0003735, GO:0019843, GO:0006412 | -3.60     |
|          | FP2048     | RNA polymerase sigma factor RpoD | rpoD, FPSM_00742 | GO:0003977, GO:0003700, GO:0016987, GO:0006352, GO:0006355, GO:0003043 | -3.02     |
|          | FP2052     | Glutamyl-tRNA amidotransferase | FPSM_00739, FP2052 | GO:0016740, GO:0016884, GO:0008152 | -2.16     |
|          | FP2124     | Epoxyqueuosine reductase (tRNA modification) | queG, FP2124 | GO:0005737, GO:00046872, GO:0051539, GO:0052693, GO:0008033, GO:0008616, GO:0055114 | 3.97      |
|          | FP2216     | 50S ribosomal protein L27 | rpmA       | GO:0005840, GO:0003735, GO:0006412 | -3.29     |
|          | FP2217     | 50S ribosomal protein L21 | rplU       | GO:0005840, GO:0003735, GO:0006412 | -3.41     |
|          | FP2380     | 50S ribosomal protein L32 | rpmF       | GO:0015934, GO:0003735, GO:0006412 | -2.38     |

** DNA repair and antioxidant activity **

|          | FP0160     | Superoxide dismutase | FPSM_00176, sodA | GO:0004784, GO:00046872, GO:0019430, GO:0055114 | -2.38     |
|          | FP0209     | Crossover junction endodeoxyribonuclease | ruvC | GO:0000287, GO:00003676, GO:0005140, GO:0006281, GO:0006310 | 2.61      |
|          | FP0269     | DNA repair protein RecO | recO | GO:0005828, GO:00053610, GO:0006310 | 3.78      |
|          | FP0568     | Cytochrome C peroxidase | FP0568 | GO:0004130, GO:0009055, GO:0020037, GO:0055114 | 5.37      |
|          | FP0670     | Chaperone protein DnaJ | dnaJ | GO:0005737, GO:00055524, GO:0005270, GO:0031072, GO:0051082, GO:0006260, GO:0006457, GO:0009408 | -3.16     |

(Continued)
| Category       | Locus tag* | DECG description                      | Hit name**               | GO number                                                                 | Log₂ FC*** |
|----------------|------------|---------------------------------------|--------------------------|---------------------------------------------------------------------------|------------|
|                | FP0671     | Chaperone protein GrpE                | grpE                     | GO:0005737, GO:0000774, GO:00051087, GO:0006457, GO:00050790              | −2.87      |
|                | FP0702     | Probable peroxiredoxin                | FPSM_01619, FP0702       | GO:0004601, GO:0051920, GO:0055114                                       | 2.37       |
|                | FP0864     | Chaperone protein DnaK                | dnaK                     | GO:0005524, GO:0051082, GO:0006457                                       | −4.00      |
|                | FP1014     | Deoxyribodipyrimidine photolyase PhrB1| phrB1, IA01_04785        | GO:0003904, GO:0018298                                                   | 3.99       |
|                | FP1100     | Thiol peroxidase                      | FPSM_02014, FP1100       | GO:0004601, GO:0051920, GO:0055114                                       | −2.05      |
|                | FP1195     | DNA polymerase III subunit epsilon    | dnaQ, FPSM_02106         | GO:0003876, GO:0003987, GO:0004527, GO:0071897, GO:0090305              | 3.53       |
|                | FP1417     | Glutathione peroxidase                | FPSM_01888, bsA           | GO:0004602, GO:0006979, GO:0051920, GO:0055114                         | −2.63      |
|                | FP1509     | Chaperone protein HtpG                | htpG, IA01_07270         | GO:0005737, GO:0005524, GO:0051082, GO:0006457                          | −3.22      |
|                | FP1594     | 2-Cys peroxiredoxin                  | tpx                      | GO:0005623, GO:0008379, GO:0045454, GO:0055114                          | −2.01      |
|                | FP1984     | groS chaperonin GroES                 | groS                     | GO:0005737, GO:0005524, GO:0006457                                       | −3.48      |
|                | FP1985     | groL chaperonin GroEL                 | groL, groEL              | GO:0005737, GO:0005524, GO:0051082, GO:0042026                          | −3.03      |
|                | FP2103     | Probable thioredoxin                  | yyal., FPSM_00685        | GO:0004798, GO:00046939                                                  | 2.82       |
|                | FP2235     | Thioredoxin family protein precursor  | FPSM_02461               | GO:0003756, GO:0006457                                                  | −3.72      |
|                | FP2236     | Thioredoxin family protein            | FPSM_00124               | GO:0005623, GO:0045454, FPSM_02461                                       | −2.76      |
|                | FP2437     | DNA processing Smf protein            | FPSM_02567, smf           | GO:0003877, GO:0006281, GO:0009294                                      | 3.77       |

(Continued)
the extracytoplasmic function-type sigma factor (FP1466), an rRNA methyltransferase (FP1867), and the epoxyqueuosine reductase (FP2124).

