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Long-term serial culture of *Piscirickettsia salmonis* leads to a genomic and transcriptomic reorganization affecting bacterial virulence

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ABSTRACT

The propagation of intracellular bacteria in culture mediums implies physiological adaptation to an artificial environment. If these conditions are persistent enough time, a permanent adaptation to the new environment can be expected. This study aimed to explore the genomic and transcriptomic rearrangements that the intracellular bacterium *Piscirickettsia salmonis* undergo during a long-term serial culture experiment and the potential impact on virulence. Here, PacBio and Illumina sequencing were conducted after 200 passages (~2 years) of *P. salmonis* in a cell-free culture medium. The results evidenced genomic rearrangements of the *P. salmonis* genome during the continuous culture period, where a chromosome segment of 35 Kb was translocated from the P0 bacterial chromosome to a P200 plasmid. Notably, the genomic structure of this segment revealed the presence of the Dot/Icm secretion system, which has been previously associated with *P. salmonis* pathogenesis. On the other hand, a reduced transcriptomic response was evidenced in *P. salmonis* after 200 passages affecting the expression of different pathways including iron acquisition and metabolism. Furthermore, *in vitro* infections revealed that after 200 passages *P. salmonis* is less capable of generating cytopathic effects than the original P0 form. Overall, our results evidence that the continuous propagation of *P. salmonis* leads to genomic and transcriptomic rearrangement that impact on bacterial virulence. These results open new perspectives about the adaptation of intracellular bacteria to artificial culture conditions, providing useful information to develop live attenuated vaccines against *P. salmonis* in salmon aquaculture.

1. Introduction

Bacteria are highly dynamic organisms that can survive and adapt to a wide variety of environments. In laboratory conditions, these microorganisms are routinely propagated in different culture settings. If these conditions are persistent enough time, bacteria will eventually become adapted (domesticated) to this non-natural environment (Eydallin et al., 2014; Kram et al., 2017). Together with their easy handling and short generation times, bacteria have been used as study models to address key aspects of adaptation and evolution in response to challenging environments. Medium adaptation has been mainly explored in *Escherichia coli*, where this bacterium has been grown through serial passages in different stressful conditions, including nutrient, acid or antibiotic stress (Barrick and Lenski, 2013; Conrad et al., 2009; Herring et al., 2006; Spagnolo et al., 2016; Toprak et al., 2012; Wiser et al., 2013). These studies have evidenced that *E. coli* displays different genotypic and phenotypic modifications, providing comparative advantages to survive or fully take advantage of the conditions where bacteria are maintained. For instance, through whole-genome sequencing, 13 *de novo* mutations were identified in *E. coli* grown in a glycerol-based growth medium for 44 days (Herring et al., 2006). Here, the authors demonstrated that these spontaneous modifications conferred to the mutants an improved fitness compared with the wild type population. Even during a short period of adaptation (2–3 days), *E. coli* can display phenotypic changes as a domestication response (Eydallin et al., 2014).

For pathogenic agents, several studies have reported that medium adaptation through serial passages improves the performance in new culture conditions but comes with the development of a decreased virulence. This fact has been extensively studied in viruses, where serial passages have been used as a classical approach for the development of live attenuated vaccines against viral infections (Hanley, 2011). For instance, vaccines against measles virus, yellow fever and varicella virus among others, have been developed through the serial passage of

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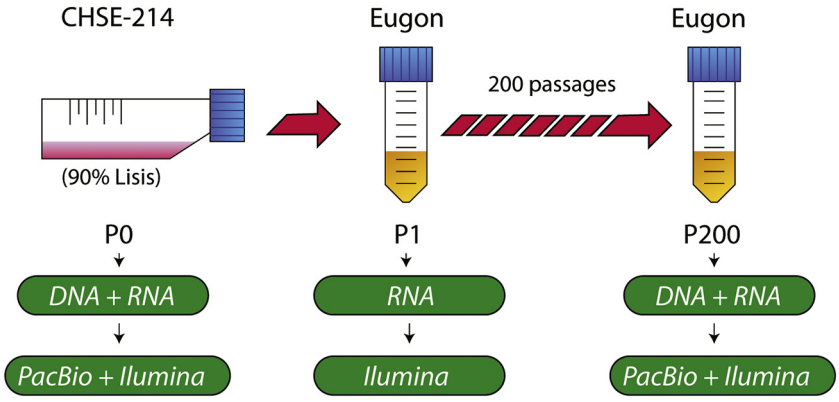

Fig. 1. Experimental design for the serial passages of *Piscirickettsia salmonis*. CHSE-214 cells were infected with 10^6*P. salmonis* (P0). When 90% of lysis was achieved, bacterial cells were collected and growth in the cell free Eugon medium. When the culture reached the exponential phase at A_{600} > 0.3 (P1), 300 µl of that culture where transferred to 2.7 ml of fresh medium. The process was repeated 200 times (P200) for a total period of broth culture of around 2 years. Nucleic acids isolated and sequencing strategy for each passage is also shown.

the virus in a non-natural host (Hanley, 2011). A reduced virulence after medium adaptation has also been reported in bacteria. A reduction in the pathogenesis of *Staphylococcus aureus* after a 6 week *in vitro* serial passage has been reported, probably associated with single mutations occurred in the coding region of the *agrC* gene (Somerville et al., 2002). Other classical examples of pathogenic attenuation through serial passages include the development of live attenuated vaccines against *Salmonella typhi* and *Mycobacterium bovis* infections (Germanier, 1975; Toida, 2000). However, most of the time these adaptations are focused into the occurrence of random single mutations that will modify a restricted number of proteins, conferring to the mutants a comparative advantage when compared with the wild type populations. Due to this, we explore the landscape of genomic and transcriptomic adaptation that *Piscirickettsia salmonis* displays through a long-term cell-free medium adaptation exposure.

Piscirickettsia salmonis is a Gram-negative, non-motile and coccoid bacteria responsible for the salmonid rickettsial septicemia (SRS), a systemic disease associated with high mortalities in salmon aquaculture (Rozas and Enriquez, 2014). Besides being one of the main threats for the sustainable development of aquaculture in Chile, *P. salmonis* has gained scientific attention due to its ability to infect and replicate within host immune cells (Ramirez et al., 2015; Rojas et al., 2010). These characteristics position *P. salmonis* as an interesting model to understand the immune evasion strategies during host-pathogen interactions (Valenzuela-Miranda and Gallardo-Escarate, 2016, 2018; Valenzuela-Miranda et al., 2017). For many years, *P. salmonis* was considered to be an obligate intracellular bacterium. However, the development of cell-free culture media changed the paradigm about the nutritional requirements for this pathogen (Henriquez et al., 2013; Yanez et al., 2012). This study aimed to explore the genomic reorganization of the intracellular bacterium *P. salmonis* through a long-term serial culture, and also evaluate changes in the bacterial virulence. Here, PacBio and Illumina sequencing were conducted over 200 passages (~2 years) in a cell-free culture medium to detect changes at chromosome and transcription levels. In turn, our results evidenced a genomic rearrangement and a smaller transcriptomic activity in medium adapted bacteria (P200), affecting some relevant pathogenic pathways, such as the Dot/Icm secretion system and mechanisms involved in iron acquisition. We suggest that these modifications can be responsible for the *in vitro* pathogenic attenuation displayed by *P. salmonis* at 200 passages.

2. Materials and methods

2.1. Serial propagation of P. salmonis in cell-free medium

Eugon broth culture was selected as a cell-free medium for the serial propagation of *P. salmonis*. This medium was prepared by dissolving 30,4 g of Eugon broth (Bacto™) in 1 l of autoclaved (121 °C for 20 min) distilled water. The dissolved Eugon was autoclaved again and let cool to room temperature. At this point, the medium was supplemented with 1% (m/v) of Casamino acids (Bacto™) and with $FeCl_3$ (MERCK) in a final concentration of 2 mM. Both Casamino acids and $FeCl_3$ were filtered through a 0,22um membrane and added to supplement the medium.

A previously field isolated *P. salmonis* (EM-90 strain) was used in this experiment (Valenzuela-Miranda and Gallardo-Escarate, 2018). This strain was isolated during an outbreak of *P. salmonis* in 2017 from Atlantic salmon. The isolated strain was first propagated and host-adapted in salmon cell lines. For this, CHSE-214 cells were cultured in T75 flasks at 20 °C with 20 ml of DMEM (HyClone) supplemented with 10% of heat-inactivated fetal bovine serum (Biological Industries) and 1% of non-essential amino acids (HyClone). When cell cultures reached 70% confluency, the flasks were inoculated with 10^6*P. salmonis*. The cultures were incubated at 20 °C until the appearance of cytopathic effects (CE) produced by *P. salmonis* infection in around 80% of the CHSE-214 cells. At this point the culture was disrupted with a cell scraper and the supernatant was collected and centrifugated to pellet CHSE-214 cell debris. The collected supernatant containing *P. salmonis* was considered as the initial passage (P0) (Fig. 1). For serial passages experiment, 1 ml of P0 bacteria was used to inoculate a 15 ml Falcon tube containing 2 ml of Eugon broth culture and maintained at 20 °C with a constant shaking of 100 rpm. When this culture reached the exponential phase (A_{600} 0,3-0,4), 300 µl were transferred to a new 15 ml falcon containing fresh 2 ml of Eugon broth culture (P0). This transference was considered as one passage and the process was repeated 200 times (Fig.1). Given the slow growth rates of *P. salmonis* the exponential phase was achieved every 3 to 4 days, thus it was necessary to maintain a continuous culture of the bacteria for around 2 years. Samples for DNA and RNA isolation were collected at P0, P1, and P200.

2.2. Whole-genome sequencing of P. salmonis

Whole-genome sequencing was conducted to compare the genome dynamics between the host-adapted bacteria (P0) and the 200 passage (P200) in Eugon broth culture. For this, total DNA was isolated from P0 and P200 cultures using the DNeasy Blood & Tissue Kits (Qiagen) according to the manufacture's instructions. The integrity of the isolated DNA was confirmed through a 1% agarose gel and purity was evaluated through the 260/280 and 260/230 absorbance ratio estimated through NanoDrop 1000 Spectrophotometer (Thermo Scientific). DNA samples with no smear and absorbance ratios above 1.8 were sent to Macrogen Inc. (Korea) for whole-genome PacBio Single-Molecule Real-Time (SMRT) sequencing, considering a pool of DNA isolated from three different *P. salmonis* colonies for both groups. After quality control and trimming, long reads were *de novo* assembled using the Hierarchical Genome Assembly Process (HGAP3) workflow with default options. The genomes and plasmids obtained for both P0 and P200 were then annotated with the RAST server (http://rast.nmpdr.org/rast.cgi) (Overbeek et al., 2014) using as reference the *P. salmonis* genomes

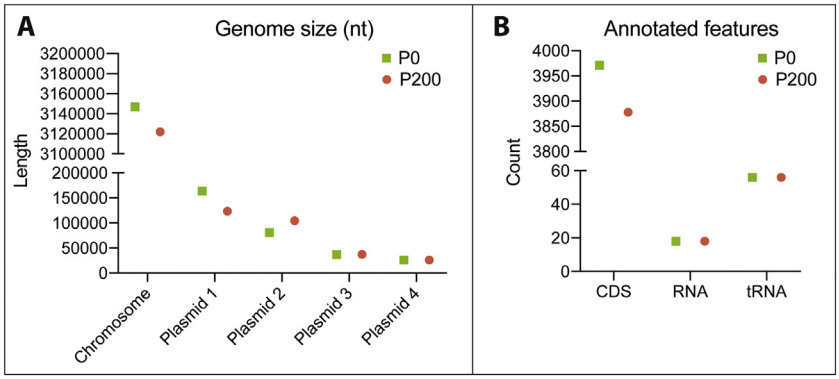

Fig. 2. A) Genome size changes in the chromosome and plasmids of *P. salmonis* from the initial bacteria (P0) and after 200 passages (P200). **B)** Number of annotated features (CDS, RNAs and tRNAs) in P0 and P200 bacteria.

(NCBI taxonomy ID: 1238) and default parameters. The quality of the assembled genomes was confirmed using CheckM (Parks et al., 2015). Thus, both P0 and P200 genomes assemblies reached completeness of 98,81% with genome contamination of 0% and 2,3% respectively, evidencing the high quality and completeness of each one of the assembled genomes (Fig. S1 and S2). Full genome sequences are publicly available in figshare (Valenzuela-Miranda et al., 2020). To identify genomic rearrangement, synteny blocks were detected between P0 and P200 genomes and plasmids. For this purpose, the Synteny Block ExpLoration tool (Sibelia) (http://bioinf.spbau.ru/sibelia) was used to identify shared regions between both genomes. The results were then visualized using the Circos software version 0.69–6 (http://circos.ca/) (Krzywinski et al., 2009).

2.3. Transcriptome sequencing of P. salmonis

RNA-seq analyses were conducted to identify differentially expressed genes among P0, P1, and P200 bacteria. For this purpose, total RNA was isolated from the three passages obtained as described above using RiboPureTM Kit (Ambion, USA) and treated with DNAse I, RNAse-free (Thermofisher, USA) according to manufacturer instruction. The quality and purity of the isolated RNA were confirmed using the 2200 TapeStation (Agilent Technologies, USA) using R6K screen tape and through NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Samples with RNA Integrity Values (RIN) above 8, with 260/280 and 260/230 absorbance ratios above 1,8 were shipped to Macrogen Inc. (Korea) for library preparation and Hi-seq Illumina sequencing. Three biological replicates were considered for sequencing in each group. Bioinformatic analyses were conducted using CLC Genomics Workbench software (V10, CLC Bio, Denmark). Raw reads were trimmed and filter by quality and adapters were removed. Filtered reads were used for RNA-seq analysis using as reference the previously annotated genomes for P0 and P200 respectively. RNA-seq parameters were set including a minimum read length fraction and a minimum read similarity fraction of 0.8, unspecific read match limit of 10, and expression values were calculated as transcripts per million reads (TPM). Differential expression analysis was conducted by comparing TPM means between all replicates for each pair of groups and an ANOVA test was used to identify statistically significant differences between all groups. *P*-values were FDR corrected and genes with an absolute fold change above 4 and with FDR corrected *p*-value above 0,05 were considered as differentially expressed transcripts. Confirmatory qPCRs were conducted to confirm the findings according to previously described protocols (Valenzuela-Miranda and Gallardo-Escarate, 2018).