Four DECGs encoding proteins required for DNA repair (FP0209/RuvC, FP0269/RecO, FP1014/PhrB1, and FP2437/Smf) and the editing exonuclease subunit of DNA polymerase III (epsilon, FP1195/DnaQ) were significantly up-regulated in mature biofilms of the two F. psychrophilum strains (Table 1). Similarly, DECGs encoding cytochrome C peroxidase (FP0568), a probable peroxiredoxin (FP0702), and a probable thioredoxin (FP2103) were significantly up-regulated in biofilm cells, although other antioxidant-coding DECGs (FP0160, FP1100, FP1417, FP1594, FP2335, and FP2336) were under-expressed (Table 1). DECGs associated with stress-induced responses due to heat and/or osmotic shocks (FP0670, FP0671, FP0864, FP1509, FP1984, and FP1985) were also down-regulated in mature biofilms (Table 1). Finally, eight DECGs encoding electron transport proteins with roles in the formation of transmembrane electrochemical potential were down-regulated in biofilm cells, just as were seven DECGs with roles in ATP synthesis (as part of the ATP synthase complex) (Table 1).

### Evaluation of the Virulence of Sessile and Planktonic Cells of F. psychrophilum

LDH-based assays showed that F. psychrophilum biofilms had higher cytotoxic effects than free-living bacteria in general (Figure 3A). The cytotoxic effect of bacteria on the fish cell line tended to increase with the time post-infection, except for infections performed with NCMB1947T planktonic cells (Figure 3A). Statistical models computed by GAMLSS indicated that ∼18% of the variability in the cytotoxicity caused by F. psychrophilum NCMB1947T and LM-02-Fp was significantly explained by the growth state (biofilm vs. plankton) and time post-infection, but not by the bacterial strain (model 1, Supplementary Table 4). The time post-infection was the most significant predictor variable for the strain LM-02-Fp (model 2, Supplementary Table 4), although an important increase in the cytotoxicity induced by LM-02-Fp biofilm cells was detectable after 24 hpi compared with planktonic cells (Figure 3A). In addition, the growth state as well as its interaction with time post-infection, were the most significant predictor variables for the strain NCMB1947T (model 3, Supplementary Table 4).

There were no significant intrastrain differences in generating rainbow trout fry mortality between planktonic and sessile cells of F. psychrophilum (Figure 3B). The same was true for differences in fish mortality found between NCMB1947T and LM-02-Fp strains, except when the type strain did not produce mortalities (P < 0.05, Gehan-Breslow-Wilcoxon and Log-rank tests), situation observed in biofilm tank 1 and plankton tank 2 (Figure 3B). No fish mortality was observed during experiments in control tanks and microbiological/PCR analyses confirmed F. psychrophilum-caused mortality in challenged fish (data not shown).

### DISCUSSION

Transcriptomes of F. psychrophilum LM-02-Fp and NCMB1947T grown under biofilm and planktonic conditions were made up of high-quality reads for a total of 2327 genes, 1918 of which (i.e., ∼82% of total number of genes expressed in transcriptomes) did not show statistically significant differences in gene expression between growth states (Levipan and Avendaño-Herrera, 2017). The present study reports 349 DECGs (Figure 1) accounting for ∼15% of total number of genes expressed in transcriptomes, since other 60 DECGs have been described previously as differentially expressed virulence- and biofilm-related genes (Levipan and Avendaño-Herrera, 2017). A similar number of differentially expressed genes (n = 440) was determined through DNA arrays in Escherichia coli K-12 TG1 by comparing 8-day-old biofilms with planktonic cells in the late exponential stage (Beloin et al., 2004). In our study, DECGs encoding ribosomal proteins, some transcriptional factors, different subunits of the DNA-directed RNA polymerase and ATP synthase, as well as translation and elongation factors were significantly down-regulated in mature biofilms as compared to free-living cells (Figure 1 and Table 1). These findings might have important

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**Table 1 | Continued**

| Category | Locus tag* | DECG description | Hit name** | GO number | Log2 FC*** |
|----------|------------|------------------|------------|-----------|-----------|
| FP2459   | ATP synthase subunit delta | atpH | GO:0005886, GO:0045261, GO:0046933, GO:0042777 | −2.06 |
| FP2460   | ATP F0F1 synthase subunit B | atpF | GO:0005886, GO:0016021, GO:0045263, GO:0046933, GO:0042777 | −4.45 |
| FP2461   | ATP synthase subunit C | atpE, FPSM_02597 | GO:0005886, GO:0016021, GO:0045263, GO:0015078, GO:0016787, GO:0015986, GO:0015991 | −3.22 |

Fold-change values (Log2 FC) for any differentially expressed candidate gene (DECG) in the mature biofilm state are written with positive and negative numbers to represent up- and down-regulation, respectively.

* Locus tags according to the reference genome (F. psychrophilum strain JIP02/86) used for high-quality read mapping.

** Hit sequences collected using the BLAST algorithm run against the non-redundant database at NCBI.

*** Padj-values ≤ 0.001.
implications in selecting the candidate antigens for vaccine design, since certain antigenic phenotypes could change over time through the cell cycle, depending on whether bacteria are growing in planktonic or biofilm modes. Thus, a vaccine designed from planktonic cells (killed and/or live-attenuated; e.g., Álvarez et al., 2008) or purified macromolecules may be ineffective to deal with biofilm-related infections, where the planktonic phenotype is only a transient state (Harro et al., 2010). For instance, elongation factor-Tu and ATP synthase subunit beta, previously reported as immunogenic proteins (LaFrentz, 2007), were unsuitable as vaccine against BCWD/RTFS (Plant et al., 2011), which could be related with the fact that these proteins are coded by DECGs between biofilm and planktonic states in F. psychrophilum (Table 1). Indeed, the antigenic
variability between biofilm and planktonic states represents a so far unexplored mechanism (Gómez et al., 2014) that could explain the persistence of this bacterium in aquaculture settings.

Some regulator-coding genes of cellular persistence-related genes (e.g., AraC family transcriptional regulators and extracytoplasmic function-type sigma factor) with putative functions in carbon metabolism, virulence, and diverse stress responses, such as metal-ion tolerance (ArsR-like transcriptional factor), were significantly up-regulated in mature biofilms (Table 1). Indeed, biofilm lifestyle is well known for providing bacteria with improved protection against varied physical-chemical stresses, that is, nutrient depletion, UV radiation, reactive oxygen species, and antibiotics (Flemming and Wingender, 2010). For instance, the overexpression of a rRNA methyltransferase in *F. psychrophilum* biofilms (FP1867, Table 1) could confer greater resistance to antibiotics targeting the ribosomes (Doi and Arakawa, 2007) to sessile cells than free-living cells. Moreover, the improved protection of bacteria in biofilm growth mode is usually accompanied by enhanced virulence and pathogenicity (He and Ahn, 2011; Flemming et al., 2016). However, there are few studies on *F. psychrophilum* comparing free-living vs. biofilm lifestyles (e.g., De la Fuente et al., 2013), and research based on mutants with defects in motility (Álvarez et al., 2006; Pérez-Pascual et al., 2015) has reported an attenuation of virulence in spread-deficient mutants compared to wild-type strains (Pérez-Pascual et al., 2017). These findings suggest that *F. psychrophilum* biofilm cells could be less virulent than motile free-living cells; however, this suggestion overlooks the fact that *F. psychrophilum* infections rely on initial attachment to mucosal external surfaces of fish (Papadopoulou et al., 2017) for cell colonization and long-term persistence of adhered cells. We have previously reported that strong and weak biofilm producers (LM-02-Fp and NCMB1947T strains in the present study, respectively) share a genetic potential for virulence that is transcriptionally enhanced with respect to free-living cells (Levipan and Avendaño-Herrera, 2017). Here we have found that biofilm and planktonic states of *F. psychrophilum* have strain-independent cytotoxic effects on CHSE-214 cells (Figure 3A), with this being particularly true for the sessile lifestyle (Supplementary Figure 1 and Supplementary Table 4). Chinook embryonic cells have successfully been used for studying adhesion to host of extracellular fish pathogens such as *Streptococcus phocae* (González-Contreras et al., 2011), and there is at least one previous study describing *F. psychrophilum*-induced degenerative changes on CHSE-214 cells (Valdebenito and Avendaño-Herrera, 2009). Moreover, even though our intraperitoneal challenge model did not support the idea that differences between sessile and planktonic lifestyles are associated with state-dependent intrastain differences in virulence, the strong biofilm producer tended to produce higher mortality in rainbow trout fry than the type strain (Figure 3B). This trend and LDH-based cytotoxicity were associated with lower transcriptional/translational activities for growth in biofilm cells than in free-living cells (Figure 1) at the moment of initial infection. This was also reflected as a lower percentage contribution of up-regulated candidate genes in the biofilm state to the top-level GO term “cellular component,” as compared to down-regulated candidate genes (Figure 2). However, it is also important to note that the growth of 96-h-old biofilms was reduced but not arrested, since an increased percentage contribution of up-regulated candidate genes was observed for “molecular function” and “biological processes” ontologies (Figure 2). For instance, the *uspA* gene encoding universal stress protein (FP0467), that is usually over-expressed under growth arrest for cell survival (Nachin et al., 2005), was significantly down-regulated in mature biofilms of *F. psychrophilum* LM-02-Fp and NCMB1947T (Figure 1).