2.4. Attenuation of P. salmonis

To test the potential impact of a serial passage culture of *P. salmonis* in a cell-free medium an *in vitro* experiment was conducted. For this, the salmon cell line CHSE-214 were grown in T75 flasks at 20 °C with 20 ml of MEM medium (HyClone) supplemented with 10% of heat-inactivated fetal bovine serum (Biological Industries) and 1% of non-essential amino acids (HyClone). When cell cultures reached 70% confluency, the flasks were inoculated with 10^6*P. salmonis* of either P0 or P200 bacteria. The flasks were incubated for 14 days at 20 °C and cytopathic effects were registered after this period. Besides, a small-scale *in vivo* experiment was performed to test P0 and P200 pathogenicity. To do this, 60 Atlantic salmon were IP injected either with P0 or P200 bacterium at a concentration previously established by our group (Tarifeno-Saldivia et al., 2017; Valenzuela-Miranda and Gallardo-Escarate, 2018; Valenzuela-Miranda et al., 2017; Valenzuela-Munoz et al., 2019). Mortalities were molecularly and clinically confirmed as a result of Piscirickettsiosis.

3. Results

Medium adaptation is defined as the process were bacteria might undergo genotypic and phenotypic changes to improve fitness in new culture conditions. Here, one of the aims of this study was to describe genomic changes that the salmonid pathogen *P. salmonis* displays during 200 serial passages. Later, through whole-genome sequencing of P0 and P200, a full-length chromosome and each one of the 4 natural plasmids commonly associated with *P. salmonis* were identified. In general, a slight diminution on both the genome size and coding sequences were evidenced in P200 genetic material regarding the original P0 bacteria. The P200 genome size was estimated as 24,965 bp smaller than the P0 genome and with 93 fewer coding sequences than the original bacteria (Fig. 2). A comparative analysis on the lost and gain genes during the 200 passage evidenced that 115 genes were present in P0 but not in P200 (supplementarysupplementary file 1). Some of these genes included Oligopeptide transport system permease protein (OppC), the Acetyl-coenzyme A carboxyl transferase alpha chain, GDP-mannose 4,6-dehydratase, the Leucyl/phenylalanyl-tRNA–protein transferase and different mobile genetic elements, transposases, and hypothetical proteins.

Through synteny analysis, it was possible to identify all the different conservation blocks in the *P. salmonis* genome. Notably, when the host-adapted (P0) and the medium adapted (P200) bacterial genomes are compared, an exchange in the position between the third and fifth synteny block was evidence (Fig. 3A), while the remaining blocks maintained their positions on both genomes. The genetic content of both blocks revealed that most of the annotated genes within these regions corresponded to mobile genetic elements or transposases, follow by flagellar related proteins, metabolic genes and secretion system proteins (Fig. 3B). RNA-seq analysis was performed to identify differential expression patterns among these genes. Results evidence

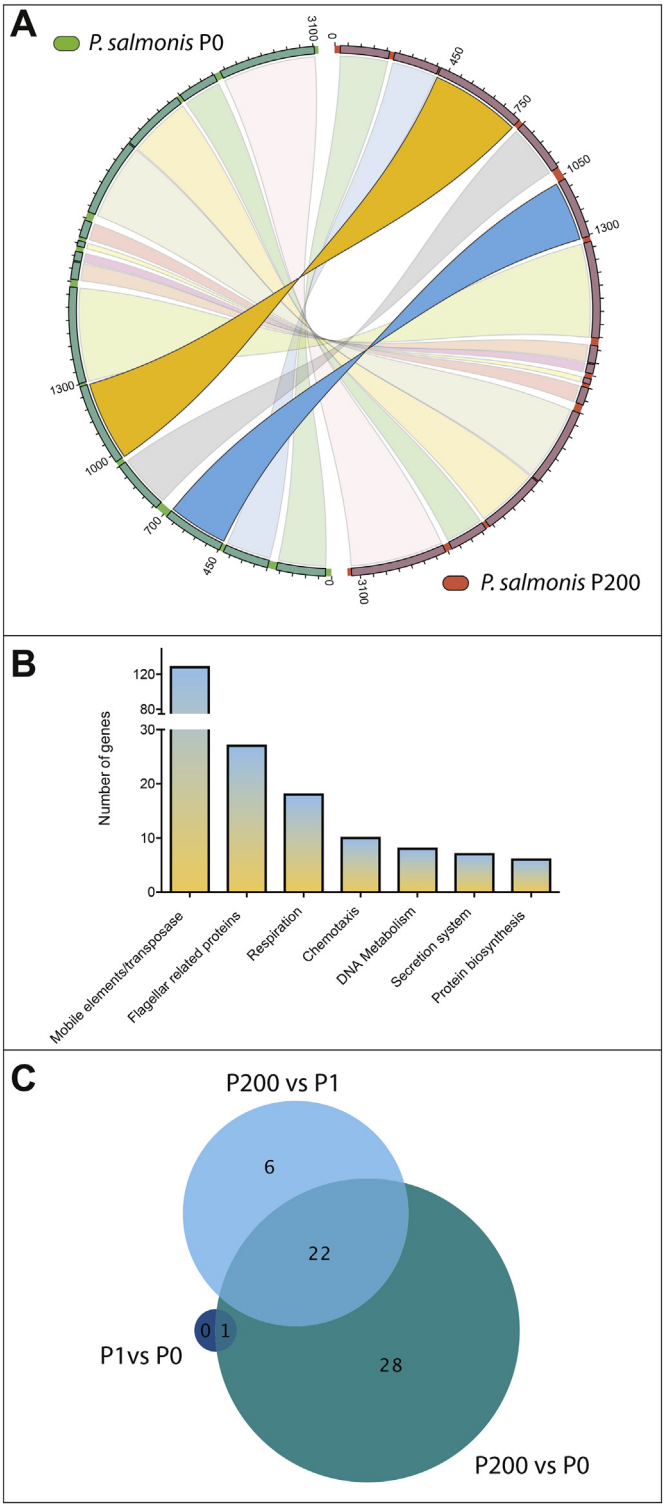

Fig. 3. A) Circos image comparing the organization of P. salmonis P0 and P200 genome blocks. Blocks with different position between the two passages are highlighted. **B)** Genomic content of the translocated blocks. Genes were classified according to the predicted function. **C)** Venn diagram showing the differentially expressed genes (Absolute fold change above 4 and p value < .05) in the translocated blocks among sequenced passages (P0, P1, P200).