Mature *F. psychrophilum* biofilms showed a significant up-regulation of all DECGs with roles in DNA repair, namely, *ruvC*, *recO*, *smf*, *phrB1*, and *dnaQ* (Table 1). Overexpression
of genes and proteins involved in DNA repair has also been reported for *F. johnsoniae* biofilms (Flemming, 2010), suggesting shared strategies between fish pathogenic bacteria in the biofilm state to face environmental changes. The *rvuC* and *recO* genes are involved in RecA-dependent homologous recombinational repair (via the RecF pathway or RecFOR) to handle single-stranded gaps in DNA (Persky and Lovett, 2008). In turn, the *smf* gene product is important for extracellular DNA uptake and protection during recombinational repair (Smeets et al., 2006). DNA transformation and recombinational repair are processes consistent with the biofilm lifestyle of *F. psychrophilum*, since extracellular DNA is a major structural component in most bacterial biofilms and substrates for lateral gene transfer events (Flemming and Wingender, 2010; Levipan and Avendaño-Herrera, 2017). The *phrB1* gene encodes a photolyase that corrects UV radiation-induced cyclobutane pyrimidine dimers in the *F. psychrophilum* genome (Goosen and Moolenaar, 2008), while *dnaQ* encodes the epsilon-subunit of DNA polymerase III, which mediates 3′-5′ proofreading in this enzyme. Up-regulation of *dnaQ* decreases the bacterial mutation rate during the SOS response (e.g., Jonczyk et al., 1988). Thus, considering that SOS response plays a key role in biofilm formation and maturation (van der Veen and Abee, 2010; Leiker and Weitao, 2016), overexpression of the *dnaQ* gene would be expected in mature *F. psychrophilum* biofilms. In practice, these findings could be associated with the relative ineffectiveness of UV radiation-based technologies for *F. psychrophilum* removal from and the prevalence of BCWD/RTFS in aquaculture settings, since a 5-log reduction in cell abundance of this bacterium can need UV doses as high as 126 mW s cm⁻² (Hedrick et al., 2000).