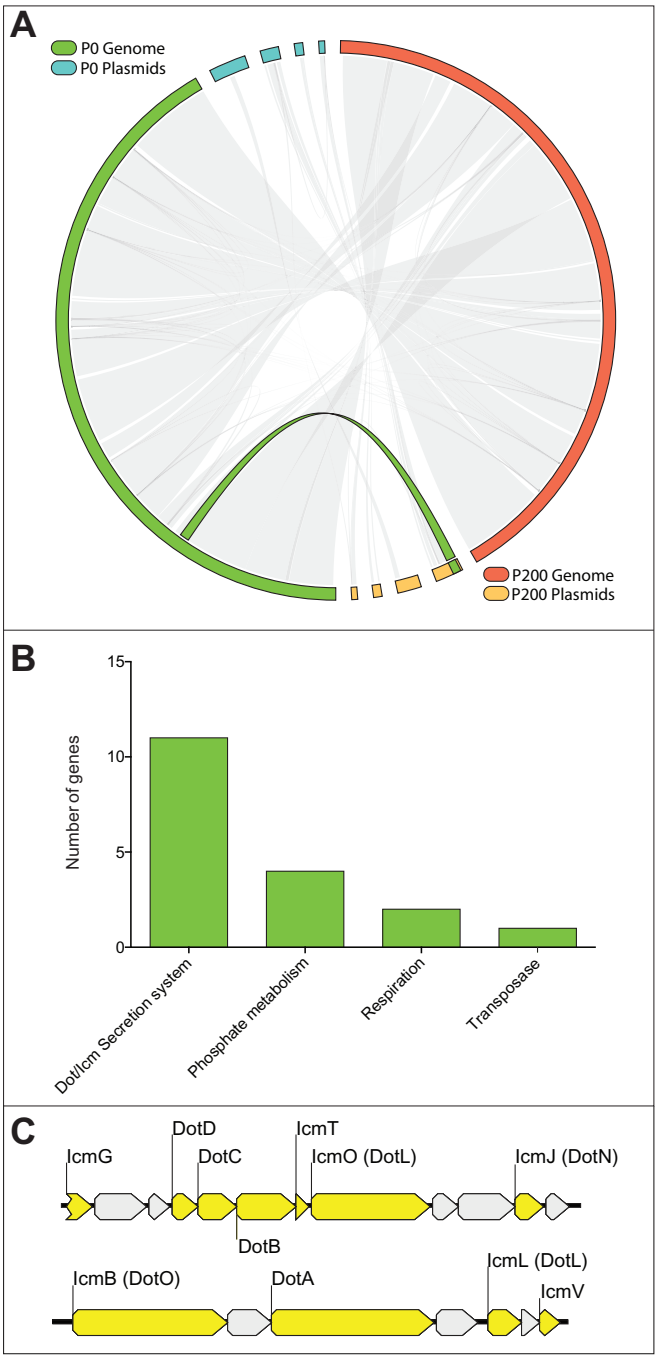

Fig. 4. A) Circos image highlighting a block of DNA that was originally located in the genome of P0 but that after 200 passages was found in one of the 4 natural plasmids of *P. salmonis*. **B)** Genomic content of the translocated region. Genes were classified according to the predicted function. **C)** List of the different genes belonging to the Dot/Icm secretion system identified within this region.

that just 57 of these genes were differentially regulated and that the major differences were found against P200 bacteria (Fig. 3C). Mostly respiration and metabolic genes were found in these genes, including *Cytochrome d ubiquinol oxidase*, *Fatty acid desaturase*, *Lactoylglutathione lyase*, and *ADP-ribose pyrophosphatase* among others (Table S1).

Second comparative genome analysis was performed including the 4 natural plasmids of *P. salmonis*. The results evidenced that a DNA segment from the *P. salmonis* genome was translocated to one of the four plasmids after two years of continuous free-cell culture (Fig. 4A). Most of the genes associated with this region were related to the Dot/Icm secretory system, phosphate metabolism, respiration, and transposases (Fig. 4B). To confirm these findings, we conducted a PCR amplification on the DotA gene, a member of the Dot/Icm secretion system. To do this, DNA and plasmids were isolated from P0 and P200 strains. The

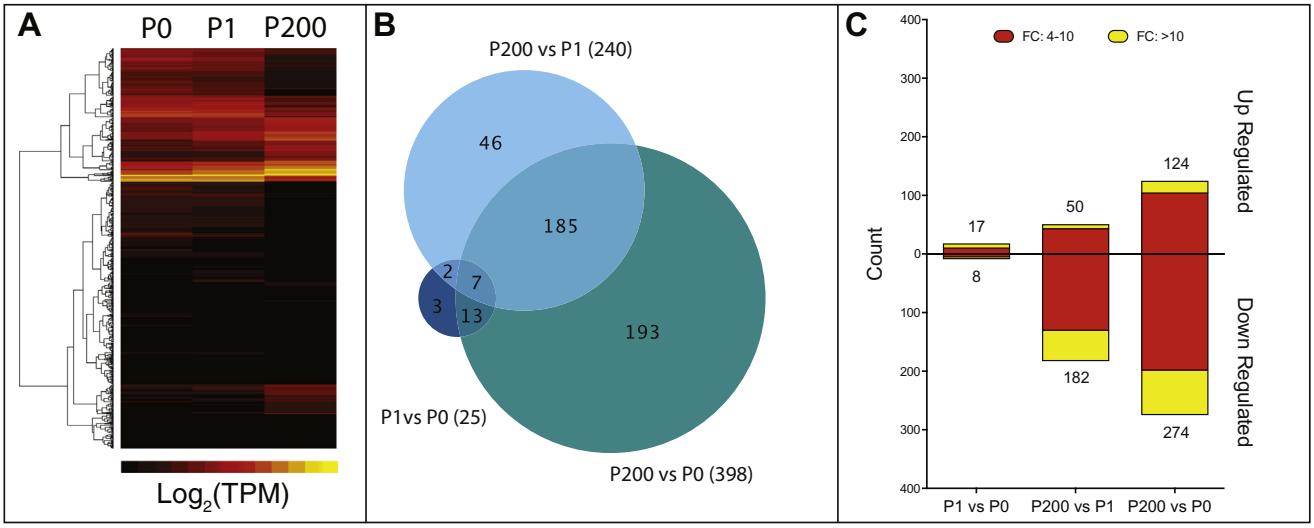

Fig. 5. A) Global transcriptomic patterns during different passages of *P. salmonis* in broth culture. **B)** Venn diagram showing the differentially expressed genes (Absolute fold change above 4 and p value < .05) among sequenced passages (P0, P1, P200). **C)** Distribution between differentially up and down regulated genes for each one of group comparisons. Red bars represent transcripts with and absolute fold change value between 4 and 10 and yellow bars represents absolute fold change values above 10. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results evidenced the amplification of these genes in the DNA from the P0 chromosome, but not on samples from the plasmid isolations. On the other hand, in P200 bacteria, the DotA gene was amplified in both DNA and plasmid isolation, evidencing that one of the two copies of this gene was translocated to one of the plasmids (Figure Supplementary fig 3). Although multiple genes associated with the secretion system of *P. salmonis* were found (Fig. 4C), just two genes were differentially expressed in the translocated segments from the genome to the plasmid when those were compared through different passages.

Global transcription patterns were also studied to uncover the transcriptome reorganization driven by medium adaptation of *P. salmonis*. In general, reduced transcriptome activity was found in medium adapted bacteria regarding P0 and P1 (Fig. 5A). Among them, a total of 499 genes were differentially expressed in at least one of the tested conditions (P0, P1, and P200). In general, most of these changes occurred in P200 bacteria. Thus, a total of 378 genes were differentially regulated in P200 compared with P0 and 231 when the comparison is made against P1 bacteria (Fig. 5B). The distribution between up and downregulated genes evidenced that most of this differential expression is explained due to a down-regulation in gene expression in medium adapted bacteria (P200), where 274 and 182 genes were down-regulated in P200 with respect to P0 and P1, respectively (Fig. 5C). The differentially expressed genes for each comparison were further classified by functions (subsystem) using RAST annotations. Here, key molecular pathways were regulated in response to medium adaptation, including respiration and metabolism of different molecules including DNA, lipids, and carbohydrates. Notably, one of the most enriched terms in P200 bacteria regarding P0 and P1 were iron adquisition and metabolism (Fig. 6).

Due to the importance of iron in bacterial pathogenesis, we further investigate this process. Thus, different genes associated with the iron siderophore vibrioferrin, including *PvuA*, *PvsA*, *PvsB*, *PvsC*, *PvsD*, *PvsE* and *TonB* (Fig. 7A). Results showed a marked decrease in the transcription activity of all these genes (Fig. 7B). These results were further confirmed by qPCR, were the analyzed genes evidenced the same decreased transcriptomic activity in P200 regarding P0 and P1 bacteria (Fig. S4). Moreover, to test if the reorganization and regulation of pathogenic related genes in P200 affects the virulence, we further explored the capacity of P0 and P200 passages to produce cytopathic effects and lysis in salmon cell lines (Fig. 8). The results evidenced a clear difference in the lysis of SHK-1 cells as a result of *P. salmonis* infection. While 70% of lytic effects were estimated in cells infected with P0 bacteria, no major lysis was evidenced in groups infected with the medium adapted bacteria (Fig. 8A and Fig. 8B). On the other hand, the *in vivo* analysis revealed a reduction in the cumulative mortality of individuals IP injected with *P. salmonis* when they were injected with the P200 strain (Fig. 8C).

4. Discussion

The continuous culture of bacteria in an artificial medium can lead to genotypic and phenotypic changes, allowing the microorganism to fully take advantage of the available nutrients in the medium. If these conditions are persistent enough time, some of these changes can become permanent (Eydallin et al., 2014; Kram et al., 2017). For intracellular pathogens, it has been well established that serial propagation in a cell-free media can lead to a decreased pathogenesis. However, most of the time these studies rely on the identification of point mutations as a result of media adaptation. Here, this study aimed to explore the genomic reorganization of the intracellular bacterium *P. salmonis* thought a long-term serial culture, and also evaluate changes in the bacterial virulence. Notably, whole-genome comparative analysis revealed a genome size and genetic content reduction after a long-term free-cell culture passage. It is known that bacterial genomes are highly dynamic both in size and composition (Puigbo et al., 2014). Genetic loss and genome reduction have been previously reported as a result of experimental evolution both in extracellular and intracellular pathogens (Dufresne et al., 2005; Koskiniemi et al., 2012; Nilsson et al., 2005). The serial culture of *E. coli* in a nutrient-rich environment has demonstrated to drive a genetic loss and the development of metabolic dependency on the medium supplemented metabolites (D'Souza and Kost, 2016). This phenomenon has been explained from the cellular energetic efficiency perspective, where loosing no longer required genes might imply an increased cellular fitness and a reduction in replication and production costs (D'Souza and Kost, 2016; D'Souza et al., 2014; Dufresne et al., 2005; Zamenhof and Eichhorn, 1967).

Together with the genome size reduction, a genomic reassortment was also evidenced after the serial culture of *P. salmonis*. These modifications included the translocation of two chromosome blocks, and also the translocation of a genomic DNA segment to the plasmid in bacteria adapted to the free-cell medium. The genetic content of these segments revealed the presence of genetic mobile elements,

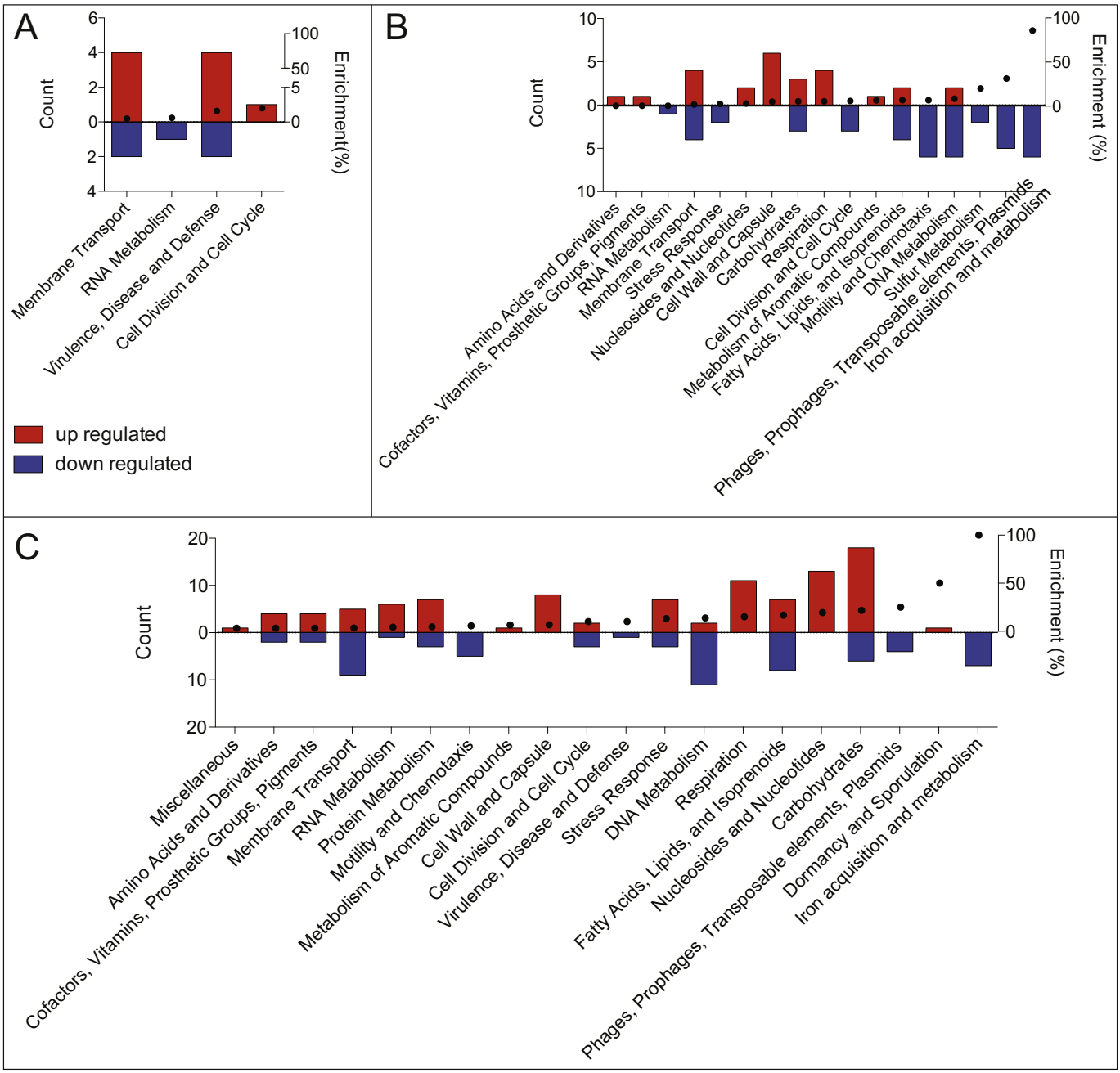

Fig. 6. Functional classification of the differentially regulated transcripts (Absolute fold change above 4 and p value < .05) between P1vsP0 **(A)** P200vsP1 **(B)** and P200vsP0 **(C)**. Red bars represent up regulated genes while the blue bars represent down regulated genes. Each functional classification is sorted by the percentage of differentially regulated genes regarding the total number of genes annotated for the pathway (Enrichment). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transposases, flagellar related proteins, and secretion system-related genes among others. Mobile genetic elements (MGEs) promote gene exchange and reassortment (Craig et al., 2002) and *Rickettsia* genomes are known to be composed of a large number of these elements (Gillespie et al., 2012). More than 40 MGEs have been described to be expressed in *P. salmonis* transcriptome (Machuca and Martinez, 2016) and some MGEs like ISPsa2 genes have been well characterized in *P. salmonis* genome (Marshall et al., 2011). Although this evidence suggested that the *P. salmonis* genome was a highly dynamic and fluid entity, this is the first report confirming that these rearrangements are occurring in the *P. salmonis* genome and the extent of these changes. Further investigations must be destined to evaluate how frequent these changes occur and what is the actual role MGEs in this reassortment.