Among nine of the DECGs encoding for molecular markers of oxidative stress (*Table 1*), only cytochrome C peroxidase (FP0568), a probable peroxiredoxin (FP0702), and a probable thioredoxin (FP2103) were significantly up-regulated in mature biofilms to deal with reactive oxygen species (ROS, e.g., H₂O₂). This finding suggests two possibilities, (i) that the degree of oxidative damage during biofilm formation was insufficient to induce a joint overexpression of these 9 DECGs as a single and coordinated scavenging system of ROS, or (ii) that a different set of proteins copes with oxidative stress in biofilm cells, as compared to planktonic cells. Nevertheless, oxidative damage should have a bacteriostatic rather than bactericidal effect in bacterial cells whether stress-defense mechanisms are fully functional (e.g., DNA repair; Imlay, 2015). Recently, it has been hypothesized that biofilms exploit ROS to activate signaling pathways that affect the production of extracellular polymeric substances and biofilm heterogeneity to adapt to changing conditions (Gambino and Cappitelli, 2016). For instance, sub-inhibitory concentrations of Ag-NPs stimulated the production of ROS in *Bacillus subtilis* biofilms but not in planktonic cells, which was also accompanied by the expression of proteins involved in quorum sensing and oxidative stress response in sessile cells, including thioredoxin (Gambino et al., 2015). Therefore, if thioredoxin-like proteins (in our case FP2103) are involved (via ROS) in signaling that controls the biofilm formation by *F. psychrophilum*, for example, through two-component systems (Hesami et al., 2011; Levipan and Avendaño-Herrera, 2017), is a topic that remains to be elucidated. On the other hand, DECGs encoding proteins with roles in the adaptive response of *F. psychrophilum* to temperature shifts (Duchaud et al., 2007) such as chaperone proteins DnaJ (FP0670), GrpE (FP0671), DnaK (FP0864), HtpG (FP1509), and GroS/GroL chaperonin GroES/GroEL (FP1984 and FP1985, respectively) were significantly down-regulated in mature biofilms (*Table 1*). These stress-induced proteins ensure cell protein homeostasis by preventing polypeptide aggregation and misfolding (Hartl et al., 2011). Therefore, under a condition of overall reduction of protein synthesis machinery in *F. psychrophilum* biofilms (*Table 1*), a significant expression of DECGs encoding products with roles in polypeptide homeostasis would mean an unnecessary energetic cost for sessile cells, which could explain these findings.

DECGs encoding respiratory complex-related proteins and different subunits of the ATP synthase were significantly down-regulated in mature biofilms (*Table 1*), suggesting a negative relationship between oxidative phosphorylation for energy conservation and maintenance of biofilm structure. In agreement with this finding, a study based on *ycfR*-deletion mutants of *E. coli* K-12 reported a transcriptional repression of several genes encoding ATP synthase subunits that coincided with an enhanced capacity to form biofilms (Zhang et al., 2007). This work along with our study suggest that a decreased capacity for ATP energy conservation does not necessarily leads to the early and total disaggregation of bacterial biofilms, as could be deduced from other bacterial models (Sule et al., 2009) or experiments with multi-species biofilms where the ATP synthesis was chemically inhibited (Xu et al., 2012). In fact, the earliest cell detachment in our experiments began at approximately 144 h of incubation post-inoculation, and biofilm cells at 268 h of incubation were still detectable (Levipan and Avendaño-Herrera, 2017). This latter suggests that a down-regulation of DECGs related to energy conservation represents a blocking cue for energy-demanding processes (e.g. protein synthesis) rather than triggering cell detachment from mature *F. psychrophilum* biofilms.

In summary, this study unveiled the major patterns of shift in overall gene expression induced by plankton-to-biofilm transition in *F. psychrophilum*. This report represents the first transcriptomic baseline toward obtaining insights into the biofilm lifestyle of this freshwater fish pathogen. Specifically, a potential stress response in mature biofilms was identified, as characterized by a generalized under-expression of DECGs with roles in protein synthesis machinery and ATP-based energy conservation via oxidative phosphorylation, along with an overexpression of several DECGs with roles in preserving cell integrity. This pattern suggests that mature *F. psychrophilum* biofilms employ an energetically economical strategy to ensure structural maintenance and metabolic functioning, relying on practically no growth and the induction of cellular homeostasis processes such as DNA repair. Finally, our results propose new avenues of research that may help to understand and/or face the prevalence of *F. psychrophilum* in aquaculture settings: (1) pathogenicity and virulence of the biofilm lifestyle, (2) changes
between planktonic and biofilm states that lead to intra-strain antigenic variations with potential impact on vaccine design, (3) role of oxidative stress-related proteins in biofilm formation, and (4) energy conservation and its implication for biofilm deterioration.

ETHICS STATEMENT

This study adhered to animal welfare procedures and was approved by the bioethical committees of the Universidad Andrés Bello and the National Commission for Scientific and Technological Research of the Chilean government.

AUTHOR CONTRIBUTIONS

HL and RA-H conceived and designed the study. JQ performed cytotoxicity experiments. HL conducted the rest of experiments, performed the analysis and interpretation of data, and wrote the manuscript. RA-H participated in the critical review of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00018/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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