Genome reorganizations can directly impact the function, evolution and regulation of gene expression in bacterial models (Hendrickson et al., 2018; Krogh et al., 2018; Ptacin and Shapiro, 2013). Although DNA translocation between genome and plasmids is a well-known phenomenon (Frost et al., 2005; Heffron et al., 1975; Russell and Dahlquist, 1989; Wozniak and Waldor, 2010), it is usually studied under the scope of antibiotic resistance. However, the role of recombination and the impact on pathogenesis remains largely unknown. Flagellar related proteins and secretion system-related genes were also found in the translocated DNA. *P. salmonis* is defined as a non-motile bacterium and no structural flagellum has been reported for the bacteria (Fryer and Hedrick, 2003). However, the presence of flagellar related proteins has been suggested as a possible immunomodulator of

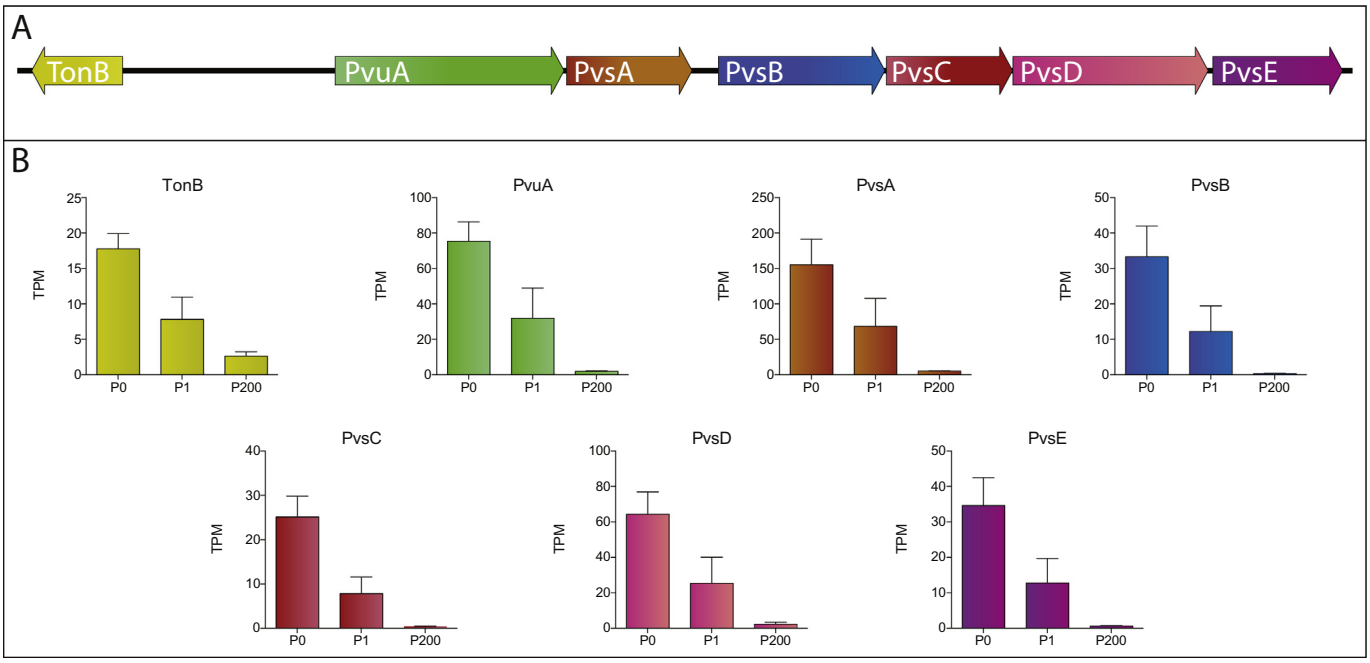

Fig. 7. A) Vibrioferrin related genes identified in *P. salmonis* genomes. **B)** Individual gene expression of each vibrioferrin related gene differentially expressed among the sequenced passages (P0, P1 and P200).

host response to promote *P. salmonis* macrophage infection (Carril et al., 2017). On the other hand, the secretion system is a well-known and conserved mechanism for the delivery of virulence factors among pathogenic bacteria (Fronzes et al., 2009). Particularly in *P. salmonis*, a functional Dot/Icm type IV-B secretion system has been reported (Gomez et al., 2013) and recent evidence suggests that the mutagenesis of this system in *P. salmonis* leads to a pathogenic attenuation of the bacteria (Mancilla et al., 2018). Our results evidenced a change in the loci of both pathogenic related genes, probably affecting the genomic architecture in medium adapted *P. salmonis*. Although no major differences in gene expression were evidenced in these genes during broth culture for P0 and P200 bacterium, it is unknown how this scenario differs during the infective process of *P. salmonis* in host cells.

Global gene expression patterns between different passages of *P. salmonis* (P0, P1, and P200) evidence a smaller transcriptomic repertory in medium adapted bacteria when compared with the first passages in the cell-free medium. We hypothesized that in the absence of competition with the host for available nutrients for more than 200 generations, *P. salmonis* displays a reduced and cost-effective transcriptomic response to take advantage of the nutrient availability in the medium. We have previously shown that nutritional immunity plays a pivotal role in *P. salmonis*-Salmon interaction, where host and pathogen can compete for the amino acid and iron availability (Valenzuela-Miranda and Gallardo-Escarate, 2016, 2018; Valenzuela-Miranda et al., 2017). This reduced transcriptomic responses involved the negative regulation of iron homeostatic related genes, including the group of vibrioferrin. Vibrioferrin (VF) is a class of carboxylate marine siderophore involved in iron transport (Amin et al., 2009). Siderophores have been characterized as key elements for pathogenic bacteria to compete for iron acquisition from their hosts (Miethke and Marahiel, 2007; Wilson et al., 2016). Previously, it has been reported that *P. salmonis* produces siderophore-like molecules and encodes vibrioferrin related genes (Calquin et al., 2018; Machuca and Martinez, 2016). It is likely that in the absence of a host to infest and with no competition for iron availability, vibrioferrin is no longer required to compete for iron uptake, therefore explaining the transcriptomic downregulation of these genes.

Due to all the important changes observed in media adapted bacteria regarding the reorganization and transcriptomic regulation of pathogenic genes, we further compare the virulence of P0 and P200 *P. salmonis* in the salmon head kidney cell lines SHK-1 and during a *in vivo* infection. Although exploratory, our experiment showed that cells infected with the media adapted bacteria evidenced less cytopathic

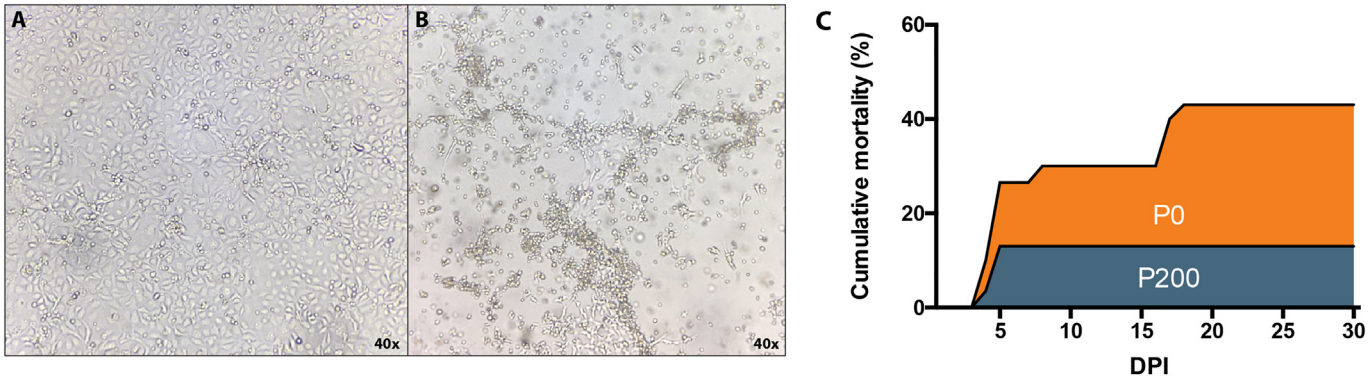

Fig. 8. *In vitro* infection of CHSE cells line with the media adapted (P200) bacteria **(A)** and the original (P0) *P. salmonis***(B)**. Both images were capture at 40× magnification. **C)** Cumulative mortality of Atlantic salmons IP injected with *P. salmonis* P0 and P200.

effects and lysis than cells infected with the original P0 bacteria. Same results were found during the *in vivo* experiment, were individuals IP injected with P200 bacteria evidenced less mortality than the control group. These results agree with the existing evidence suggesting that multiple passages of pathogenic bacteria leads to attenuated virulence (Cabral et al., 2017). In fact, until the development of effective bacterial genome editing, the serial passage of pathogenic bacteria was the gold standard method for the development of live attenuated vaccines (Hanley, 2011). Considering that almost all developed vaccines to control *P. salmonis* infection rely on inactivated bacterium (bacterin) with a low protection level (Maisey et al., 2017), alternative strategies can be developed. Herein, the present study opens novel pivotal questions to investigate how the genome reorganization of *P. salmonis* can be useful for the generation of effective live attenuated vaccines. Future investigations will apply CRISPER-Cas9 to evaluate if the genes and DNA segments translocated as a consequence of the cell-free medium adaptation will be applied to attenuate the bacterial virulence *in vivo* assays.

5. Conclusions

We have shown that the serial propagation of *P. salmonis* in a cell-free broth culture leads to a genomic and transcriptomic reorganization, affecting the organization and regulation of pathogenic related genes, including the Dot/Icm secretion system and iron homeostasis pathways among others. Our findings also suggested that these modifications can be associated with the reduced virulence observed in adapted bacteria to the cell-free culture. Future investigations will be conducted to develop novel live attenuated vaccines against Piscirickettsiosis, improving the sustainability of the salmon industry in Chile.

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Author statement

The study was conceived by DV, VV, GN and CG. DV performed the experiments and analyzed the data under CG's supervision. The cell infection experiment was conducted by VV. DV and CG drafted the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Amin, S.A., Green, D.H., Küpper, F.C., Carrano, C.J., 2009. Vibrioferrin, an unusual marine Siderophore: Iron binding, photochemistry, and biological implications. Inorg. Chem. 48, 11451–11458.

Barrick, J.E., Lenski, R.E., 2013. Genome dynamics during experimental evolution. Nat. Rev. Genet. 14, 827–839.

Cabral, M.P., Garcia, P., Beceiro, A., Rumbo, C., Perez, A., Moscoso, M., Bou, G., 2017. Design of live attenuated bacterial vaccines based on D-glutamate auxotrophy. Nat. Commun. 8.

Calquin, P., Ruiz, P., Oliver, C., Sanchez, P., Haro, R., Oliva, H., Vargas-Chacoff, L., Avendano-Herrera, R., Yanez, A.J., 2018. Physiological evidence that Piscirickettsia salmonis produces siderophores and uses iron from different sources. J. Fish Dis. 41, 553–558.

Carril, G.P., Gomez, F.A., Marshall, S.H., 2017. Expression of flagellin and key regulatory flagellar genes in the non-motile bacterium Piscirickettsia salmonis. Dis. Aquat. Org. 123, 29–43.

Conrad, T.M., Joyce, A.R., Applebee, M.K., Barrett, C.L., Xie, B., Gao, Y., Palsson, B.O., 2009. Whole-genome resequencing of Escherichia coli K-12 MG1655 undergoing short-term laboratory evolution in lactate minimal media reveals flexible selection of adaptive mutations. Genome Biol. 10.

Craig, N.L., Craigie, R., Gellert, M., Lambowitz, A.M., 2002. Mobile DNA ii. Washington, DC. 18. ASM Press, pp. 32.

D'Souza, G., Kost, C., 2016. Experimental evolution of metabolic dependency in Bacteria. PLoS Genet. 12, e1006364.

D'Souza, G., Waschina, S., Pande, S., Bohl, K., Kaleta, C., Kost, C., 2014. Less is more: selective advantages can explain the prevalent loss of biosynthetic genes in Bacteria. Evolution. 68, 2559–2570.

Dufresne, A., Garczarek, L., Partensky, F., 2005. Accelerated evolution associated with genome reduction in a free-living prokaryote. Genome Biol. 6.

Eydallin, G., Ryall, B., Maharjan, R., Ferenci, T., 2014. The nature of laboratory domestication changes in freshly isolated Escherichia coli strains. Environ. Microbiol. 16, 813–828.

Fronzes, R., Christie, P.J., Waksman, G., 2009. The structural biology of type IV secretion systems. Nat. Rev. Microbiol. 7, 703–714.

Frost, L.S., Leplae, R., Summers, A.O., Toussaint, A., 2005. Mobile genetic elements: the agents of open source evolution. Nat. Rev. Microbiol. 3, 722–732.

Fryer, J.L., Hedrick, R.P., 2003. Piscirickettsia salmonis: a gram-negative intracellular bacterial pathogen of fish. J. Fish Dis. 26, 251–262.

Germanier, R., 1975. Isolation and characterization of gal E mutant Ty 21a of Salmonella typhi: a candidate strain for a live. Oral Typhoid Vaccine. The Journal of Infectious Diseases. 131, 553–558.

Gillespie, J.J., Joardar, V., Williams, K.P., Driscoll, T., Hostetler, J.B., Nordberg, E., Shukla, M., Walenz, B., Hill, C.A., Nene, V.M., Azad, A.F., Sobral, B.W., Caler, E., 2012. A rickettsia genome overrun by Mobile genetic elements provides insight into the Acquisition of Genes Characteristic of an obligate intracellular lifestyle. J. Bacteriol. 194, 376–394.

Gomez, F.A., Tobar, J.A., Henriquez, V., Sola, M., Altamirano, C., Marshall, S.H., 2013. Evidence of the presence of a functional dot/Icm type IV-B secretion system in the fish bacterial pathogen Piscirickettsia salmonis. PLoS One 8, e54934.

Hanley, K.A., 2011. The double-edged sword: how evolution can make or break a live-attenuated virus vaccine. Evolution: Education and Outreach. 4, 635–643.

Heffron, F., Rubens, C., Falkow, S., 1975. Translocation of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specificity of insertion. P Natl Acad Sci USA. 72, 3623–3627.

Hendrickson, H.L., Barbeau, D., Ceschin, R., Lawrence, J.G., 2018. Chromosome architecture constrains horizontal gene transfer in bacteria. PLoS Genet. 14.

Henriquez, M., Gonzalez, E., Marshall, S.H., Henriquez, V., Gomez, F.A., Martinez, I., Altamirano, C., 2013. A novel liquid medium for the efficient growth of the salmonid pathogen Piscirickettsia salmonis and optimization of culture conditions. PLoS One 8, e71830.

Herring, C.D., Raghunathan, A., Honisch, C., Patel, T., Applebee, M.K., Joyce, A.R., Albert, T.J., Blattner, F.R., van den Boom, D., Cantor, C.R., Palsson, B.O., 2006. Comparative genome sequencing of Escherichia coli allows observation of bacterial evolution on a laboratory timescale. Nat. Genet. 38, 1406–1412.

Koskiniemi, S., Sun, S., Berg, O.G., Andersson, D.I., 2012. Selection-driven gene loss in Bacteria. PLoS Genet. 8.

Kram, K.E., Geiger, C., Ismail, W.M., Lee, H., Tang, H.X., Foster, P.L., Finkel, S.E., 2017. Adaptation of Escherichia coli to long-term serial passage in complex medium: evidence of parallel evolution. Msystems. 2.

Krogh, T.J., Moller-Jensen, J., Kaleta, C., 2018. Impact of chromosomal architecture on the function and evolution of bacterial genomes. Front. Microbiol. 9, 2019.

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., Marra, M.A., 2009. Circos: an information aesthetic for comparative genomics. Genome Res. 19, 1639–1645.

Machuca, A., Martinez, V., 2016. Transcriptome analysis of the intracellular facultative pathogen Piscirickettsia salmonis: expression of putative groups of genes associated with virulence and Iron metabolism. PLoS One 11, e0168855.

Maisey, K., Montero, R., Christodoulides, M., 2017. Vaccines for piscirickettsiosis (salmonid rickettsial septicaemia, SRS): the Chile perspective. Expert Review of Vaccines. 16, 215–228.

Mancilla, M., Saavedra, J., Grandon, M., Tapia, E., Navas, E., Grothusen, H., Bustos, P., 2018. The mutagenesis of a type IV secretion system locus of Piscirickettsia salmonis leads to the attenuation of the pathogen in Atlantic salmon, *Salmo salar*. J. Fish Dis. 41, 625–634.

Marshall, S.H., Henríquez, V., Gómez, F.A., Cárdenas, C., 2011. ISPsa2, the first mobile genetic element to be described and characterized in the bacterial facultative intracellular pathogen Piscirickettsia salmonis. FEMS Microbiol. Lett. 314, 18–24.

Miethke, M., Marahiel, M.A., 2007. Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol R. 71 413 − +.

Nilsson, A.I., Koskiniemi, S., Eriksson, S., Kugelberg, E., Hinton, J.C.D., Andersson, D.I., 2005. Bacterial genome size reduction by experimental evolution. P Natl Acad Sci USA. 102, 12112–12116.

Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A.R., Xia, F.F., Stevens, R., 2014. The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). Nucleic Acids Res. 42, D206–D214.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and

metagenomes. Genome Res. 25, 1043–1055.
Ptacin, J.L., Shapiro, L., 2013. Chromosome architecture is a key element of bacterial cellular organization. Cell. Microbiol. 15, 45–52.
Puigbo, P., Lobkovsky, A.E., Kristensen, D.M., Wolf, Y.I., Koonin, E.V., 2014. Genomes in turmoil: quantification of genome dynamics in prokaryote supergenomes. BMC Biol. 12.
Ramirez, R., Gomez, F.A., Marshall, S.H., 2015. The infection process of Piscirickettsia salmonis in fish macrophages is dependent upon interaction with host-cell clathrin and actin. FEMS Microbiol. Lett. 362, 1–8.
Rojas, V., Galanti, N., Bols, N.C., Jimenez, V., Paredes, R., Marshall, S.H., 2010. Piscirickettsia salmonis induces apoptosis in macrophages and monocyte-like cells from rainbow trout. J. Cell. Biochem. 110, 468–476.
Rozas, M., Enriquez, R., 2014. Piscirickettsiosis and Piscirickettsia salmonis in fish: a review. J. Fish Dis. 37, 163–188.
Russell, C.B., Dahlquist, F.W., 1989. Exchange of chromosomal and plasmid alleles in Escherichia coli by selection for loss of a dominant antibiotic sensitivity marker. J. Bacteriol. 171, 2614.
Somerville, G.A., Beres, S.B., Fitzgerald, J.R., DeLeo, F.R., Cole, R.L., Hoff, J.S., Musser, J.M., 2002. In vitro serial passage of Staphylococcus aureus: changes in physiology, virulence factor production, and agr nucleotide sequence. J. Bacteriol. 184, 1430–1437.
Spagnolo, F., Rinaldi, C., Sajorda, D.R., Dykhuizen, D.E., 2016. Evolution of resistance to continuously increasing streptomycin concentrations in populations of Escherichia coli. Antimicrob Agents Ch. 60, 1336–1342.
Tarifeno-Saldivia, E., Valenzuela-Miranda, D., Gallardo-Escarate, C., 2017. In the shadow: the emerging role of long non-coding RNAs in the immune response of Atlantic salmon. Dev. Comp. Immunol. 73, 193–205.
Toida, I., 2000. Development of the Mycobacterium bovis BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. Tuber. Lung Dis. 80, 291.
Toprak, E., Veres, A., Michel, J.B., Chait, R., Hartl, D.L., Kishony, R., 2012. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. Nat. Genet. 44, 101–U140.
Valenzuela-Miranda, D., Gallardo-Escarate, C., 2016. Novel insights into the response of Atlantic salmon (Salmo salar) to Piscirickettsia salmonis: interplay of coding genes and lncRNAs during bacterial infection. Fish Shellfish Immunol. 59, 427–438.
Valenzuela-Miranda, D., Gallardo-Escarate, C., 2018. Dual RNA-Seq uncovers metabolic amino acids dependency of the intracellular bacterium Piscirickettsia salmonis infecting Atlantic Salmon. Front. Microbiol. 9, 2877.
Valenzuela-Miranda, D., Valenzuela-Munoz, V., Farlora, R., Gallardo-Escarate, C., 2017. MicroRNA-based transcriptomic responses of Atlantic salmon during infection by the intracellular bacterium Piscirickettsia salmonis. Dev. Comp. Immunol. 77, 287–296.
Valenzuela-Miranda, D., Valentina, V.-M., Gustavo, N.-A., Cristian, G.-E., 2020. Long-term serial culture of Piscirickettsia salmonis leads to a genomic and transcriptomic reorganization affecting bacterial in vitro virulence. Figshare. https://doi.org/10.6084/m9.figshare.12047238.v1. Dataset.
Valenzuela-Munoz, V., Valenzuela-Miranda, D., Gallardo-Escarate, C., 2019. Iron overload alters the immune response in Atlantic salmon and increases the susceptibility to Piscirickettsia salmonis infection. Fish Shellfish Immun. 91, 451.
Wilson, B.R., Bogdan, A.R., Miyazawa, M., Hashimoto, K., Tsuji, Y., 2016. Siderophores in Iron metabolism: from mechanism to therapy potential. Trends Mol. Med. 22, 1077–1090.
Wiser, M.J., Ribeck, N., Lenski, R.E., 2013. Long-term dynamics of adaptation in asexual populations. Science. 342, 1364–1367.
Wozniak, R.A.F., Waldor, M.K., 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat. Rev. Microbiol. 8, 552–563.
Yanez, A.J., Valenzuela, K., Silva, H., Retamales, J., Romero, A., Enriquez, R., Figueroa, J., Claude, A., Gonzalez, J., Avendano-Herrera, R., Carcamo, J.G., 2012. Broth medium for the successful culture of the fish pathogen Piscirickettsia salmonis. Dis. Aquat. Org. 97, 197–205.
Zamenhof, S., Eichhorn, H.H., 1967. Study of microbial evolution through loss of biosynthetic functions: establishment of “defective” mutants. Nature. 216, 456–458.